ASSISTANCE TO STATES FOR
CONTROL OF ANIMAL DISEASES (ASCAD)

TRAINING COURSE ON

ADVANCED APPROACHES FOR
DIAGNOSIS OF LIVESTOCK DISEASES
(25-30 November 2013)

Department of Veterinary Microbiology
College of Veterinary Science & Animal Husbandry
Junagadh Agricultural University, Junagadh-362001, Gujarat
ASSISTANCE TO STATES FOR CONTROL OF ANIMAL DISEASES (ASCAD) TRAINING COURSE

On

“Advanced Approaches for Diagnosis of Livestock Diseases”

25th - 30th November, 2013

Course Director
Dr. P. H. Vataliya

Course Co-ordinator:
Dr. B. B. Javia

Editors:
Dr. D. B. Barad
Dr. B.S. Mathapati
Dr. A. R. Bhadaniya
Dr. R. J. Padodara
Dr. S. H. Sindhi
Dr. Binod kumar

Organized By

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College of Veterinary Science & Animal Husbandry
Junagadh Agricultural University, Junagadh-362001, Gujarat, India
College of Veterinary Science & Animal Husbandry
Junagadh Agricultural University, Junagadh

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Message

From Desk of Course Director

Animal health is under constant threat from various dreaded infectious diseases, which result in significant economic losses to livestock owners. Maintenance of good health status of animals relies on early, precise and rapid diagnosis of a range of animal diseases which largely consists of infectious diseases. The conventional diagnostic methods are time consuming and labour intensive. Recent advances in the molecular biology and biotechnology has opened new avenues in disease diagnosis. These advanced techniques of disease diagnosis are rapid, specific and less labour intensive which helps in early detection of causative pathogen. All these advanced techniques and methodologies are not exploited to the fullest extent. There is a need to generate qualified, trained and competent manpower for further improvement in existing disease diagnostic methods. Due to paucity of trained man power in different institutions of public undertakings, it is highly desirable that the knowledge be disseminated from experts to users of these techniques so that the expertise gained can be harnessed in more effective and efficient manner.

In this direction, Centrally sponsored “Assistance for states to control of animal diseases (ASCAD)” training course on “Advanced approaches for diagnosis of livestock diseases” with an aim to transform field veterinarians to trained manpower in disease diagnosis is organized in the College of Veterinary Science and Animal Husbandary, Junagadh Agricultural University, Junagadh. The young and energetic faculty members with recent knowledge on new technologies are most appropriate personnel to impart this kind of hands-on for training animal disease diagnosis.

I congratulate Dr. B.B. Javia, Course Co-ordinator and his team for taking this initiative and compiling training manual which covers a range of advanced techniques in disease diagnosis. I hope that this training course will be of immense help in improving knowledge and skills of veterinarians in disease diagnosis. The livestock farmers will be benefited of early, quick and accurate diagnosis of diseases affecting their animals due to timely measures for control of the diseases.

I wish this training programme to be successful and proved to be rewarding to all concerned.

Date: 25/11/2013

(P.H. Vataliya)
Course Director
The Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, JAU, Junagadh is fully equipped with advance diagnostic facilities like Thermocycler(PCR), ELISA Reader, Immune-electrophoresis, Phase Contrast Microscope, Fluorescent Microscope, Cell culture equipments like Biosafety Cabinet, CO2 Incubator, Inverted Microscope with necessary supporting instruments.

The training courses on "Advanced approaches for diagnosis of livestock diseases" include all the advanced diagnostic techniques such as PCR, Real time PCR, Various types of ELISA, Fluorescent Microscope, Phase Contrast Microscopy, Cell Culture Techniques, Imaging Techniques with latest software besides the basic microbial identification tests. The compendium has been meticulously compiled by the teachers of the department which I hope, will be a useful document and guide for the further reference to veterinarians.

I am highly thankful to Dr. N. C. Patel, Honorable Vice Chancellor, Junagadh Agricultural University, Junagadh. I extend my thanks to Dr. A. J. Kachhia Patel, Director, Animal Husbandry. Government of Gujarat, Gadhinagar and Dr. P. H. Vataliya, Principal & Dean, College of Veterinary Science & Animal Husbandry, JAU, Junagadh for giving full support for conducting the training course.

(B. B. Javia)
Course Co-ordinator
Head, Department of Veterinary Microbiology
College of Veterinary Science & A. H.
JAU, Junagadh
Participants: Training on "Advanced Approaches for Diagnosis of Livestock Diseases"
25th-30th November, 2013

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<tr>
<td>1</td>
<td>Dr. Hiteshbhai Shamjibhai Kher Veterinary Officer, District Panchayat, Bhavnagar</td>
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<td>9638404840</td>
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<td>Dr. Revabhai Ganeshbhai Mali Veterinary Officer, District Panchayat, Bhavnagar</td>
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<td>4</td>
<td>Dr. Hareshkumar Laxmidas Kacha Veterinary Officer, District Panchayat, Junagadh</td>
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<tr>
<td>6</td>
<td>Dr. Hiteshkumar Gobarbhai Vamja Veterinary Officer, Forest Department, Dhari</td>
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<td>7</td>
<td>Dr. Bhavinkumar Dineshbhai Patel Veterinary Officer, District Panchayat, Surendranagar</td>
<td><a href="mailto:drbhavinpatel.patel@gmail.com">drbhavinpatel.patel@gmail.com</a></td>
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<tr>
<td>8</td>
<td>Dr. Bhagavantsinh Malaji Solanki Veterinary Officer, District Panchayat, Banaskantha</td>
<td><a href="mailto:bmsolanki14@gmail.com">bmsolanki14@gmail.com</a></td>
<td>9429407564</td>
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# ASCAD Training on

**“Advanced Approaches for Diagnosis of Livestock Diseases”**

## Training Schedule

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<td>25/11/13</td>
<td>9.00 to 9.30</td>
<td>Registration and Inauguration</td>
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<tr>
<td></td>
<td></td>
<td>9.30 to 10.30</td>
<td>Role of molecular techniques in diagnosis of animal diseases</td>
<td>Dr. P. H. Vataliya</td>
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<td></td>
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<td>10.30 to 11.30</td>
<td>Advanced microscopy and its utility in animal disease diagnosis</td>
<td>Dr. D.B.Barad</td>
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<td>11.30 to 12.30</td>
<td>Importance of hematology in animal disease diagnosis</td>
<td>Dr. R. J. Padodara</td>
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<td>Antibiotic resistance: practices and management</td>
<td>Dr. U. D. Patel</td>
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<td>Molecular methods for diagnosis of HS in cattle and buffaloes</td>
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<td>Recent approaches for diagnosis of foot and mouth disease</td>
<td>Dr. B. S. Matapathi</td>
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<td>LAMP: An advanced low cost tool for animal disease diagnosis</td>
<td>Dr. V. K. Singh</td>
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<td>Dr. A. R. Bhadania</td>
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<td>Differential diagnosis of abortion causing infection in animals</td>
<td>Dr. K. B. Vala</td>
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Role of Molecular Techniques in Diagnosis of Animal Diseases

P. H. Vataliya, A. R. Ahlawat, G. S. Sonawane and V.B. Dongre
Department of Animal Genetics & Breeding
College of Veterinary Science & Animal Husbandry
Junagadh Agricultural University, Junagadh-362001

Laboratory diagnostics for Veterinary pathogens have traditionally relied on methods of detecting the pathogen by culture or antibodies, using a variety of techniques such as neutralisation, enzyme-linked immunosorbent assay, agar gel immunodiffusion and complement fixation. The effective control and treatment of diseases of animals requires access to diagnostic tests that are rapid, reliable and highly sensitive. However, these methods are time-consuming and costly.

Efforts to overcome these problems have lead to the development of several diagnostic methods including fluorescent antibody tests (FAT), enzyme-linked immunosorbent assays (ELISA), radioimmunoassay (RIA), in situ hybridization (ISH), dot blot hybridization (DBH) and polymerase chain reaction (PCR) amplification techniques. The use of DNA-based methods derives from the premise that each species of pathogen carries unique DNA or RNA sequences that differentiate it from other organisms. The techniques offer high sensitivity and specificity, and diagnostics kits allowing rapid screening for the presence of pathogen DNA are moving rapidly from development in specialized laboratories to routine application. DNA probes are expected to find increasing use in routine disease monitoring and treatment programs in veterinary medicine, in field epidemiology and in efforts to prevent the international spread of pathogens. The development and use of DNA-based diagnostic techniques will assist international efforts to control the introduction of exotic diseases into new geographic areas. Reliable and rapid techniques are needed by national and regional diagnostic laboratories to screen for important pathogens.

Nucleic acid diagnostics

The use of nucleic acid-based diagnostics in veterinary medicine has increased exponentially in recent years. These techniques have redefined the level of information available for animal disease control programmes. In addition, modifications of nucleic acid detection techniques based on polymerase chain reaction (PCR) have lead to the development of rapid, specific assays. The molecular technique with the widest variety and application in veterinary diagnostics is PCR. The strength of this technique is its ability to make millions of copies of a deoxyribonucleic acid (DNA) target. This amplification enables the desired target to be readily detected by other techniques such as electrophoresis and sequencing. Initial use of PCR in veterinary diagnostics was for specific genomic detection e.g. bovine viral diarrhoea virus, foot and mouth disease virus, Infectious bovine rhinotracheitis, Buffalopox, Ephemeral Fever virus, etc.

Following are the list of the different molecular diagnosis methods in animal disease:

1. Reverse transcription PCR
2. Nested PCR
3. Real-time PCR
4. Multiplex PCR
5. Fluorescent in situ hybridisation (FISH)
6. Nucleic acid sequence-based amplification (NASBA)
7. Proteomics
8. Microarray technology
9. Nanotechnology

Reverse transcription PCR: This assay initially makes a complementary DNA copy of the original viral RNA before final amplification and is more sensitive than the traditional Northern blot method of RNA detection. A potential approach to RNA detection is to use binding stretches of RNA (aptamers). This type of application does not require amplification and is currently used in studies of gene expression in human cells and tissues. Most of the disease causing RNA viruses are diagnosed now a days using reverse transcriptase PCR converting their fragile RNA genome into stable cDNA.

Nested PCR: It refers to the application of a second set of primers targeting a shorter area on the first-stage amplified product (DNA). Using this approach increases the sensitivity of the PCR and generates two amplified products for confirmation purposes. This technique has been used to detect a number of agents of veterinary interest including West Nile virus (1). A disadvantage of the nested PCR is the increased risk of cross-contamination due to the opening of amplification tubes to add an additional set of primers.

Real-time PCR is the latest improvement in the standard PCR technique to be implemented in veterinary laboratories. This technique is a single-capillary, closed assay that greatly decreases the problem of cross-contamination between samples. The fluorescence readings are plotted by computer software and results can be transmitted electronically, eliminating the need for post-PCR reaction analysis by electrophoresis (2). The development of extraction methods such as the magnetic bead technique has made it possible to use real-time PCR to test large numbers of samples in a matter of hours during disease outbreaks. In addition, real-time PCR has been adapted for use in the field through the use of portable thermocyclers and lyophilised reagents. This approach may allow for more rapid decision-making during potential disease outbreaks. The PCR is also used extensively for the genotyping and phylogenetic analysis (relatedness) of veterinary pathogens.

Non-PCR methods of nucleic acid detection:

New methods of nucleic acid amplification have been developed and may eventually be used for veterinary diagnostics. Examples of these methods include the rolling circle amplification technique and direct signal amplification systems. These techniques are currently being used in human diagnostics for the detection of human cytomegalovirus and human immunodeficiency viruses; veterinary applications are currently being developed.

Fluorescent in situ hybridisation (FISH) is a technique that can localize nucleic acid sequences within cellular material. Peptide nucleic acids are molecules in which the sugar
backbone has been replaced by a peptide backbone. These molecules are perfect mimics of DNA with high affinity for hybridisation that can be used to improve FISH techniques.

**Nucleic acid sequence-based amplification (NASBA)** is a promising gene amplification method. This isothermal technique is comprised of a two-step process whereby there is an initial enzymatic amplification of the nucleic acid targets followed by detection of the generated amplicons. The entire NASBA process is conducted at a single temperature, thereby eliminating the need for a thermocycler.

**Proteomics**

In addition to the use of proteomics to identify and characterize the protein produced by pathogenic agents, proteomic technologies have great potential in veterinary diagnostic applications because they target the patterns of protein expression of the target analyte whether it is viral, bacterial, parasitic, etc. The standard proteomic approach involves the separation of proteins by two-dimensional gels with the staining of the proteins and molecular weight control. This protein ‘pattern’ or fingerprint is then analyzed by performing image analysis. Proteome maps can be compared in order to find proteins that may be up- or down-regulated due to disease. A protein of interest can be cut from the gel and fully characterized using peptide-mass fingerprinting and/or mass spectrometry methods. In the future, veterinary diagnostics may make use of proteomics to identify or look for known disease markers or patterns with biochip technology and instrumentation that combines mass spectrometry with other separation chromatography or molecular techniques. These instrumentations are designed to specifically select, separate by molecular mass, and identify the complex mixture of proteins in a sample, which can then be compared to known samples for diagnostic purposes. This type of technology may be useful for identifying animals infected with agents that do not induce predictable serologic reactions, such as bovine tuberculosis (4).

**Microarray technology**

Originally developed for the mapping of genes, it is being used to detect a wide variety of veterinary pathogens (5). Specific oligonucleotides are bound to small solid supports such as glass slides, silicon chips or nylon membranes. Extracted DNA or complementary DNA is labelled with a fluorescent dye and then hybridized with the microarray. Specific patterns of fluorescence are detected by a microarray reader which allows the identification of specific gene sequences found only in the veterinary pathogen of interest. This technology has the potential to identify the presence of agents of interest at the serotype or subspecies level, or to differentiate agents that cause similar clinical signs, for example, vesicular lesions.
Nanotechnology

The term ‘nanotechnology’ is broadly defined as systems or devices related to the features of nanometre scale (one billionth of a metre). This scale of technology as it applies to diagnostics would include the detection of molecular interactions. The small dimensions of this technology have led to the use of nanoarrays and nanochips as test platforms (3). One advantage of this technology is the potential to analyze a sample for an array of infectious agents on a single chip. Applications include the identification of specific strains or serotypes of disease agents, such as the identification of specific influenza strains, or the differentiation of diseases caused by different viruses but with similar clinical signs, such as vesicular viral diseases. Many research groups are considering the use of chip assays that detect a number of agroterrorism agents in each sample. Small, portable platforms are being designed to allow pen-side testing of animals for diseases of concern. Another facet of nanotechnology is the use of nanoparticles to label antibodies. These labelled antibodies can then be used in various assays to identify specific pathogens, molecules or structures. Examples of nanoparticle technology include the use of gold nanoparticles, nanobarcodes, quantum dots (cadmium selenide) and nanoparticle probes.

Impediments to the Use of DNA-Based Diagnostic Techniques

Although offering considerable potential, the routine use of DNA-based diagnostic techniques is hampered by a number of potential problems.

- The extreme sensitivity of these methods allows the detection of target DNA present at very low levels. However, positive results provide little quantitative assessment of the infection level, and do not indicate whether the pathogen is replicating or causing disease in the species tested. Thus, carrier status and viability of the pathogen are not determined using DNA-probes.
- The extremely high specificity of these tests, coupled with the ability of many viruses to rapidly change in genetic structure, can result in failure to detect a virus that has altered its genetic profile.
- Large differences in sensitivity are related to the PCR method used
- PCR methodologies are highly susceptible to contamination. Contamination during processing may result in false positives, particularly in 2-step PCR methods. PCR tests must be conducted in very well managed, clean laboratories.
- "False negatives" are easily caused by the selection of inappropriate host tissue sources for detection of the pathogen in question, incorrect choice of DNA extraction method, or low pathogen prevalence in the population sampled.

DNA-based detection and diagnostic methods have the potential for widespread application in Animal diseases diagnosis. As the technology is already being adopted rapidly in developing countries in Asia, there is an urgent need to address these issues and to develop an action plan for research and training activities that will facilitate more effective utilization.
References:


Molecular methods for diagnosis of hemorrhagic septicemia in cattle and buffaloes

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Introduction

Hemorrhagic septicemia is a major disease of cattle and buffalo occurring as catastrophic epizootics in many Asian and African countries resulting into high mortality and morbidity (AHIS, 1997; Mustafa et al., 1978; Singh et al., 1996). The disease has been also recorded in poultry, rabbit, pig and wild mammals (Carigan et al., 1991; Rosen, 1981). *Pasteurella multocida* is associated with hemorrhagic septicaemia in cattle and buffaloes, pneumatic pasteurellosis in sheep and goats, fowl cholera in poultry, atrophic rhinitis in pigs and snuffles in rabbits (De Alwis, 1996). An annual economic loss in India due to *Pasteurella multocida* is Rs. 225/- millions (Singh, 2008). Fowl cholera has been recognized as an important disease in domestic poultry for more than 200 years that causes devastating economic losses to poultry industry worldwide (Aye et al, 2001).

Haemorrhagic septicaemia is caused by *Pasteurella multocida* type B:2, B:2.5 and B:5 in Asian countries and type E:2 in African countries. *Pasteurella multocida* have five types of capsular serotype i.e. type A, B, D, E and F. *Pasteurella multocida* type A produce cholera in fowl; pneumonia in cattle, sheep, and pig. Capsular type D of *Pasteurella multocida* produces atrophic rhinitis in pig and snuffles in rabbits.

Two typing systems for serotyping of *Pasteurella multocida* isolates are adopted. One for capsular typing by Carter’s IHA system and other somatic typing by the method of Namioka and Murata or Heddleston. Diagnosis of the disease is mainly based on the clinical sign and symptom, post mortem findings. Confirmatory diagnosis is done by the isolation and identification of causative agent. A variety of laboratory diagnostic techniques have been developed over the years for pasteurellosis and used routinely in the laboratory. The organism is identified directly through examination of blood smear from affected animal and can be isolated in suitable culture medium in the laboratory. Various biochemical and serological tests are used for the identification and serotyping of the organism. Rapid slide agglutination test is performed on slide for rapid diagnosis; in which floccular agglutination appear within 30 seconds in the positive cases. Indirect haemagglutination test is carried out for the determination of capsular types of Pasteurella multocida.

With development in biotechnological techniques for the detection of nucleic acid, the identification and characterization of etiological agents has become quick, easy and accurate (Dutta et al., 2005).

1. **PCR based diagnosis and typing**: Numerous studies for diagnosis and characterization of *Pasteurella multocida* have been carried out with variable results. The phenotypic characterization systems by means of morphology, biochemical typing, serotyping etc. are
very much laborious and timeconsuming. Even after capsular and somatic antigen determination, still few isolates react similarly in both the antigens. The PCR based techniques have provided the alternative methods of characterization to overcoming the limitations of phenotyping.

a. *Pasteurella multocida* specific PCR assay: The species specific PCR assay can be applied for detection of *Pasteurella multocida* by using template as either genomic DNA or bacterial colony or by using the direct field samples such as nasal swab, morbid materials like spleen, one marrow, and heart blood. Earlier the PCR needed additional hybridization step for increasing the specificity but later with improved PCR technique it became possible to detect as minimum as 10 organisms per reaction. The *Pasteurella multocida* can identify all subspecies of Pasteurella multocida. The sensitivity and specificity of this PCR offer the most compelling argument for the use of PCR technology in laboratory to investigate the suspected HS cases using the primer set as

KMT1T7- 5’-ATC CGC TAT TTA CCC AGT GG-3’ and KMT1SP6 5’-GCTGTAAAC GAACTC GCCAC-3’ (Townsend et al,1998,) by the amplification of a 460bp fragment of DNA. This technique has reduced the time for diagnosis of the disease and also it is specific than traditional one.

b. HS causing type B specific PCR assay: The PCR amplification can also detect the serotype B specific *Pasteurella multocida* directly HS causing type B specific PCR is 100% specific for type B serotypes of *Pasteurella multocida* isolates. Serotype B cultures with the any combination of somatic antigen are identified by the amplification of a 620 bp fragment with the KT SP61: 5’- ATC CGC TAA CAC ACT CTC- 3’ and KTT72: 5’- AGG CTC GTT TGG ATT ATG AAG- 3’ primers (Townsend et al., 1998).

c. *Pasteurella multocida* type A specific PCR:

Primers for typing of serogroup A strains which causing number of infection in livestock and poultry with several somatic types have been reported to be useful in specific identification of isolates. The primers RGPMA5: 5’- AATGTTTG CGATAG YCC GTTAGA- 3’ and RGPMA6: 5’- ATT TGG CGC CAT ATC ACAGTC- 3’ gives PCR amplicon size of 564 bp which confirms the presence of *Pasteurella multocida* serotype A.

d. Multiplex PCR for *Pasteurella multocida* Capsular typing

Alternative to the conventional capsular serotyping system and used for capsular types determination. The serogroup specific primers used in this assay were designed following identification, sequence determination and analysis of the capsular biosynthetic loci of each capsular group. The multiplex capsular PCR assay is highly specific and its result correlated well with conventional serotyping results with the exception of those for some serogroup F strains (Townsend et al., 1998).

The capsular typing of all the isolates were determined by multiplex PCR using capsular types A,
B, D, E and F specific primers as mentioned below:

1. CAPA- F 5’- 3’TGCCAAAATCGCAGTCAG
2. CAPA- R 5’- 3’TTGCCATCATTTGCACTG
3. CAP B- F 5’- 3’CATTTATCCAGCTCCACC
4. CAP B- R 5’- 3’GCCCGAGAGTTTCAATCC
5. CAP D- F 5’- 3’TTACAAAAGAAAGACTTAGGAGCCC
6. CAP D- R 5’- 3’CATCTACCACACTCAACCATATCAG
7. CAP E- F 5’- 3’TCCGCAGAAAAATTATTGACTC
8. CAP E- R 5’- 3’GCTTGCTTGTATTTTGT
9. CAP F- F 5’- 3’AATCGGAGAAGCGAGAAATCA
10. CAP F- R 5’- 3’TTCGCAGCAATTACTCTG

Sizes of the multiplex PCR amplicons are as follows:

<table>
<thead>
<tr>
<th>Amplicon size</th>
<th>Capsular type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1044 bp</td>
<td>A</td>
</tr>
<tr>
<td>760 bp</td>
<td>B</td>
</tr>
<tr>
<td>657 bp</td>
<td>D</td>
</tr>
<tr>
<td>511 bp</td>
<td>E</td>
</tr>
<tr>
<td>851 bp</td>
<td>F</td>
</tr>
</tbody>
</table>

e. REP- PCR and ERIC- PCR: Recently Repetitive Extragenic Palindromic (REP) and Enterobacterial Repetitive Insertion Consensus (ERIC) PCR have been developed for the characterization of *Pasteurella multocida* isolates. REP elements (33 to 40 base pair repeats) are present in 500- 1000 copies accounting for up to 1% of the genome (Stern et al., 1984) and are present in a wide range of bacteria (Olive and Bean, 1999). As the REP elements are distributed widely across the genome, it produces a multiple banding pattern. ERIC- PCR has been successfully used to differentiate strains of *Pasteurella multocida*. The visual analyses of banding pattern were in range of 100-900 bp. The band patterns provide DNA fingerprints which allows distinction between species and between strains within species.

f. Detection of toxigenic *Pasteurella multocida*: The *Pasteurella multocida* capsular type D strain has been identified as causative agent of atrophic rhinitis in pigs and snuffles in rabbits. The toxA gene of *Pasteurella multocida* encodes the dermanecrotic toxin responsible for atrophic rhinitis. The toxA gene based PCR can be used for direct analysis of toxigenic capsular typing: A multiplex PCR assay is a rapid *Pasteurella multocida* without additional
hybridization. The assay appears to be the most sensitive and expensive electrophoresis equipment, which is effective method for large scale analysis of nasal and generally not available in normal diagnostic tonsillar swabs (Kamp et al., 1996).

2. Restriction endonuclease analysis (REA): Restriction endonucleases cleave the DNA at specific nucleotide sequences and produce a set of DNA fragments which, upon electrophoresis separate into a characteristic banding pattern or fingerprint of the respective genome. Restriction endonuclease analysis has been successfully used as a tool for differentiation of strains in a variety of bacterial infections including that cause by Pasteurella multocida. Several restriction enzymes (HhaI, HpaII, Smal, BglII, PstI, EcoRI) have been taken into consideration for characterization the different isolates of Pasteurella multocida.

3. Ribotyping: Ribotyping in conjunction with REA has been widely used to characterize and differentiate the Pasteurella multocida isolates (Blackall et al., 1995). REA followed by additional hybridization with a labeled DNA probe made easy to read the banding pattern and give the necessary interpretation. The probe may be labeled either by radio active or non radioactive materials. rRNA probe is widely accepted for hybridization and subsequent interpretation (Blackall, 2000).

4. Colony hybridization assay: A colony lift hybridization assay using a commercially available multicolour detection kit was recently developed for rapid and simultaneous detection of toxigenic Pasteurella multocida and Bordetella bronchiseptica (Register et al., 1998). The major advantage of this assay is the ability to screen the suspect colonies in primary isolation plate so, there is no need of pure cultures and it can analyze the large number of samples in a very short period.

5. Filed alternation gel electrophoresis (FAGE):

This technique is also known as ‘Pulsed Field Gel Electrophoresis’ (PAGE) and it is a method of fingerprinting with high specificity and precision. Conventional electrophoresis, which used a constant current that cannot resolve the large fragments generated by rare cutting restriction enzymes. But in PFGE, where the electric field across the gel is changed periodically can effectively separate the large size DNA fragments on size basis. PFGE analysis has consistently shown the greater discrimination in identification of bacterial species than ribotyping but, it has limited application in the typing of Pasteurella multocida isolates (Townsend et al., 1997a). The major drawbacks of this technique are the requirements of highly purified intact DNA and specialized and expensive electrophoresis equipment, which is generally not available in normal diagnostic laboratories (Dutta et al., 2005).

6. Detection of Pasteurella multocida by Real Time PCR: This latest method for detection of Pasteurella multocida in field sample. This highly sensitive and specific test than PM PCR and Multiplex PCR.
References

Advanced Microscopy and its Utility in Animal Disease Diagnosis
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Introduction

Depending on how magnification is produced microscopes can be categorized as light or electron microscope. In light microscopes light waves are used to magnify the specimen. In electron microscope electron beams are used to produce a magnified image of the specimen. Bright field, dark field, phase contrast and fluorescence are the common light microscopy methods used in microbiology.

Bright-field microscopy

It is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample. Bright-field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes and its simplicity makes it a popular technique. The typical appearance of a bright-field microscopy image is a dark sample on a bright background, hence the name.

Dark field microscopy

In darkfield microscopy the condenser is designed to form a hollow cone of light (see illustration below) which is a light opaque disc. This disc blocks the light that enters the objective directly. Only light that is reflected off the specimen enters the objective lens, as opposed to brightfield microscopy that illuminates the sample with a full cone of light. In darkfield microscopy, the objective lens sits in the dark hollow of this cone and light travels around the objective lens, but does not enter the cone shaped area. The entire field of view appears dark when there is no sample on the microscope stage. However, when a sample is placed on the stage it appears bright against a dark background.

It is used for examining live organisms that are either invisible in ordinary light microscope or cannot be stained by standard methods. Only light that is reflected off the specimen enters the objective lens. Because of this the background appears black. This
technique is frequently used to see unstained microorganisms in liquid. Spirochetes are also examined by this method.

**Phase contrast microscopy**

Phase contrast microscopy is normally used to study the internal structures of living microorganisms. In this method the specimen need not be fixed and stained. The principle is based on slight variation in the refractive index. As light rays pass through the specimen, their velocity may be altered by differences in the thickness and physical properties of various parts of the specimen. Light rays passing through the specimen are diffracted differently and travel different paths to reach the eye. These phase differences are seen through the microscope as different degrees of brightness. Details of the specimen also become more sharply defined. The internal details appear as degrees of brightness against a dark background. A phase contrast microscope uses a special condenser with annular ring shaped diaphragm which allows a ring of light to pass through the condenser, thus focusing light on the specimen and on a ring shaped diffractive plate in the objective lens. The diffracted and undiffracted rays are then brought back in to phase with each other to produce image.
Fluorescence microscopy

Fluorescence is the emission of a higher wave length by some substances after absorbing light of a particular wave length. This phenomenon is used in fluorescence microscope. In this microscope instead of normal light blue light is used. The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The specimen is stained by fluorescent dyes like 4',6-diamidino-2-phenylindole (DAPI) Tetramethylrhodamine Isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC). These dyes fluoresce when exposed to blue light. DAPI produces blue fluorescence, Rhodamine produces red fluorescence and FITC produces green fluorescence. This microscopy is normally used in immunology to study antigen antibody reaction like Immunofluorescence (IF) or Fluorescent antibody test (FAT)
Electron Microscopy

The electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail. They are large, expensive pieces of equipment, generally standing alone in a small, specially designed room and requiring trained personnel to operate the

There are two types of Electron microscopes namely, Transmission electron microscope (TEM) and Scanning electron microscope (SEM).

Transmission Electron Microscopy (TEM)

The original form of electron microscopy, Transmission electron microscopy (TEM) involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses. The electron beam that has been partially transmitted through the very thin (and so semitransparent for electrons) specimen carries information about the structure of the specimen. The spatial variation in this information (the "image") is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a monitor or computer.

Transmission electron microscopes produce two-dimensional, black and white images.

Scanning Electron Microscopy (SEM)

In scanning electron microscope a narrow beam of electrons moves over the surface of the specimen. This leads to release secondary electrons and other types of radiation from the specimen surface. The intensity secondary electrons vary based on the shape and the chemical composition of the specimen. A detector produces signals by collecting the secondary electrons. These signals are used to produce an image on cathode ray tube.

SEM images are therefore considered to provide us with 3D, topographical information about the sample surface but will still always be only in black and white. The resolving power of scanning electron microscope is lesser than that of transmission electron microscope.
CCHF: A current threat and its diagnosis  
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Introduction:

Crimean Congo hemorrhagic fever (CCHF) has recently been in the news with first ever reports of its outbreak in India from the village of Kolat which is 30 Kms southwest of Ahmedabad in Gujarat. A 30 year old woman and a doctor and nurse treating her in Ahmedabad succumbed to this illness creating panic in the local population and the country as well. General public including the medical fraternity was not fully aware of this disease, thus a fear of unknown was spread initially.

History:

CCHF is not new disease as Congo hemorrhagic fever (CCHF) was first described in the 12th century as a hemorrhagic syndrome in present day Tajikistan (Ergönül, 2006). During that era, it was speculated that the disease’s transmission was associated to louse or ticks that normally parasite black birds. However, in the modern era, the first outbreak of the CCHF was reported in 1944-1945 in the Crimea region when more than 200 cases occurred and at that time the disease was called Crimean Hemorrhagic Fever. Ten years later and specifically in 1956, the virus was isolated from a febrile patient in Belgian Congo and this isolate was noted to have the same antigenic structure with the Crimean strains. For this reason, the virus was called Crimean Congo Hemorrhagic Fever (Simpson et al., 1967; Ergönül, 2006). Nowadays, outbreaks of CCHF have been documented in Africa, the Middle East, Eastern Europe, and Western Asia (Hoogstraal, 1979).

Etiology:

CCHF is a severe hemorrhagic fever with a case fatality rate of up to 50%. The virus that causes the disease is a tick-borne virus belonging to the family Bunyaviridae, genus Nairovirus (Martin et al., 1985). Like other nairoviruses, CCHF virus is an enveloped single stranded negative-sense RNA virus.

Transmission:

The virus is transmitted to humans through the bite of infected ticks or by direct contact with viremic animals or humans (Ergönül, 2006). Infected humans can spread the disease via close contacts which may result in community outbreaks and nosocomial infections (Jamil et al., 2005). The potential human to human transmission along with the high mortality rates, the fears that the virus could be used as a bioterrorism agent and the increase of the incidence and geographic range of the Crimean Congo hemorrhagic fever make the virus an important human pathogen.

Epidemiology:
Like other tick borne zoonotic agents, CCHF virus circulates in nature in an enzootic tick-vertebrate-tick cycle. Humans are being infected mainly through direct contact with blood or tissues from infected livestock or through tick bites. CCHF virus is transmitted by Hyalomma genus ticks and in particular by Hyalomma marginatum marginatum. Ticks of the genus Hyalomma serve indeed as vectors and reservoir of the CCHF virus and the geographic distribution of the disease coincide with the global distribution of Hyalomma ticks (Vorou et al., 2007).

The virus is reported in over than 30 countries in Africa (Democratic Republic of Congo, Uganda, Mauritania, Nigeria, S. Africa, Senegal, etc), Southeast Europe (Russia, Bulgaria, Kosovo, Turkey, Greece, etc), the Middle East (Iraq, Iran, Saudi Arabia, Oman) and Asia (China, Kazakhstan, Tajikistan, Uzbekistan, Pakistan) (Morikawa et al., 2007; Koutis, 2007). In this regard, the geographical distribution of CCHF virus is the greatest among all tick-borne viruses. CCHF virus has been isolated from adult Hyalomma genus ticks in the ‘60s and transovarial and transstadial transmissions have been already suggested since viral isolates have been also found in field collected eggs and unfed immature stages of H. marginatum, respectively (Watts et al., 1988). CCHF virus has been also isolated in laboratory from other tick genera eg. Rhipicephalus, Ornithoros, Boophilus, Dermacentor and Ixodes spp.

Although, the virus persists in ticks, vertebrates are needed to provide blood meals for the ticks and a variety of livestock can become infected with the CCHF virus. In fact, numerous domestic and wild vertebrates have been reported to present antibody response and/or viremia (Vorou et al., 2007). This livestock includes cattle, goats, sheep, horses, pigs hares, ostriches, camels, donkeys, mice and domestic dogs. In contrast to human infections the livestock’s infections generally result in unapparent or subclinical disease (Whitehouse, 2004). However, the infected livestock during the viremic phase is dangerous for the disease transmission in humans. In this regard, domestic ruminant animals such as cattle, sheep and goats will present viremia for one week after becoming infected (Athar et al., 2003). Although it has been shown that the majority of birds is resistant to infection (Whitehouse, 2004) the potential role of migratory birds in the disease dissemination could not be ignored.

Clinical features and pathogenesis:

CCHFV infections are asymptomatic in animals and birds are thought to be resistant. Humans are the main victims to this disease. The course of the disease can be divided into four phases- incubation, prehemorrhagic, hemorrhagic and convalescence (Hoogstraal, 1979). The incubation period depends on the mode of infection. Infections acquired via tick bites usually become Infections acquired via tick bites usually become apparent after 1-3 days (Whitehouse, 2004). Exposure to blood or tissues results in longer incubation period. In Indian cases, the incubation period ranged from 7-12 days through the later mode (Patel et al., 2011).

Pre-hemorrhagic symptoms are non specific and include fever, chills, severe headache, dizziness, photophobia, myalgia and arthralgia. This phase may last for 1-7 days (Saijo and Morikawa, 2010). The hemorrhagic phase develops suddenly lasting for 2-3 days. A petechial rash may be the first symptom both on the internal mucosal surfaces such as mouth and throat
and on the skin. They are followed by ecchymoses and other hemorrhagic phenomenon such as hematemesis, melena, epistaxis, hematuria, and hemoptysis. Hepatomegaly and splenomegaly can be seen in some patients (Appannavar and Mishra, 2011). There may be rapid kidney deterioration. Death may occur in many cases. The mortality rate is 30% and the case fatality rate is up to 40% (WHO, 2013). In Indian cases death occurred due to cardio respiratory arrest, multiorgan failure and disseminated intravascular coagulation (DIC) and gastrointestinal bleeding in one case. In patients who survive recovery begins 10-20 days after onset of illness. Recovery may take up to a year (Patel et al., 2011).

No clear pathogenesis is described for CCHF. Endothelial damage is a common feature leading to capillary fragility and accounts for the characteristic rash and contributes to hemostatic failure by stimulating platelet aggregation and degranulation (Whitehouse, 2004). Thrombocytopenia occurs and dysregulation of the coagulation cascade leads to DIC. Proinflammatory cytokines are important in pathogenesis and the IL-6 and TNF-α level are significantly higher in fatal CCHF (Ergönül, 2006). A study shows that viral genome can be detected from saliva and urine of infected patient. In CCHF there is increased serum ferritin level which can be used as a marker for disease activity and prognosis (Barut et al., 2010).

Public health importance:

Humans readily succumb to CCHFV infection. However domestic animals are either refractory or undergo mild infection with transient viremia sometimes, but they act as a main source of infection for humans (Prajapati, 2011). Persons living in close contact with animals are at the high risk of getting CCHF. Veterinarians and farmers may castrate, dehorn, attach ear tags and immunize young animals and thus expose themselves to the virus infected blood. They may have broken skin or scratch on the skin through which they may get infected. Consumption of unboiled or uncooked meat and milk of infected animal may be a potential source of infection. There is lack of evidence of disease in urban consumers of meat but the infected animal may reach to abattoir to pose a potential threat for workers and meat consumers. Exposure to aerosols while working with infected animals and in the hospital setting are the potential hazards. The population in the infected or infection prone area should be aware of the potential routes of infection and the safety measures to be taken to avoid the infection. CCHFV may be used for bioterrorism or as a biowarfare agent. Due to this it is included in CDC/NIAID Category C Pathogen (CFSPH, 2007).

Diagnosis:

To save the patient and to prevent the further transmission of disease, early diagnosis is essential. The key indicators to suspect CCHF infection includes compatible clinical manifestations like fever and bleeding, history of tick bite, travel to endemic area and contact with infected cases and tick infested animals. The disease should be differentiated from the other VHFs, malaria, dengue, yellow fever, Kyasanur forest disease, rickettsiosis and leptospirosis (Ergonul, 2013). The knowledge of ecology and endemicity of CCHFV should be kept in mind to proceed with further diagnosis. The methods of diagnosis include virus isolation, immunological assays like ELISA and molecular diagnostic methods like reverse transcription- polymerase chain reaction (RT-PCR) (CFSPH, 2007).
CCHFV can be isolated from the blood, plasma and tissue of infected patient for the diagnosis. Virus isolation should be performed in a high bio-containment laboratory (CFSPH, 2007). A variety of cell lines including vero, BHK-21, LLC-MK2 and SW-13 can be used for virus culture. Cell culture can detect only high virus concentration and only useful during first five days of disease. Generally the virus produces no or little cytopathic effects so it can be identified by immunofluorescence assay using specific monoclonal antibodies (Whitehouse, 2004). The traditional method of animal inoculation of newborn mice is more sensitive than cell culture and also detects the virus for longer period. The virus isolation by cell culture is of limited value because it needs a biosafety level-4 laboratory (BSL-4) which is unavailable in most of the endemic areas (Ergonul, 2013). In the first few days of illness usually the patients do not develop a measurable antibody response so the serological tests are useful in the second week of illness. There are various serological tests available for detection of CCHFV but these tests are of limited use in fatal cases as patients generally die without developing antibodies. The conventional serological test for CCHFV like Complement fixation, hemagglutination inhibition and immunodiffusion suffered lack of sensitivity and reproducibility (Hoogstraal, 1979). This problem was solved by Indirect Immunofluorescence assay (IFA) and Enzyme-linked immunosorbent assay (ELISA) for the detection of IgM and IgG antibodies. Both IgM and IgG can be detected up to 7-9 days of illness by indirect FIA (Donets, 1982). ELISA has replaced the conventional methods for antibody detection. IgM can be detected up to 4 months and IgG persist for 5 years post-infection but its level decrease (Hoogstraal, 1979).

Molecular diagnostic assays such as reverse transcriptase polymerase chain reaction now serve as the front-line tool in the diagnosis of CCHF. PCR based methods are sensitive, specific, rapid and can be done without the need to culture the virus which requires BSL-4 facility (Hoogstraal, 1979). Molecular epidemiology can also be performed by this technique. A further improvement on the conventional RT-PCR assay has been the advent of automated real-time PCR based assays. The real-time PCR is more advantageous over conventional RT-PCR methods with respect to sensitivity, specificity and time taken for detection. Real-time PCR also offers less contamination rate. There are various detection chemistries available for the real time PCR like SYBR green, TaqMan and molecular beacon etc. There are several real-time RT-PCR assays reported till now for CCHFV detection. Some important assays developed for CCHFV detection are SYBR green, TaqMan and TaqMan-Minor Groove Binding (MGB) probe based assays (Wölfel et al., 2007).

**Treatment:**

In case of CCHF, treatment is mainly supportive. It includes careful management of fluid and electrolyte balance depending upon the severity of illness. Currently there is no specific antiviral therapy for CCHF approved by United States Food and Drug Administration (FDA) for human use. Ribavarin, a guanosine analogue is found effective against CCHFV (Bajpai and Nadkar, 2011). CCHFV is susceptible to ribavirin in vitro (Watts et al., 1989). According to some reports oral and intravenous ribavirin is effective for treating CCHFV infections (Christova et al., 2009). In India one case recovered by the oral administration of ribavirin and discharged after ten days. Passive immunotherapy using
specific immunoglobulin CCHF-Venin is also found beneficial in CCHFV treatment (Khan et al., 2011).

Prevention and control:

The prevention and control should be both at community level as well as in nosocomial set up. Minimizing human contact with suspected livestock and reducing the tick burden in the animals are the primary and most important preventive measures (WHO, 2013). Animals should be carefully monitored for tick infestation and treated by appropriate acaricidal agents particularly before slaughter or export. Wearing fully covered clothes and use of tick repellent is recommended to prevent tick attachment on the body surface. The unpasteurized milk and uncooked meat should not be taken. Human- to human infection mainly occurs in the nosocomial setup by the contact of infected blood or tissue. So use of protective clothing, gloves, goggles and face-masks reduces the chances of exposure. Safe burial practices with proper use of disinfectants should be followed. Veterinarians, research workers, slaughter house workers and medical professionals should take utmost care to reduce the contact with suspected material. They should take the prophylactic treatment after high risk exposure. Laboratory and research workers are advised to follow stringent biosafety precautions during handling the pathogen and the work should be carried out under BSL-4 facilities. Virus can be inactivated by using 1% hypochlorite and 2% glutaraldehyde. Heating at 56°C for 30 minutes also destroy the virus (CFSPH, 2007).

Vaccination:

Vaccine against CCHF is not available in most of the countries. However a formalin inactivated vaccine derived from suckling mouse brain has been used in Bulgaria and former Soviet Union (Papa et al., 2011). There is no vaccine available for animal use (WHO, 2013).

Conclusion:

CCHF is an emerging disease in India. Its zoonotic potential and fatality have created a great havoc in the general population as well as in health care community. Since animals play an important role in the transmission of virus to human, the persons associated with animals are at the great risk of CCHFV infection. This disease is new to India so people should be aware of the various aspects of this fatal disease mainly its modes of transmission, clinical manifestations, public health importance and preventive measures.

References:


Recent Approaches for Diagnosis of Foot-And-Mouth Disease

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease affecting *artiodactylae*, mostly cattle, swine, sheep, goats, and many species of wild ungulates. FMD affects extensive areas worldwide and is included in the list of diseases notifiable to the World Organization for Animal Health. It is recognized as a significant epidemic disease threatening the cattle industry since the sixteenth century and till date it is a major global animal health problem. FMD generally involves mortality rates below 5%, but even so it is considered the most important disease of farm animals since it causes huge losses in terms of livestock productivity and trade. Although FMDV rarely causes death in adult animals, the virus can cause severe lesion in the myocardium of young animals, leading to high mortality rates. The main constraints in controlling this disease and why it is considered as the most dreaded viral disease are its high contagiousness, wide geographical distribution, broad host range, ability to establish carrier status, antigenic diversity leading to poor cross-immunity, and relatively short duration of immunity. Poor surveillance and diagnostic facilities as well as inadequate control programs are major problems in control of this disease in the country. FMD is still a leading cause of loss of livestock economy in India. Outbreaks are still being reported from time to time around the year. Besides causing direct losses to livestock economy it also causes indirect losses in terms of severe trade restrictions, impact which may be higher than direct losses.

The etiological agent, foot-and-mouth disease virus (FMDV) is classified within the genus *Aphthovirus* in the family *Picornaviridae*. The virus exists in the form of seven serologically and genetically distinguishable types, namely, O, A, C, Asia1, SAT1, SAT2, and SAT3, but a large number of subtypes have evolved within each serotype. Serotype O and A reported in France by Valee and Caree and in 1926, Waldmann and Trautwein reported serotype C. Serotypes SAT1, SAT2, and SAT3 of FMDV was observed in sample collected from the FMD outbreak in South Africa. The seventh serotype, Asia 1, was reported from Pakistan. FMDV is a single stranded (ss) positive sense RNA virus with the whole virus particles having sedimentation coefficient of 146S and genome of 8.5 Kb size.

Although, the disease has been controlled successfully in many parts of the world by regular vaccination of susceptible animals and slaughtering of infected animals, no country has been considered safe, because of the highly contagious nature and rapid spread of the infection. For the effective control of the disease, outbreaks should be detected at an early stage and persistent infections should also be recognized to prevent further transmittance. These can be achieved when vaccination is regular and effective and when diagnostic tools available are specific and sensitive and at the same time rapid. Lots of work has been carried out to develop and validate diagnostic tests in regard to this disease. Conventional techniques
such as complement fixation test (CFT), serum neutralization tests (SNT) and enzyme-linked immunosorbent assay (ELISA) are still in use for the routine detection of FMDV in clinical samples. Sandwich ELISA is being carried out for the detection of specific FMDV antigens in epithelial tissue suspensions which is usually accompanied by concurrent cell culture isolation and the application of ELISA to any samples showing a cytopathogenic effect. Virus isolation in primary cultures is laborious, expensive, and requires days/weeks (cell passages) before the results are obtained. However, with the introduction of molecular techniques in the field of diagnosis, several techniques based on viral genome detection such as hybridization using DNA probes and the advent of Polymerase Chain Reaction (PCR) technique in the recent past have led to development of several reverse transcription PCR (RT-PCR) procedures for specific detection of FMDV RNA. Because of the reported sensitivity and specificity, RT-PCR has been evaluated as a diagnostic tool for FMDV detection in parallel with ELISA and virus isolation. Another form of PCR, multiplex PCR (mPCR), has also been evaluated for differentiating FMDV serotypes as well as for differential diagnosis with other vesicular diseases such as Vesicular Stomatitis, Swine Vesicular Disease. The most recent development in the field of diagnosis by nucleic acid detection is the use of thermal cyclers capable of measuring fluorogenic PCR amplification in real-time have become available, making precise quantitation of nucleic acids possible over a wide concentration range. The fluorogenic RTPCR provides relatively fast result, enables a quantitative assessment to be made of virus amount, and can handle more samples and/or replicates of samples in a single assay than the conventional RT-PCR procedure. Therefore it is seen as a valuable tool to complement the routine diagnosis procedure for FMD virus diagnosis.

Currently, the FMD diagnosis in our country (India) is being carried out using techniques developed at Project Directorate on FMD (PD FMD), Mukteswar which includes Virus isolation (VI), Sandwich-ELISA (S-ELISA), Liquid-Phase Blocking ELISA (LPBE), Multiplex-PCR (mPCR) and recently DIVA test which is an indirect ELISA for detection of antibody against nonstructural proteins. LPBE is being used for the detection of antibody titers against the FMD vaccinated animals. The S-ELISA is being used for the antigen detection using the material from the lesions but because of its low sensitivity currently mPCR is being used. The real-time PCR-based detection method is used in many reference laboratories in the world for the purpose.

**Diagnostic approaches**

The accurate diagnosis of infection with FMDV is of prime most importance for both control and eradication campaigns in FMD endemic areas and as a supportive measure to the stamping out policy in FMD-free areas. The history of research and diagnosis in foot-and-mouth disease falls into several distinct areas. The search for experimental laboratory animals, producing the disease culminated in the demonstration by Waldmann and Pape, the susceptibility of the guinea pig, and the suckling mouse by Skinner in 1951. Early work by Hecke and the Maitlands in the early 1930s, followed by the crucial demonstration by Frenkel in 1947 that large amounts of the virus could be produced in surviving tongue epithelium, formed the basis for the vaccination programmes initiated in Europe in the 1950s. The subsequent development of cell lines has brought a remarkable degree of sophistication
to the study of virus growth. The recognition of more than one serotype has led to the development of various techniques for serotyping of the virus.

**Complement Fixation Test (CFT)**

In 1929, Ciucu was first to use CFT for typing antiserum and FMDV of guinea pig origin. Later, virus of bovine origin was successfully typed by CFT using guinea pig antiserum. Since then CFT has been used extensively for distinguishing different strains of FMDV. Subsequently, a modification of CFT, micro-CFT was developed; in which 96 well microtiter plates were used instead of tubes. In the years 1964-1965 CFT (tube test) was used to replace the virus type identification by guinea pig cross-protection test. Subsequently, the micro-CFT was adopted for this purpose. Although CFT was a fast method it needed high virus load and results were sometimes affected by pro-and anticomplementary activities of the test sample.

**Virus Isolation**

Primary cell culture of bovine, ovine and porcine origin has exhibited susceptibility to FMDV from infected tissues. However, the most sensitive culture system for virus isolation is primary bovine thyroid cells but cryopreservation of bovine thyroid cells directly after trypsinization results in the loss of susceptibility to FMDV. Some stable cell lines, like IBRS-2, MVPK-1 clone 7, LFBK cell line and BHK-21 are also susceptible to FMDV and so are most desirable for diagnostic system but these are less sensitive than primary cells for detecting low amount of infectivity. The availability of cell culture techniques and the realization that FMDV can be grown in in vitro cultured cells made possible the adaption of neutralization test for routine type identification of FMDV isolates and were found to be more specific than CFT. In particular, virus isolation requires a laboratory cell culture facility, which can be difficult and expensive to maintain, besides requiring 4 to 6 days for test completion. Subsequently microneutralization test (MNT) was used for the assessment of antigenic variation in field strains, as it correlated well with cattle protection test. But it was observed that, minimal heterotypic contamination in the sample could interfere with precise type identification of the virus; on the other hand VN test depends on tissue culture and is more prone to variability than ELISA and also require biocontainment facilities.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA came into use as diagnostic methods for many infectious diseases around the year 1975; till then it has been used as one of the most accepted serological techniques. The first report of use of an indirect ELISA to screen cattle for antibodies against FMDV was that of Abu Elzein and Crowther. Subsequently, a sandwich ELISA using convalescent bovine immunoglobulin (Igs) as capture and anti-146S guinea pig sera as tracing sera was found suitable for detection and quantification of FMD virus in infected tissue culture fluid and epithelial tissue samples. The use of anti-146S rabbit immunoglobulin in place of convalescent bovine immunoglobulin as capture antibody increased the sensitivity of sandwich ELISA. At the FAO/WRL for FMD, the preferred procedure for the detection of FMDV antigen and identification of viral serotypes is ELISA. The results can be obtained
within 3-4 hours after sample is received by the laboratory; a negative sample is confirmed by inoculation of sample into sensitive cultures followed by confirmation of the virus serotype by ELISA. Indirect ELISA was initially used for detection of FMDV antigen in infected cell culture fluid, mice carcass, and cattle tongue as well as antibodies in sera samples. Later a sandwich ELISA was used for subtype analysis of FMDV isolates. Subsequently, a sandwich ELISA was developed for detection and typing of FMDV directly from field materials. Although ELISA is far finer to CFT, a large number of samples failed to give positive results and such negative sample has to be confirmed by inoculation of sample into sensitive cultures followed by confirmation of the virus serotype by ELISA taking 4 more days, a time frame compatible with the need to rapidly detect disease and initiate and appropriate disease control strategy.

Reverse Transcription-Polymerase Chain Reaction (RTPCR)

Polymerase chain reaction was the most widely use nucleic-acid-based diagnostic techniques since its invention. With the development of RT-PCR to amplify RNA targets many workers have assessed the usefulness of it as a reliable tool for FMD diagnosis and in parallel with conventional assays. A particularly high sensitivity was reported by RT-PCR ELISA. A specific RT-PCR was developed and validated for the detection of the polymerase gene (3D) of FMD with an analytical sensitivity equal to 1000 times higher than that of a single passage virus isolation. In a study to compare the sensitivity of assays for the diagnosis of FMD, a cell suspension plaque test on BHK21-CT cells and a reverse transcription nested PCR (RT-nPCR) were used to examine nasal swabs and probang samples obtained from FMDV infected cattle, it has been observed that examination of nasal swab revealed a higher number of infected animals using RTnPCR than by the used the plaque tests and for probing samples both test gave approximately equivalent result. PCR products generated have currently only been analyzed by gel electrophoresis exposing to the chance of post-PCR contamination. However PCR offers potential advantages over other conventional tests. The risk of false negative associated with poor sample handling is limited. Because virus, if present, would be inactivated by RNA extraction, it would be acceptable to use lower level biosafety. Detection of all seven serotypes of the virus with each of the serotype specific primers in selected RTPCR protocols at OIE/FAO World Reference Laboratory for FMD(WRL), Pirbright demonstrated suitable specificity and detected cell culture passage isolate with some success but were not adequate for the serotyping of suspension prepared from clinical samples of epithelium. RT-PCR though has paved the way to more sensitive and rapid test in the field of molecular diagnosis, serotyping of FMDV has been of difficult and this has been solved with the advent of multiplex PCR (mPCR).

Multiplex Polymerase Chain Reaction (mPCR)

To overcome the inherent disadvantage of cost and to improve the diagnostic capacity of the test, multiplex PCR, a variant of the test in which more than one target sequence is amplified using more than one pair of primers, has been developed. This modified technique was originally developed to detect distinct/genetic alterations in large regions of human genome. Afterwards, mPCR was used for the detection and differentiation of multiple
pathogens/different strains of the same pathogen. It has additional advantages such as cost effectiveness and rapidity. There are reports on the use of mPCR for differential diagnosis of FMDV serotypes. To date, these mPCR assays are limited to partial serotyping of FMDV because no existing multiplex PCR assay offer complete coverage for all seven serotypes. Multiplex PCR for differential diagnosis of various vesicular diseases like Vesicular Stomatitis, Swine Vesicular Disease, Vesicular Exanthema, and FMDV have also been described. Recently, mPCR for differentiation of Indian FMDV serotypes has been developed and evaluated on tongue epithelium and cell culture samples.

**Real-Time Polymerase Chain Reaction**

The use of polymerase chain reaction (PCR) in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origin and it has become an essential tool in the research laboratory. The potential of this format to provide sensitive, specific, and swift detection and quantification of viral RNAs has made it an indispensible tool for state-of-the-art diagnostics of important human and animal viral pathogens. Real-time PCR has engendered wider acceptance of PCR due to its improved rapidity and sensitivity overcoming poor precision, low sensitivity, low resolution, absence of automation, only size-based discrimination, absence of expression of results in numbers, poor quantitative performance (Ethidium bromide for staining is not very quantitative), and post-PCR processing, rendering the conventional PCR not very suitable for accurate diagnosis. There are currently five main chemistries used for the detection of PCR product during real-time PCR. These are the DNA-binding fluorophores, the 5` endonuclease, adjacent linear and hairpin oligoprobes, and the self-fluorescing amplicons. This approach is a highly sensitive technique enabling simultaneous amplification and quantification of specific nucleic acid sequences. In addition to enhanced sensitivity, the benefits of real-time PCR assays over conventional endpoint detection methods include their large dynamic range, a reduced risk of cross-contamination, an ability to be scaled up for high-throughput applications and the potential for accurate target quantification. Real-time PCR assays recommended by the World Organization for animal health (OIE) for detection of FMDV incorporate universal primers and fluorescent-labeled probes that recognized conserved region within the 5` UTR or conserved gene regions within the RNA-dependent RNA polymerase gene (3Dpol). Recently, TaqMan technology has combined the 5` nuclease activity of the Taq DNA polymerase and forster resonance energy transfer to detect and quantify amplification product in a closed tube format. Using this technology real-time PCR has been developed to detect the nucleic acid. This is most sensitive and rapid method to detect the nucleic acid. The viral RNA can be consistently detected over a seven log range, the lowest of which corresponded to as few as 10–100 RNA per volume tested. The test can be performed in 2 hours or less on a portable instrument and sample can be held at ambient temperatures. In addition to the widely exploited 5`nuclease (TaqMan) system using dual labeled probes and modified MGB probes, assays have also been developed using other rRT-PCR formats such as those using hybridization probes and PriProET.

The development of multicolour real-time PCR cyclers and “ready-to-use” commercial multiplex real-time PCR kits has also made it possible to combine several assays
within a single tube. Major advantages of multiplexing include a reduced sample requirement, which is especially important when sample material is scarce, and the ability to combine assays with an internal control system. However, it is important to optimize these assays in order to limit competitive interactions that may significantly impact upon assay sensitivity. The combined properties of high sensitivity and specificity, low contamination risk, and speed have made real-time PCR technology a highly attractive alternative to tissue culture or immuneassay based methods for diagnosing many infectious diseases.

**Recombinant Antigen-Based Diagnosis**

The 3AB protein of FMDV was expressed in *E. coli* or in *P. pastoris* and has been used for the diagnosis of FMD infection in cattle. Similarly, 3ABC proteins expressed in heterologous systems were used in ELISA (3ABC ELISA) for serodiagnosis of FMD. Further, four serotypes of FMDV structural proteins expressed in *P. pastoris* and its potential utility either as immunogen or antigen has been successfully assessed in animal model. A recombinant FMDV polyprotein (P1) with 3C expressed in insect cells was evaluated for detecting antibodies to FMDV serotype Asia 1 in ELISA and has the potential to replace the liquid phase blocking (LPB)-ELISA using an inactivated FMDV antigen as a simple and robust serological tool for screening antibodies to FMDV serotype Asia 1.

**DIVA-Based Companion Diagnostic Approach**

The ability to identify and selectively delete genes from a pathogen has allowed the development of “marker vaccines” that, combined with suitable diagnostic assays, allow differentiating infected from vaccinated animals (DIVA) by differentiation of antibody responses induced by the vaccine (no antibodies generated to deleted genes) from those induced during infection with the wild-type virus. A number of antigenic non-structural proteins (NSPs) of FMD were identified and out of which 3ABC gene appears to be the most reliable marker of FMD virus replication. The deletion of NSP (3ABC) gene has been used for enabling DIVA approach for FMD (Cedivac-FMD inactivated vaccine). For detection of NSP antibodies, the Ceditest FMD-NS ELISA is commercially available. Indirect ELISA test for the detection of antibodies against nonstructural proteins will play an essential role in the serological survey of livestock herds in future post outbreak situations.

**Microarray-Based Diagnosis**

DNA microarrays are becoming increasingly useful for the analysis of gene expression and single nucleotide polymorphisms (SNPs). The application to discriminate among variants of FMDV is added to a number of microarray procedures used in virology to analyze multiple viral pathogens that belong to different virus families, to detect specific viruses or to define genetic variations undergone by viruses. The distinction among mutants of the same virus is becoming increasingly necessary in view of the extensive variation among representatives of most virus groups, the quasispecies population structure of RNA viruses and some DNA viruses, and the increasing recognition that one or a limited number of mutations in a viral genome can have a profound effect in its biological behavior.
**Biosensors for Detection of FMDV**

An immunobiosensor using a piezo electric (PZ) crystal was developed and standardized for foot- and- mouth disease (FMD) diagnosis and virus typing [213]. Allosteric biosensors allow detection of antibodies against different viruses by accommodating peptide sequences from surface viral proteins, acting as antibody receptors, into permissive sites of allosterically responsive recombinant β-galactosidases. Among the advantages of such biosensors as diagnostic tools is the homogeneous nature of the assay, the short time required for the enzymatic reaction and antibody detection, and the potential for handling large number of samples and for automatic processing, as shown for human immunodeficiency virus. In the serological diagnosis of infectious diseases, the use of allosteric biosensors, namely, hybrid enzymes that respond enzymatically to antibodies directed to foreign peptides displayed on the enzyme surface, is highly promising.

**Nucleic-Acid-Based Diagnostic Methods**

FMDV serotypes A, O, and C was possible, using cDNA probes from individual serotypes that corresponded to structural protein VP1, where thirteen complementary DNA (cDNA) probes labelled with 32P were used to detect the presence of foot-and-mouth disease virus (FMDV) enabled the detection of 1 pg of viral-RNA, or 1 virus copy per cell. A nucleic acid sequence-based amplification (NASBA) assay for the detection of foot-and-mouth disease virus (FMDV) was developed. Two detection methods, NASBA electro chemiluminescence (NASBA-ECL) and a newly developed NASBA-enzymelinked oligonucleotide capture (NASBA-EOC), were evaluated and compared with other laboratory-based methods, data analysis support the use of NASBA as a rapid and sensitive diagnostic method for the detection and surveillance of FMDV.

**Phage Display-Based Diagnosis**

Due to the high antigenic variability of FMDV, it is important to undertake mutation analysis under immunological pressure. To study the bovine antibody response at a molecular level, phage display technology was used to produce bovine anti-FMDV Fabs where CH1-VH chains with FMDV specific binding was isolated after selection from a library made from vaccinated cattle. Recently, screening a phage displayed random 12-peptide library, it was found that positive phages displaying the consensus motif ETTXLE (X is any amino acid (aa)), which is highly homologous to 6ETTLLE11 at the N-terminus of the VP2 protein (structural protein) of the FMDV, a minimal epitopic region require to bind a monoclonal antibody of serotype independent FMDV (MAb 4B2) and thus can be used as a universal diagnostic candidate against.
**Pen Side Diagnostic Approach**

Routine diagnosis of FMD is made at several laboratories by the combined use of enzyme-linked immunosorbent assay (ELISA), virus isolation techniques, supplemented by reverse transcriptase PCR (RT-PCR), and so forth, which has been already discussed. However, most of these diagnostic methods require the availability of a dedicated laboratory facility, highly trained laboratory personnel, stable reagents, multistep sample handling or preparation, and management of the logistical considerations associated with sample collection and transport is also required. A rapid and easy-to-perform test, which would allow for on-site diagnosis to be made in the case of a suspected disease outbreak, would circumvent problems associated with the transportation of samples to the laboratory and would be especially useful for a faster diagnosis in areas where the disease is endemic. Availability of “point of care” or “pen-side” diagnostic tests would have the advantage of rapid, user friendly, correct identification of a particular strain and economically feasible diagnosis of FMD in field condition. The LFA is an appropriate technology on which to base a rapid assay. The technique permits rapid diagnosis, allowing time for the early implementation of control measures to reduce the possibility of spread of FMD. The LFA has been developed widely to support clinical diagnosis of different diseases, including FMD. A rapid lateral-flow assay (LFA) based on FMDV antigen detection, which is easy to use and can be utilized on the farm to reduce the time required for transport and laboratory diagnosis. The detection of FMDV antigens by direct application of vesicular fluids and epithelial suspensions from animals of an infected farm may reduce the chances of diagnostic error arising from nonspecific reactions.

An alternative molecular technique called Loop Mediated Isothermal Amplification (LAMP) was developed for detection of viruses, where target DNA or RNA specifically amplify using four specific primers under isothermal conditions. A new version LAMP called Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) was developed for rapid, specific and sensitive detection of viruses including FMDV in laboratory and in field condition.

**Reference:**


Importance of Hematology in Animal Disease Diagnosis

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In hematology, CBC (complete blood count) is a medical analysis for assessing the content of hemoglobin in the red blood, the number of red blood cells, a color indicator, the number of white blood cells, platelets. Clinical analysis of blood allows us to consider leukocyte counts and erythrocyte sedimentation rate (ESR).

With this analysis we can identify anemia, inflammatory processes, the state of the vascular wall, a suspicion of helminthic infestations and suspicion of malignant processes in the body. CBC is widely used in radiobiology in the diagnosis and treatment of radiation sickness.

Blood parameters

Currently, most of the indicators are carried out on automated hematology analyzers that determine 5 to 24 parameters simultaneously. Of these, the main ones being the number of leukocytes, the concentration of hemoglobin, hematocrit, number of erythrocytes, mean erythrocyte volume, the average concentration of hemoglobin, the average content of hemoglobin, the half-size distribution of red blood cells, platelet count, mean platelet volume etc.

- **WBC** (White blood cells) - The absolute content of white blood cells (normal 4.5-11 X 10^9 cells / L). White blood cells are responsible for recognition and neutralization of alien components of the immune defence against viruses and bacteria and are also involved in the removal of dead cells of the body.
- **RBC** (Red blood cells) - The absolute content of red blood cells (normal 4.3-5.7 X 10^12 cells / L). Red blood cells contain haemoglobin that transports oxygen and carbon dioxide.
- **HGB** (Hb, hemoglobin) - The concentration of hemoglobin in whole blood (normal 13.2-17.3 g %). Measured in moles or grams per liter or per deciliter.
- **HCT** (hematocrit) - Hematocrit (normal 0.39-0.49), part of the total blood volume, attributable to blood cells. Blood by 40-45% consists of formed elements (erythrocytes, platelets, white blood cells) and 60-65% of the plasma. Hematocrit is the ratio of corpuscles to plasma. It is believed that the hematocrit reflects the ratio of red blood cells to the volume of blood plasma as mostly red blood cells make up the volume of blood cells.
- **PLT** (platelets) - The absolute content of platelets (normal 150-400 X 10^9 cells / L) in blood cells. It is involved in haemostasis.

Erythrocyte indices (MCV, MCH, MCHC):

- **MCV** - Mean volume of erythrocytes in cubic micrometers (microns) or femto litre (Fl) (normal 80-95 Fl). In the old analysis indicate: microcytosis, normotsitoz, macrocytosis.
- **MCH** - Mean content of hemoglobin in single erythrocytes in absolute units (normal 27-31 pg), is proportional to the relative "hemoglobin / red blood cells." Color index of blood in the old analysis. CPU = MCH * 0.03
- **MCHC** - Mean concentration of hemoglobin in erythrocytes (normal 320-370 g / L), reflects the degree of saturation of the red blood cell hemoglobin. Reduced MCHC observed in diseases with a violation of hemoglobin synthesis. Nevertheless, it is the most stable haematological parameters. Any inaccuracy associated with the determination of
hemoglobin, hematocrit, MCV, leads to an increase in MCHC, so this parameter is used as an indicator of the instrument error or an error in preparing samples for study.

**Platelet indices (MPV, PDW, PCT):**

- **MPV** (mean platelet volume) - The average volume of platelets (normal 10.7 PL).
- **PDW** - The relative width of the distribution of platelets in volume index of the heterogeneity of platelets.
- **PCT** (platelet crit) – Thrombo crit (normal 0.108-0.282), the proportion (%) of whole blood occupied by platelets.

**Erythrocyte indices:**

- **RBC / HCT** - Average volume of red blood cells.
- **HGB / RBC** - The average content of hemoglobin in erythrocytes.
- **HGB / HCT** - The average concentration of hemoglobin in erythrocytes.
- **RDW** - Red cell Distribution Width - the distribution width of red blood cells "so-called" red cell anisocytosis - an indicator of heterogeneity of red blood cells, calculated as the coefficient of variation of the average volume of red blood cells.
- **RDW-SD** - The relative distribution width of red blood cells by volume, standard deviation.
- **RDW-CV** - The relative distribution width of red blood cells by volume, coefficient of variation.
- **P-LCR** - Ratio of large platelets.
- **ESR** (erythrocyte sedimentation rate) - A nonspecific indicator of a pathological condition of the body.

**Hemoglobin**

Hemoglobin (Hb, Hgb) in the blood, is the main component of red blood cells that carries oxygen to organs and tissues. It is measured in moles or grams per liter or per deciliter. His determination has not only diagnostic but also prognostic significance, as the pathological conditions that lead to a decrease in hemoglobin, leading to oxygen starvation of tissues.

Increasing hemoglobin seen with:

- Primary and secondary erythremia
- Dehydration (spurious effect due to haemo-concentration);
- Excessive smoking (the formation of functionally inactive NSO).

Reduced hemoglobin revealed by:

- Anemia;
- Hyperhydration (spurious effect due to hemo-dilution - "dilution" of blood, increased plasma volume relative to total corpuscles).

**Anemia**

Anemia is defined as subnormal hemoglobin level two standard deviations below the normal for the age and sex of the patient. From the CBC report, one can classify anemia as
microcytic, normocytic or macrocytic if the MCV is low, normal or high, respectively. Common etiologies of these anemias are as follows:

**Microcytic:**
- Fe deficiency
- Thalassemias
- Some patients with anemia of chronic disorder of inflammation

**Normocytic:**
- Anemia of chronic disorder
- Anemia of renal failure
- Anemia due to endocrine disorders

**Macrocytic:**
- B12 deficiency
- Folate deficiency
- Preleukmias
- Some cases of hypothyoidism
- Hemolytic anemias (because of high reticulocyte count)

RDW is a very useful measure in the assessment of anemia. Combined with red cell indices, it can narrow down the diagnostic possibilities. For example, a patient with microcytic anemia and high RDW is very likely to have iron deficiency. If the RDW is normal thalassemia become much more likely

**Erythrocytes**

*Erythrocytes* (E) in the blood - Red blood cells that are involved in the transport of oxygen in the tissue and maintain body processes of biological oxidation.

Increase (Erythrocytosis, polycythemia), red blood cell count is at:
- Neoplasms ;
- Polycystic kidney disease ;
- Edema renal pelvis;
- Effects of corticosteroids ;
- Disease of Cushing's syndrome ;
- Treatment with steroids

A small relative increase in the number of red blood cells may be associated with thickening of blood due to burns, diarrhoea, receiving diuretics.

Decrease (Erythrocytopenia) of red blood cells observed at:
- Blood loss;
- Anemia;
- Pregnancy;
• Reducing the intensity of formation of red blood cells in the bone marrow;
• Accelerated destruction of red blood cells;
• Hyperhydration.

**Leukocytes**

*Leukocytes* (L) is the blood cells produced in bone marrow and lymph nodes. Distinguish 5 types of leukocytes: granulocytes (neutrophils, eosinophils, basophils), agranulocytes (monocytes and lymphocytes). The main function of white blood cells is to protect the body from foreign antigens (including microorganisms, tumor cells, the effect are manifested in the direction of cell transplant).

**Increased (Leukocytosis) is at:**

- Acute inflammatory processes;
- Purulent processes, sepsis;
- Many infectious diseases of viral, bacterial, fungal and other etiologies;
- Malignancies;
- Tissue injuries;
- Myocardial infarction;
- In pregnancy (last trimester);
- After calf hood/childbirth - a period of feeding the udder/breast milk;
- After strenuous exercise (physiological leukocytosis).

**To decrease (Leukopenia) leads to:**

- Aplasia or hypoplasia of the bone marrow;
- Effects of ionizing radiation, radiation sickness;
- Typhoid fever;
- Viral disease;
- Anaphylactic shock;
- Addison's disease - Biermer;
- Collagen/rheumatic diseases;
- Under the influence of certain drugs (sulfonamides and some antibiotics, non-steroidal anti-inflammatory drugs, thyreostatics, antiepileptic drugs, antispasmodic oral drugs);
- Damage to bone marrow chemicals, drugs;
- Hypersplenism (primary, secondary);
- Acute leukemia;
- Myelofibrosis;
- Myelodysplastic syndromes;
- Plasmacytoma;
- Metastatic tumors in bone marrow;
- Pernicious anemia;
- Typhoid and paratyphoid fever;

**Increased numbers of various cell types are associated with the following conditions:**

**Neutrophilia:**

- Acute infections by bacterial infection
• Inflammation
• Acute hemorrhage
• Acute hemolysis
• Chronic granulocytic leukemia
• Malignancy
• Medications (steroids, lithium)
• Vigorous exercise

Lymphocytosis:
• Viral infections
• Toxoplasmosis
• Pertussis (whooping cough)
• Chronic lymphocytic leukemia

Monocytosis:
• Infections like TB, Sub-acute bacterial endocarditis
• Malignancy
• Inflammatory bowel disease

Eosinophilia:
• Allergic disorders
• Parasitic infestations
• Malignancy (Hodgkin's disease)
• Myeloproliferative disorders

Decrease in various cell types can be associated with the following disorders:

Neutropenia:
• Certain bacterial infections like Brucellosis, Typhoid
• Viral infections
• Medications like chemotherapy, anti-arthritis medications
• Aplastic anemia
• B12 and Folic acid deficiency
• Sequestration due to splenomegaly

Lymphopenia:
• Viral infections (HIV)
• Steroids
• Radiation, chemotherapy
**Wbc**

Wbc (Leukogram) is the percentage of different types of white blood cells, determined by counting them in a stained blood smear under a microscope.

**Color index**

**Color index (CP)** is the degree of saturation of erythrocyte verses hemoglobin:

- 0.90-1.10 - normal;
- Less than 0.80 - hypochromic anemia;
- 0.80-1.05 - red cells are normochromic;
- Greater than 1.10 - hyperchromic anemia.

In pathological states observed in parallel and approximately equal decrease in both the number of red blood cells and hemoglobin.

Reducing CPU (0.50-0.70) happens when:

- Iron deficiency anemia;
- Anemia due to lead intoxication.

Increased CPU (1.10 or more) is at:

- Deficiency of vitamin B12 in the body;
- Folic acid deficiency;
- Cancer;
- Polyposis of the stomach.

For a correct assessment of the color indicator is necessary to consider not only the number of red blood cells, but their volume.

**ESR**

**Erythrocyte sedimentation rate (ESR)** is a nonspecific indicator of a pathological condition of the body or it is the rate of settle down of RBC at the bottom in room temperature condition.

Increase in ESR occurs when:

- Infectious and inflammatory diseases;
- Collagen/rheumatic diseases;
- Kidney disease, liver and endocrine disorders;
- Pregnancy, postpartum period, menstruation;
- Fractures;
- Surgery;
- Anaemia
It can grow and physiological states such as food intake (up to 25 mm / h) and pregnancy (up to 45 mm / h).

Reducing the ESR is at:

- Hyperbilirubinemia;
- Raising the level of bile acids;
- Chronic circulatory failure;
- Erythremia;
- Hypofibrinogenaemia

**PLATELET ABNORMALITIES**

Platelet count may be elevated (Thrombocytosis) or decreased (Thrombocytopenia). These may result from a variety of disorders.

**Thrombocytosis:**

Reactive:

- Vigorous exercise
- Acute hemorrhage
- Infections by bacteria
- Malignancy

Autonomic:

- Chronic myeloproliferative disorders (polycythemia vera, chronic granulocytic leukemia, early phase myelofibrosis, essential thrombocytemia)

**Thrombocytopenia:**

Decreased Marrow Production:

- Aplastic anemia
- Chemotherapy, radiation
- B12, folate deficiency
- Leukemia, Preleukemia

Increased Destruction/Sequestration

- Immune thrombocytopenia
- Disseminated intravascular coagulation
- Splenomegaly

**The following terms are occasionally used in reporting CBC results:**

Pancytopenia: When all three cell-lines are decreased. This can result from aplastic anemia, infiltrative processes of the bone marrow like leukemia or myeloma, preleukemia and B12 or folate deficiency.
Leukemoid Reaction: The CBC report resembles that seen in leukemia (acute or chronic). This can be seen in severe infections, malignancies and severe hemolysis.

Leukoerythroblastic Reaction: The blood report shows presence of immature erythroid as well as granulocytic cells. This is seen in infiltrative processes in the marrow which may be a malignancy, hemolytic anemia, infections, fracture of marrow containing bones.
**Immmuno-PCR – High sensitivity detection of protein by nucleic acid amplification**

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**Introduction**

Antibody-based detection systems for specific antigens are a versatile and powerful tool for various molecular and cellular analyses and clinical diagnostics. The power of such systems originates from the considerable Specificity of antibodies for their particular epitopes. Methods that combine high-sensitivity detection with fast, robust and cost-efficient protocols for routine diagnostics and basic research applications are needed.

Polymerase chain reaction (PCR) technology (Quijada et al., 1997) has become a powerful tool in molecular biology and genetic engineering (Ke and Warner, 2000). The efficacy of PCR is based on its ability to amplify a specific DNA segment flanked by a set of primers. The enormous amplification capability of PCR allows the production of large amounts of specific DNA products, which can be detected by various methods. The extremely high specificity of PCR for a target sequence defined by a set of primers should avoid the generation of false signals from other nucleic acid molecules present in samples. They reasoned that the capability of antigen detection systems could be considerably enhanced and potentially broadened by coupling to PCR. Following these ideas, scientist have developed an antigen detection system, termed immuno-PCR, in which a specific antibody-DNA conjugate is used to detect antigens. In immuno-PCR, a linker molecule with bispecific binding affinity for DNA and antibodies is used to attach a DNA molecule (marker) specifically to an antigen-antibody complex, resulting in the formation of a specific antigen-antibody-DNA conjugates. The attached marker DNA can be amplified by PCR with the appropriate primers. The presence of specific PCR products demonstrates that marker DNA molecules are attached specifically to antigen-antibody complexes, which indicates the presence of antigen. A streptavidin-protein A chimera used as the linker (McElhinny et al., 1998). The chimera has two independent specific binding abilities; one is to biotin, derived from the streptavidin moiety, and the other is to the Fc portion of an immunoglobulin G (IgG) molecule, derived from the protein A moiety. This bifunctional specificity both for biotin and antibody allows the specific conjugation of any biotinylated DNA molecule to antigen-antibody complexes.
ELISA and IPCR

fig. 1

The general setup of IPCR is similar to that of antigen (Ag) detection through ELISA. Whereas an antibody–enzyme conjugate in ELISA converts a substrate into a detectable product, while in case of IPCR a conjugate comprising an antibody and a DNA marker is utilized. Nucleotides, specific primers and a polymerase are added and the marker is amplified by PCR for signal generation. The number of PCR amplicons produced is proportional to the initial quantity of antigen to be detected (Sano et al., 1992).

Reagent require for IPCR

1. Immobilization of antibody or antigen on ELISA plates
2. Biotinylated monoclonal antibody
3. Biotinylated reporter DNA (Huining Liang et al., 2003)

Immuno-PCR Protocol

- Coating of antigen or antibody on the polyvinylchloride microtitre ELISA plate well at room temperature for overnight
- Wash each well with washing buffer-A (Phosphate buffer saline (PBS) with 0.05% Tween 20 and 0.1% SDS) for 3 times
- Block non specific protein binding sites present on plate surface with blocking buffer (fetal bovine serum or dry fat milk powder) at 37oC for 15 min
- Wash each wells reaction with washing buffer- B (PBS with Tween 20) for 3 times
- Add 100μl of biotinylated monoclonal antibody specific to antigen which we are going to detect of concentration 10^8 copies/μl
- Incubate each wells at 37oC for 2hr and then go for Washing 3 times with buffer-B to remove unbound Mab
- Add 100μl of linker molecules streptovidin to link biotinylated Ab –biotinylated marker DNA of concentration of 10^9 copies/μl
- Incubate at room temperature for 30 min and Wash 3 times with buffer-B
- Add Biotinylated reporter DNA
- Mixture is incubate at room temp for 30 min, and each Wells are wash with buffer-B for 5 times and 4 times with milli-Q water to remove unbound marker DNA
- Bound marker DNA are detach from the plate by denaturing at 96°C in oven for 5 min
- Content of wells are transferred in to a PCR tube perform PCR amplification and read out of amplified product through Agarose gel electrophoresis followed by visualization under UV transillumination
  
  (Sano et al., 1992)

**Linker reagent**

Use to Link biotinylated antibody with biotinylated marker DNA

1. STV-Protein A chimera  
2. STV-Biotin  
3. Covalent linking  
4. Nutravidin-Biotin

**Types of IPCR**

1. Direct IPCR
2. Indirect IPCR
3. Two-sided (sandwich) IPCR
4. Real time IPCR
5. Magneto IPCR
6. Phage display mediated IPCR
7. In situ IPCR
8. Combination of DNA directed immobilization and IPCR

**Direct IPCR**

![Image of Direct IPCR assay](image)

Schematic representation of direct immuno-PCR assay. Maxisorp micro titer plates were used as the antigen carrier. Streptavidin was used as the bridge to link biotinylated anti-Group A mAbs with biotinylated reporter DNA. Assay response was obtained by PCR amplification of the DNA and detection of the DNA products.

(Wu et al., 2001)

**Sensitivity**

Comparison of the sensitivities of the four antigen detection assays of BHV-1-infected cells
PFU ................................................................. 7.2 (7.0, 7.6, 7.0)
ELISA ............................................................... 3.9 (3.6, 3.6, 4.6)
PCR ................................................................. 4.0 (4.0, 4.0, 4.0)
Immuno-PCR .................................................... 10.9 (9.9, 11.9, 10.9)

Immuno-PCR/Ag detected the antigen in the culture supernatant up to a dilution of 1:10^{9.9} followed by PFU (10^7 PFU/ml). ELISA detected the antigen up to a dilution of 1:10^{4.9} (Above) of the same sample. Results obtained from three independent tests with the same sample are summarized in above Table. Immuno-PCR/Ag was up to 10^{3.7}, 10^{7.0}, and 10^{6.9} more sensitive than plaque-forming assay, ELISA, and PCR, respectively.

(Mweene et al., 1996)

Application of IPCR

- Ultrasensitive detection of pathogens, allergens and pollutants eg. detection of *Clostridium botulinum* neurotoxin A
- Drug discovery: In various phase of the value chain for pharmaceutical R&D (Zolg et al., 2004).
- (Bio-) pharma R&D : Matrix switch to detection of tumor marker
- (Pre-) clinical trial: Toxicokinetic and Pharmacokinetic
- Clinical diagnosis: Human and veterinarian
- Diagnostic research: Detection of misfolded protein, e.g. prions or Alzheimer disease
- Bio-/Home defense: Detection of toxic proteins
- Food & beverage: Analysis for quality assurance
- Doping control: Detection of doping substances
- Agriculture: Control of modified plant and many more (Chassy et al., 2002)

Merit of IPCR

1. Significantly minimize sample volume requirements
2. High tolerance against drugs and matrix effects and its adaptability for the detection of basically any antigen.
3. IPCR Offers the opportunity to freely choose the sequence of the DNA marker
4. IPCR opens up access to an almost unlimited range of specifically labeled antibodies, which even enable multiplex detection of various different antigens.
5. Due to high sensitivity of IPCR, this method enables the testing of pooled sample instead of screening single unit at a time, there by significantly reduces the time and the cost of high throughput of serological test

Demerit of IPCR

1. Production technology of Mabs remains to be a labor-intensive and high cost process
2. Preparation of MAbs-DNA conjugate involves complex covalent coupling chemistry
3. It mostly associated with the complicated multistep protocol requiring experimental expertise in both PCR and ELISA
4. In some cases exotic and hardly accessible reagents protein-DNA conjugates, if they are not properly prepared and/or are contaminated then high background signal occurs in the IPCR
5. Signal generating complex is weaker if heterogeneous system to equilibrate

Summary and outlook

IPCR is a novel technology for antigen detection, combining the versatility of established ELISA techniques with the sensitivity of nucleic acid analyses. As established by numerous examples, a 100–10,000-fold improvement in common ELISA sensitivity is generally accessible when a given ELISA is adapted to an IPCR assay. A significant magnification of the dynamic range for antigen quantification of up to six orders of magnitude is brought about by the ELISA to IPCR adjustment, still maintaining the precision and robustness of the assay. From a practical point of view, the ELISA to IPCR adjustment does not require any sophisticated instrumentation; the IPCR assay can be carried out using routine laboratory equipment for microplate handling and PCR.

Reference:


Methods of sample collection, preservation and dispatch for microbiological examination

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Introduction

The main aim of collection of material is to establish exact etiology of the disease so as to give timely and accurate diagnosis of suspected disease. The diseases most commonly encountered in animals are of bacterial, viral, parasitic, fungal and metabolic origin. Diagnosis based on symptoms and laboratory examination of the relevant materials is essential for initiating treatment at the proper time. The knowledge of pathogenesis of disease is an important factor collection of the most suitable material. In general the following points should be duly considered while collecting materials for laboratory diagnosis.

General precaution for collection of materials for microbiological examination

1. All materials collected should be accompanied by full history of disease outbreak namely species affected, duration of disease, clinical signs, morbidity and mortality rates, disease suspected etc.
2. The collected biological specimens should be transported on ice to the nearest laboratory as early as possible.
3. Materials collected for bacteriological examination should be kept at refrigeration temperature (4°C) in case of delay of transportation. If a viral etiology is suspected the material can be stored at –20 to –80°C.
4. When sero-diagnosis is required, collect paired serum samples (about 2 ml sera). One serum sample should be collected at the onset of disease and second sera after recovery (3-4 weeks) from disease preferably on 21st day.
5. If death is reported, the post-mortem examination should be conducted at the earliest as putrefied materials are unfit for laboratory examination.
6. Detailed post-mortem report should be attached along with the samples collected during postmortem.
7. The different virological transport media that can be used are 50% Phosphate Buffered Glycerine Saline and Phosphate Buffer Saline (pH 7.2-7.4). Collect samples in sterile containers when a transport media is not available and coolers them on ice as early as possible.
8. For Histopathology studies, tissues should be preserved in 10% formalin. The volume of formalin used should be approximately 10 times the volume of material. Specimen bottles with wide mouth should be used for collecting tissues.
9. The specimen bottles should be sealed well so as to avoid leakage and clearly indicating the fixative/transport media used.
10. All the impression smears before culars, should be fixed in methanol for 1-5 minutes unless otherwise specified.

11. In case of outbreaks, try to collect materials from as many ailing animals (5-6 or more) as possible at the height of body temperature /clinical signs.

**When to suspect Infectious Diseases**

1. Historical evidence of contact with infected animals and their discharge, exposure to vectors and transportation to endemic areas. Vaccination and deworming status can be of help in diagnosing an infectious diseases.

2. Physical examination findings including fever, lymphadenopathy, hepatomegaly, testicular swelling and pain, foul smelling or blood stained faeces, change in colour and consistency of nasal, lachrymal and uterine discharge.

3. Complete blood count findings indicating neutrophilia (left shift or degenerative), lymphocytosis and eosinophilia or any other change suggestive of an infectious disease.

**1. Sample collection from live animals**

**a) Blood**

Blood samples may be taken for culture and/or direct examination for bacteria, viruses, or protozoa, in which case it is usual to use anticoagulants, such as ethylene diamine tetra-acetic acid (EDTA) or heparin. They may also be taken for serology, which requires a clotted sample. Blood plasma is also used for some procedures. A blood sample is taken, as cleanly as possible, by venipuncture. In most large mammals, the jugular vein or a caudal vein is selected, but brachial veins and mammary veins are also used. Vena cava veins are also used in pigs. In birds, a wing vein (brachial vein) is usually selected. Blood may be taken by syringe and needle or by needle and vacuum tube (not easy in delicate veins but convenient in strong veins). Small quantities of blood are conveniently obtained by pricking with a triangular, solid-pointed needle. Ideally the skin at the site of venipuncture should first be shaved (plucked) and swabbed with 70% alcohol and allowed to dry.

For samples that are collected with anticoagulant, thorough mixing, using gentle agitation only, is necessary as soon as the sample has been taken. It may also be necessary to make a smear of fresh blood on a microscope slide; both thick and thin smears may be prepared. For polymerase chain reactions, EDTA is the preferred anticoagulant. For serum samples, the blood should be left to stand at ambient temperature (but protected from excessive heat or cold) for 1–2 hours until the clot begins to contract. The clot can then be ringed round with a sterile rod and the bottles placed in a refrigerator at 4°C. After several hours, or overnight, the sample can be centrifuged at about 1000 g for 10–15 minutes and the serum can be decanted or removed with a pipette. In order to establish the significance of antibody titres, paired serum samples will often need to be collected 7–14 days apart. An alternative method for collecting and transporting blood that is to be used for serology is to place a drop of blood on to filter paper, the blood is dried at room temperature and the sample can then be shipped unrefrigerated.
Contact the laboratory to enquire if this method of collection is validated for the required tests.

b) Faeces
   An alternative and sometimes preferable method is to take swabs from the rectum (or cloaca), taking care to swab the mucosal surface. Care should be taken when collecting swabs from small, delicate animals or birds to avoid injury to the animal; small swabs are commercially available that should be used. Swabs should be transported in appropriate transport medium. Faeces are best stored and transported at 4°C.

c) Skin
   In diseases producing vesicular lesions, collect, if possible, 2 g of affected epithelial tissue as aseptically as possible and place it in 5 ml phosphate buffered glycerine or Tris-buffered tryptose broth virus transport medium at pH 7.6. Additionally, the vesicular fluid should be sampled where unruptured vesicles are present; if possible, vesicular fluid should be aspirated with a syringe and placed in a separate sterile tube. Plucked hair or wool samples are useful for surface-feeding mites, lice and fungal infections. Deep skin scrapings, using the edge of a scalpel blade, are useful for burrowing mites and, in birds, feather tips can be taken for detection of viral antigen where Marek’s disease is suspected.

(d) Genital tract and semen
   Samples may be taken by vaginal or preputial washing, or by the use of suitable swabs. The cervix or urethra may be sampled by swabbing. Samples of semen are best obtained using an artificial vagina or by extrusion of the penis and artificial stimulation. The sperm-rich fraction should be present in the sample and contamination by antiseptic washing solutions should be avoided. Specific transport media and conditions are often required.

e) Eye
   Sample from the conjunctiva can be taken by holding the palpebra apart and gently swabbing the surface. The swab is then put into transport medium. Scrapings may also be taken on to a microscope slide. The handles of metal-handled swabs are useful for this, to ensure that sufficient cells are removed for microscopic examination. Mucopurulent nasal and lacrimal discharges are rarely useful.

f) Nasal discharge (saliva, tears)
   Samples may be taken with dacron, cotton or gauze swabs, preferably on wire handles as wood is inflexible and may snap. It may be helpful if the swab is first moistened with transport medium. The swab should be allowed to remain in contact with the secretions for up to 1 minute, then placed in transport medium and sent to the laboratory without delay at 4°C. Long protected nasopharyngeal swabs should be used to collect samples for some suspected viral infections.
g) Milk

Milk samples should be taken after cleansing and drying the tip of the teat, the use of antiseptics should be avoided. The initial stream of milk should be discarded and a tube filled with the next stream(s), a sample of bulk tank milk can be used for some tests. Milk for serological tests should not have been frozen, heated or subjected to violent shaking. If there is going to be a delay in submitting them to the laboratory, preservatives can be added to milk samples that are being collected for serological testing. If necessary, milk for bacterial examination can be frozen.

2. Sample collection at post-mortem

Samples of tissue from a variety of organs can be taken at post-mortem. Tissue may be collected for the histopathology, microbiological culture, parasitology, biochemistry, and/or immunohistochemistry and for detection of genomic nucleic acids. Each piece of tissue should be placed in a fully labeled plastic bag or sterile screw-capped jar. Swabs should be always be submitted in appropriate transport media. Amies transport medium with charcoal or Stuart transport medium without charcoal is used to transport the swabs. Sterile instruments should be used for collecting samples for microbiological culture and care should be taken not to contaminate tissue with intestinal contents. Disinfected should not be used on or near tissue to be sampled for bacterial culture or virus isolation. The tissue may be to the laboratory dry or in bacterial or virus transport medium, depending on the types of specimen and the examinations required; swabs should be sent in transport medium. After collection, samples for microbiological examination should be refrigerated until shipped. If shipment cannot be made within 48 hrs, the sample should be frozen; however prolonged storage at -20°C may be detrimental to virus. For certain suspected diseases, larger portions of brain are required; the brain is sectioned using a sagittal cut, half is submitted fresh on ice, and the other half is submitted in 10% buffered formalin.

General consideration for Virological samples

Storage of virus specimens

- Samples for virological investigation can be collected in Charcoal virus transport medium or
  Phosphate buffered saline (pH 7.3) or 50% Glycerol saline
- Phosphate buffered saline is a very useful general diluent and suspending fluid
- Glycerol saline is prepared by mixing equal amounts of glycerine and saline (0.85% NaCl)
- Media or collection solution may be autoclaved at 121°C for 15 min.
- Keep the specimens in tightly capped container to avoid deleterious effect of CO₂.

Materials to be collected in abortion cases

- Placenta
  o Impression smears prepared from at least 2-3 cotyledons after blotting the cotyledons with filter paper.
  o Air dry and gently heat fix (2 slides)
  o Small pieces from 2-3 cotyledons in a sterile bottle on ice.
- Pieces from 2-3 cotyledons and intercotyledonal membrane preserved in 10% formal saline

- **Foetus**
  - Smears from thoracic and abdominal fluid, air dried and gently heat fixed
  - Thoracic and abdominal fluid (5ml) in a sterile container on ice for cultural isolation
  - Small pieces from lung, liver, spleen, kidney and brain in a sterile bottle on ice
  - Foetal stomach after tying both the ends and kept in a sterile bottle on ice or 10-20 ml of stomach content in sterile vials on ice for isolation of bacteria
  - Small pieces of lung, liver, spleen, thymus, stomach, kidney, brain, heart, intestine and any gross lesion from foetus, preserved in 10% formal saline

- **Dam (aborted)**
  - Vaginal (deeper part) and uterine mucosal smears are to be prepared within 3 days of abortion, and air dried and gently heat fixed
  - Swab from uterine discharge
  - Milk in sterile container in case of brucellosis
  - Collect serum on the day of abortion and again 3 weeks after abortion
  - If leptospiral abortion is suspected, 20 ml of midstream urine from the dam preserved in 1.5 ml of 10% formalin

- **Sire**
  - Preputial washing in a sterile container on ice
  - Serum sample

**Dispatch of materials**
- Properly pack and dispatch the materials within 24 hours under refrigerated condition (+ 4 °C) or on ice in a thermos flask.
- Dry ice (solid CO₂) can also be used.
- If delay is expected store at -70 °C (-20 to -25 °C is also satisfactory but infectivity is reduced in certain cases).
- Paste the label containing specimen number, date of collection and nature of specimen
- Enclose detailed history sheet containing specimen number, date of collection, name of the owner and address, species, age, sex, particulars of illness, symptoms, treatment and lesions, herd or flock health, disease suspected etc.
Loop Mediated Isothermal Amplification (LAMP): A Rapid, Cost-Effective and Highly Sensitive Technique for Infectious Diseases Diagnosis

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Millions of animals around the world, especially in developing countries, are affected every year by infectious Pathogens. Inadequate therapeutic measures, insufficient planning and management and above all lack of accurate diagnostic measures result in repeated outbreak of such diseases. Accurate diagnosis of such diseases is important not only for the prescribing of effective drugs to the animals in adequate doses but also for preventing the evolution of resistant microorganisms, which occurs by treating non infected animals who show similar symptoms. Therefore, the development and adoption of rapid, accurate, and sensitive diagnostic methods for the identification of infectious pathogens play key role in treatment, control and even eradication of infectious disease.

Loop mediated isothermal amplification (LAMP) is a simple, rapid, highly specific and cost-effective single tube technique for the amplification of DNA. LAMP has the potential to be used as a simple screening assay in the field or at the point of disease outbreak by clinicians. It may be a useful method for infectious disease diagnosis particularly in developing countries. This technology has been developed into commercially available detection kits for a variety of pathogens including bacteria and viruses. The current focus on LAMP methodology is as a diagnostic system to be employed in resource-limited laboratories in developing countries, where many fatal tropical diseases are endemic. Classical pathogen detection and identification is generally based on culture methodology and microscopy. Though these methods provide plenty of information rather than just identifying the pathogen, they are time consuming and require specialized laboratory facilities. In the past few decades, several molecular methods like polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA) have been developed to overcome the shortcomings of the classical diagnostic methods but they are not cost effective and require expensive equipments. LAMP certainly has potential to be used as a rapid and reliable tool for disease diagnosis especially in developing countries.

Amplification and detection of gene can be completed in a single step using LAMP that take just 15-60 minutes, by incubating the mixture of samples, primers, Bst DNA polymerase with strand displacement activity and substrates at a constant temperature (about 60-65°C). LAMP is an isothermal nucleic acid amplification technique so it does not require expensive thermal cyclers. In-tube detection of DNA amplification is possible hence there is no need to run gel electrophoresis after amplification. This makes detection of amplification product very rapid and easy. It can be determined by photometry, Fluorimetry or even by naked eye. Turbidity is noticed with the increase in amplification product concentration due to formation of Magnesium pyrophosphate in solution, as a byproduct of amplification. On
addition of fluorescence dye such as SYBR green, that intercalate into double stranded DNA, a color change can be seen without equipment. The target sequence is amplified at a constant temperature of 60 - 65°C using either two or three sets of primers and a polymerase (Bst DNA polymerase) with high strand displacement activity in addition to a replication activity. Double stranded DNA is in dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA.

Lamp is mostly used as a qualitative detection method but the reaction can be followed in real-time either by measuring the turbidity or the signals produced via fluorescent dyes that intercalate the DNA, and in turn can be correlated to the number of copies initially present. Hence, LAMP can also be quantitative.

Proper primer design is crucial for performing LAMP amplification. The primers for LAMP must be designed for a region which is highly conserved and unique to desired targets. Automated primer design software are available that takes care of base composition, GC contents and the formation of secondary structures. Primer Explorer (http://primetexplorer.jp/e/) is special software to design LAMP primers provided by Fujitsu Ltd and Eitken Chemical Company. Another even more versatile LAMP primer design tool named LAVA (LAMP assay versatile analysis) is now available. Other points that should be kept in mind during primer designing for LAMP are just like the PCR primer designing and they are as follows.

- Distance between primer regions
- Tm value for primer regions
- The stability of primer end
- GC contents
- Secondary structure formation

LAMP standard procedures
Procedure for LAMP is quite simple and rapid. It takes around 1 hr to perform LAMP assay. The steps are as follows.

- Sample collection (Blood, sputum, feces or tissue)
- DNA/ RNA extraction from the tissue
- Amplification
- Detection

Advantages of LAMP

- LAMP has certain advantages over other molecular diagnostic methods.
- LAMP is an easy technique to perform as it is a single step isothermal amplification.
- It is a rapid technique and can be performed within 60 min.
• Visual detection of the results is possible.
• Amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase.
• The amount of DNA produced in LAMP (400-800 µg/ml) is considerably higher than PCR (4-40 µg/ml) based amplification.
• Lamp is highly specific and typically uses 2-3 set of primers to identify 6 distinct regions on the target sequence.
• LAMP is highly cost effective as it does not require special reagents or sophisticated equipments.

Commercial Diagnostic kits based on LAMP have been applied available for pathogens like Salmonella, Legionella, Listeria, verotoxin-producing Escherichia coli, Giardia and Campylobacter. LAMP assays have been developed for the diagnosis of other important pathogens, including measles virus, human papilloma virus, mumps virus, Cryptosporidium oocysts, Legionella, and Vibrio cholerae. LAMP assays for Tuberculosis, malaria, influenza, sleeping sickness and human immunodeficiency virus (HIV) are at different stage of development. LAMP assay are very promising especially in patients with dengue where very low copy numbers can be detected, which even outperforms the sensitivity of real-time PCR. Hence, LAMP is one of the promising tools for rapid diagnosis of diseases in human as well as animal and it could help control and even eradicate deadly diseases if used rationally and with utmost care.

References:


Immunohistochemistry techniques in animal disease diagnosis

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Introduction:

Immunohistochemistry is the localization of antigens in tissue sections by the use of labeled antibody as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold.

Albert H. Coons and his colleagues (Coons et al. 1941, 1955; Coons and Kaplan 1950) were the first to label antibodies with a fluorescent dye, and use it to identify antigens in tissue sections. With the expansion and development of immunohistochemistry technique, enzyme labels have been introduced such as peroxidase (Nakane and Pierce 1966; Avrameas and Uriel 1966) and alkaline phosphatase (Mason and Sammons 1978). Colloidal gold (Faulk and Taylor 1971) label has also been discovered and used to identify immunohistochemical reactions at both light and electron microscopy level. Other labels include radioactive elements, and the immunoreaction can be visualized by autoradiography.

Since immunohistochemistry involves specific antigen-antibody reaction, it has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, immunohistochemistry has become a crucial technique and widely used in many medical research laboratories as well as clinical diagnostics.

There are numerous immunohistochemistry methods that may be used to localize antigens. The selection of a suitable method should be based on parameters such as the type of specimen under investigation and the degree of sensitivity required.

Tissue Preparation

Fixation:

Tissue preparation is the cornerstone of immunohistochemistry. To ensure the preservation of tissue architecture and cell morphology, prompt and adequate fixation is essential. However, inappropriate or prolonged fixation may significantly diminish the antibody binding capability.

There is no one universal fixative that is ideal for the demonstration of all antigens. However, in general, many antigens can be successfully demonstrated in formalin-fixed paraffin-embedded tissue sections. The discovery and development of antigen retrieval techniques further enhanced the use of formalin as routine fixative for immunohistochemistry in many research laboratories. For best results, vertebrate tissues (especially neuronal tissues) usually require fixation by transcardial perfusion for optimal tissue preservation. The most common fixatives used for immunohistochemistry are the followings:

- 4% paraformaldehyde in 0.1M phosphate buffer
- 2% paraformaldehyde with 0.2% picric acid in 0.1M phosphate buffer
- PLP fixative: 4% paraformaldehyde, 0.2% periodate and 1.2% lysine in 0.1M phosphate buffer
- 4% paraformaldehyde with 0.05% glutaraldehyde (TEM immunohistochemistry)

Some antigens will not survive even moderate amounts of aldehyde fixation. Under this condition, tissues should be rapidly fresh frozen in liquid nitrogen and cut with a cryostat without infiltrating with sucrose. The sections should be kept frozen at -20°C or lower until fixation with cold acetone or alcohol. After fixation, the sections can be processed using standard immunohistochemical staining protocols.

**Sectioning:**

Since its introduction, paraffin wax has remained the most widely used embedding medium for diagnostic histopathology in routine histological laboratories. Accordingly, the largest proportion of material for immunohistochemistry is formalin-fixed, paraffin-embedded. Paraffin sections produce satisfactory results for the demonstration of majority of tissue antigens with the use of antigen retrieval techniques.

Certain cell antigens do not survive routine fixation and paraffin embedding. So the use of frozen sections still remains essential for the demonstration of many antigens. However, the disadvantage of frozen sections includes poor morphology, poor resolution at higher magnifications, special storage needed, limited retrospective studies and cutting difficulty over paraffin sections.

Vibratome sections have some advantages when doing immunohistochemistry since the tissue is not processed through organic solvents or high heat, which can destroy the antigenicity. In addition, the morphology of tissue sections is not disrupted due to no freezing and thawing needed. Vibratome sections are often used for floating immunostaining, especially for pre-embedding EM immunohistochemistry. The disadvantage of vibratome sections is that the sectioning process is slow and difficult with soft and poorly fixed tissues. In addiction, the chatter marks or vibratome lines are often appeared in the sections.

**Whole Mount Preparation:**

Small blocks of tissue (less than 5 mm thick) can be processed as whole mounts. The advantage of whole mount preparations is that the results provide three dimensional information about the location of antigens without the need for reconstruction from sections. However, the major limitation of using whole mounts is antibody penetration may not be complete in the tissue, resulting in uneven staining or false negative staining. So Triton X-100 or saponin treatment are used routinely for whole mount immunohistochemistry to enhance penetration of the antibody.

**Antigen Retrieval**

The demonstration of many antigens can be significantly improved by the pretreatment with the antigen retrieval reagent that break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. The techniques involved the application of heat for varying lengths of time to formalin-fixed, paraffin-embedded tissue sections in an aqueous solution (commonly referred to as the retrieval solution). This is called "Heat Induced Epitope Retrieval (HIER)". Another method uses enzyme digestion and is called "Proteolytic Induced Epitope Retrieval (PIER)".
Microwave Oven, Pressure Cooker and Steamer are the most commonly used heating devices. Other devices also include the use of autoclave and water bath. The heating length of 20 minutes appears to be the most satisfactory and the cooling usually takes about 20 minutes. Citrate buffer of pH 6.0 is the most popularly used retrieval solution and is suitable for most of antibody applications. The TRIS-EDTA of pH 9.0 and EDTA of pH 8.0 are second most used retrieval solutions. Proteinase K is effective enzyme digestion reagent for membrane antigens such as Integrins, CD31, vWF, etc.

PIER methods (such as proteinase k, trypsin, chymotrypsin, pepsin, pronase and various other proteases) has also been reported for restoring immunoreactivity to tissue antigens with different degrees of success. However, the use of enzyme digestion method may destroy some epitopes and tissue morphology. Therefore the optimal enzyme concentration and incubation time need to be tested.

Combination of Heat Mediated and Proteolytic Enzyme Method is an alternative approach to unmask antigens if other methods did not work. It is especially useful when performing double or triple labeling of two or more antigens simultaneously.

Improving antibody penetration is also important for immunohistochemical staining of frozen and vibratome sections. Triton X-100 is by far the most popular detergent for improving antibody penetration for immunohistochemistry. However, it is not appropriate for the use of membrane antigens since triton X-100 destroy membranes. Some researchers prefer the freeze and thaw method for the improvement of antibody penetration. Sodium borohydride (1% in phosphate buffer) treatment is also widely used to unmask antigens, particularly in glutaraldehyde fixed tissue to reduce the glutaraldehyde linkages.

**IHC Methods**

**Blocking:**

Background staining may be specific or non-specific. Inadequate or delayed fixation may give rise to false positive results due to the passive uptake of serum protein and diffusion of the antigen. Such false positives are common in the center of large tissue blocks or throughout tissues in which fixation was delayed.

Antibodies, specially polyclonal antibodies, are sometimes contaminated with other antibodies due to impure antigen used to immunize the host animal.

The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blocking those sites with normal serum.

Endogenous peroxidase activity is found in many tissues and can be detected by reacting fixed tissue sections with DAB substrate. The solution for eliminating endogenous peroxidase activity is by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody.

Many tissues also contain endogenous alkaline phosphatase (AP) activity and should be blocked by the pretreatment of the tissue section with levamisole if using AP as a label.

Some tissues such as liver and kidney have endogenous biotin. To avoid unwanted avidin binding to endogenous biotin if using biotin-avidin detection system, a step is
necessary for these tissues by the pretreatment of unconjugated avidin which is then saturated with biotin.

Autofluorescence or natural fluorescence exists in some tissues and can cause background problems when fluorescent dyes are used in the experiments. The simplest test is to view the tissue sections with a fluorescence microscope before any antibody incubation. If autofluorescence is detected in the tissue sections, the best solution is to avoid use of fluorescent method but choose enzyme or other labeling methods.

**Controls:**

Special controls must be run in order to test the protocol and for the specificity of the antibody being used.

Positive control is to test a protocol or procedure and make sure it works. It will be ideal to use the tissue of known positive as a control. If the positive control tissue showed negative staining, the protocol or procedure needs to be checked until a good positive staining is obtained.

Negative control is to test for the specificity of an antibody involved. First, no staining must be shown when omitting primary antibody or replacing an specific primary antibody with normal serum (must be the same species as primary antibody). This control is easy to achieve and can be used routinely in immunohistochemical staining.

Second, the staining must be inhibited by adsorption of a primary antibody with the purified antigen prior to its use, but not by adsorption with other related or unrelated antigens. This type of negative control is ideal and necessary in the characterization and evaluation of new antibodies but it is sometimes difficult to obtain the purified antigen, therefore it is rarely used routinely in immunohistochemical staining.

**Direct Method:**

Direct method is one step staining method, and involves a labeled antibody (i.e. FITC conjugated antiserum) reacting directly with the antigen in tissue sections. This technique utilizes only one antibody and the procedure is short and quick. However, it is insensitive due to little signal amplification and rarely used since the introduction of indirect method.

**Indirect Method:**

Indirect method involves an unlabeled primary antibody (first layer) which react with tissue antigen, and a labeled secondary antibody (second layer) react with primary antibody (Note: The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. In addition, it is also economy since one labeled second layer antibody can be used with many first layer antibodies (raised from the same animal species) to different antigens.

The second layer antibody can be labeled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called indirect immunofluorescence method. The second layer antibody may be labeled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called indirect immunoenzyme method.
PAP Method (peroxidase anti-peroxidase method):

PAP method is a further development of the indirect technique and it involves a third layer which is a rabbit antibody to peroxidase, coupled with peroxidase to make a very stable peroxidase anti-peroxidase complex. The complex, composed of rabbit gaba-globulin and peroxidase, acts as a third layer antigen and becomes bound to the unconjugated goat anti-rabbit gaba-globulin of the second layer. The sensitivity is about 100 to 1000 times higher since the peroxidase molecule is not chemically conjugated to the anti IgG but immunologically bound, and loses none of its enzyme activity. It also allows for much higher dilution of the primary antibody, thus eliminating many of the unwanted antibodies and reducing non-specific background staining.

Avidin-Biotin Complex (ABC) Method:

ABC method is standard IHC method and one of widely used technique for immunohistochemical staining. Avidin, a large glycoprotein, can be labeled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies.

The technique involves three layers. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by the DAB or other substrate to produce different colorimetric end products.

Labeled StreptAvidin Biotin (LSAB) Method:

Streptavidin, derived from streptococcus avidini, is a recent innovation for substitution of avidin. The streptavidin molecule is uncharged relative to animal tissue, unlike avidin which has an isoelectric point of 10, and therefore electrostatic binding to tissue is eliminated. In addition, streptavidin does not contain carbohydrate groups which might bind to tissue lectins, resulting in some background staining.

LSAB is technically similar to standard ABC method. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is Enzyme-Streptavidin conjugates (HRP-Streptavidin or AP-Streptavidin) to replace the complex of avidin-biotin peroxidase. The enzyme is then visualized by application of the substrate chromogen solutions to produce different colorimetric end products. The third layer can also be Fluorescent dye-Streptavidin such as FITC-Streptavidin if fluorescence labeling is preferred.

A recent report suggests that LSAB method is about 5 to 10 times more sensitive than standard ABC method.

Polymeric Methods:

1) EnVision Systems are based on dextran polymer technology. This unique chemistry permits binding of a large number of enzyme molecules (horseradish peroxidase or alkaline phosphatase) to a secondary antibody via the dextran backbone. The benefits are many, including increased sensitivity, minimized non-specific background staining and a reduction in the total number of assay steps as compared to conventional techniques. The
simple protocol is i) Application of primary antibody; ii) Application of enzyme labeled polymer; iii) Application of the substrate chromogen. EnVision+ was developed after EnVision to provide increased sensitivity.

2) ImmPRESS polymerized reporter enzyme staining system is based on a new method of polymerizing enzymes and attaching these polymers to antibodies. The novel approach employed to form enzyme "micropolymers" avoids the intrinsic shortcomings of using large dextrans or other macromolecules as backbones. Attaching a unique "micropolymer with a high density of very active enzyme to a secondary antibody generates a reagent that overcomes steric interference and provides enhanced accessibility to its target. The result is outstanding sensitivity, signal intensity, low background staining, and reduced non-specific binding. The simple protocol is i) Application of primary antibody; ii) Application of enzyme labeled polymer; iii) Application of the substrate chromogen.

CSA Methods From Dako:

1) CSA Systems use Tyramide Signal Amplification. It is ideal for the following applications: i) Detecting small quantities of antigen; ii) Enhancing performance of low affinity mouse and rabbit antibodies; iii) Enabling compatibility of certain "tough" mouse and rabbit antibodies with paraffin embedded tissue sections. The simple protocol is as follows:

1. Application of primary antibody.
2. Application of biotinylated linking antibody.
3. Application of the Tyramide Amplification Reagent.
4. Application of Streptavidin-HRP.
5. Application of the substrate chromogen

2) CSA II - Biotin-free Tyramide Signal Amplification System is a highly sensitive immunohistochemical (IHC) staining procedure incorporating a signal amplification method based on the peroxidase-catalyzed deposition of a fluorescein-labelled phenolic compound, followed by a secondary reaction with a peroxidase-conjugated anti-fluorescein. In the procedure, a mouse primary antibody is first detected with a peroxidase-conjugated secondary antibody. The next step utilizes the bound peroxidase to catalyze oxidation of a fluorescein-conjugated phenol (fluorescyl-tyramide) which then precipitates onto the specimen. The procedure is continued with detection of the bound fluorescein by a peroxidase-conjugated anti-fluorescein. Staining is completed using diaminobenzidine/hydrogen peroxide as chromogen/substrate, and can be observed with a light microscope. In comparison to standard immunohistochemical methods, such as labelled streptavidin biotin (LSAB) or avidin-biotin complexes (ABC), tyramide amplification methods have been reported to be many fold more sensitive. The CSA II System is a simplified version of the extremely sensitive Catalyzed Signal Amplification System (code K1500) that utilizes biotinyl-tyramide. The highly sensitive CSA II System allows for the detection of very small quantities of target protein, as well as for the use of low affinity antibodies. This reagent system utilizes fluorescyl-tyramide, rather than biotinyl-tyramide,
and does not contain avidin/biotin reagents, thus eliminating potential background staining due to reactivity with endogenous biotin.

Principles of Procedure: The specimens are first incubated with Peroxidase Block for five minutes to quench endogenous peroxidase activity. The specimens are then incubated for five minutes with a protein block to suppress nonspecific binding of subsequent reagents, followed by a 15-minute incubation with an appropriately characterized and diluted mouse primary antibody or negative control reagent (user provided). This is followed by sequential 15-minute incubations with anti-mouse immunoglobulins-HRP, fluorescyl-tyramide hydrogen peroxide (amplification reagent) and anti-fluorescein-HRP. Staining is completed by a five-minute incubation with 3,3’ diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide, which results in a brown precipitate at the antigen site.

Multiple Labeling

It is often useful to be able to stain for two or more antigens in one common tissue section. This can be achieved by immunofluorescence method using different fluorescent dyes. Multiple staining can also be done with peroxidase conjugated antibodies developed with different chromogen substrates to produce the end products of different colors. There are three basic approaches in planning multiple staining: parallel, sequential and adjacent. In addition, the antibody dilution and condition are also important factors to be considered. Finally, appropriate color combination is also crucial since improper color combination may produce poor result and fail to demonstrate multiple antigens in the same section. For best result, the careful design and test of multiple staining protocols are necessary.

References


Key concepts in diagnosis of parasitic infections/infestations in livestock

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Introduction
Parasites are a major cause of disease of man and domestic animals. More than half the human population live in misery and pain and suffer vast economic losses due to parasites. A possible solution to these problems lies in the development of new agricultural technologies for expanding food production. One of the ways in which livestock production can be increased is by reduction of losses due to disease. Estimates suggest that only a 6% reduction in disease could provide food for an additional 250 million people (Ristic & Montenegro-James, 1987). The situation is most serious in developing countries where 75% of the world's population resides. According to the Food and Agriculture Organisation, it is estimated that up to 70% of the world's livestock resources exist in these regions, yet they account for only 30% of the world's meat output (Ristic & Montenegro-James, 1987). The effective control and treatment of parasitic diseases requires rapid, reliable and highly sensitive diagnostic tests, which can also serve to monitor the effectiveness of the therapeutic and prophylactic protocol. Currently, the detection and diagnosis of parasite infections rely on several laboratory methods in addition to clinical symptoms, clinical history, travel history, and geographic location of patient. The primary tests currently used to diagnose many parasitic diseases have changed little since the development of the microscope in the 15th century by Antonie van Leeuwenhoek. Furthermore, most of the current tests cannot distinguish between past, latent, acute, and reactivated infections and are not useful for following response to therapy or for prognosis. The usual immunological tests do not always meet these requirements and are not able reliably to distinguish between closely related species. In fact, diagnostic serology is in many cases not specific. Molecular biology has provided new methods for identifying parasites and their vectors; methods that reach the genomic organisation of the organism and often replace traditional approaches because of their rapidity, sensitivity, ease of use and accuracy. Yet, perhaps more importantly, identification using DNA probes is rapidly moving beyond the laboratory environment and becoming a technique readily available for field epidemiologists and controlling bodies. Here we will discussed the application of various diagnostic techniques for the detection and classification of economically important rickettsial, protozoan and helminthic parasites of domestic animals and wild ruminants.

Diagnostic techniques
The most reliable way to diagnose a parasitic infection is by detection and identification of the infecting organism. Since, majority of parasitic stages are voided through faeces and blood, their gross and microscopical examination is essential for correct identification. Besides, post-mortem examination of dead animals is done systematically of different organs and tissues for search of parasites and their stages for correct identification and choosing morbid materials for histopathological examination. However, failure to demonstrate or recover a parasite does not exclude the possibility of infection. Many of these parasites, especially the protozoa, can be identified only by microscopic examination. This requires considerable skill and apart from being time-consuming and labour-intensive the method has limitations. This also applies to most of the commonly used serological techniques.
Table 1: Key validation parameters employed for the assessment of a diagnostic test (based on Conraths and Schares, 2006; Pfeiffer, 2010; Thrusfield, 2005).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>The proportion of animals with the disease and are test-negative.</td>
</tr>
<tr>
<td>Specificity</td>
<td>The proportion of animals without the disease and are test-negative.</td>
</tr>
<tr>
<td>Agreement</td>
<td>The agreement in results between two diagnostic tests, with one of the tests being a generally accepted diagnostic method.</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Refers to the concordance between test results and the “true” clinical state.</td>
</tr>
<tr>
<td>Reliability</td>
<td>The extent to which test results are consistent in repeat experiments.</td>
</tr>
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**Direct identification of parasites**

It has been done from samples of faeces, urine, blood and tissue etc.

*Faeces:* Evidence of intestinal parasitism, apart from the general clinical signs, is obtained from faecal or post-mortem examination. There is no "general" technique, nor is there an "ideal" technique for the microscopic examination of faeces. In fact, a reliable diagnosis can usually be made only by using a combination of several techniques, such as:

- **Direct saline smear:** This procedure provides only an indication of the parasites present and cannot be used quantitatively. To prepare a direct faecal smear a drop of saline is placed in the centre of a microscope slide and a 2 mg faecal sample is suspended in this drop without spreading it. This is then covered with a coverslip and examined.

- **Stained smears:** This type of smear is essential for accurate diagnostic detail and is also suitable for long-term storage for record purposes. The two stains generally used are haematoxylin and trichrome.

- **Parasite concentration in faeces by flotation:** This is used for the identification of oocysts of coccidia and helminth eggs. One drawback of this technique is that there is not always a direct relationship between the number of eggs in faeces and the number of parasites present.

- **Parasite concentration in faeces by sedimentation:** This technique is used to isolate eggs of flukes, acanthocephalans and some other tapeworms and nematodes whose eggs do not float readily in common floatation solutions. In the simple sedimentation test, tap water is combined with faeces and allowed to settle briefly before the supernatant is removed. This allows the removal of fine particulate material, but unlike the flotation exam, sedimentation tests have only limited concentrating ability.

*Urine:* Examination of urine sediment is used mainly for the identification of Encephalitozoon cuniculi and Schistosoma eggs.

*Blood:* Testing is used to identify the various stages of blood parasites and is routinely applied to diagnose malaria, theileriosis, babesiosis, anaplasmosis, ehrlichiosis, trypanosomiasis and most types of filariasis. Trypanosoma can also be diagnosed with wet smears. Depending on the application and purpose, two types of blood films are used. Thin blood films are useful for studying morphological changes of blood cells and blood parasites. The main disadvantage is that sample volume is small, making the detection of low parasitaemia and carrier animals difficult. Thick blood films contain 6 to 20 times as much blood per unit area as thin films. The thick film is suited for rapid diagnosis of parasitaemia that is too low to be detected with thin films.

*Tissue:* Recovery of protozoa or helminths from biopsy material is often an important aid to diagnosis. Lymph node, spleen, liver, lung, bone marrow or spinal fluid biopsies are frequently used to diagnose a variety of diseases.
Post-mortem: Post-mortem examination is currently the most effective way to accurately diagnose helminth infection. Brain-cortex smears are examined for babesiosis (*Babesia bovis*), turning sickness (cerebral theileriosis) and cowdriosis.

**Indirect identification**

All methods for the direct identification of parasites fail if the parasite density in the specimen is below the sensitivity of the method employed, or if the parasite cannot be directly demonstrated due to the life cycle in the host (e.g. toxoplasmosis, echinococcosis and cysticercosis). In such cases indirect methods must be used. Ideally, serology should allow differentiation between recent and latent infections and should be able to demonstrate whether an animal is a carrier as well as the elimination of the parasite after therapeutic measures have been applied. In practice, serology seldom achieves this. There is a lack of dependable tests for the serodiagnosis of parasitic disease in animals. Commercially available tests present problems in reliability and interpretation of results. Costly, specialised apparatus is often needed to perform the tests. With the majority of tests, specificity is not satisfactory and cross-reaction seldom allows distinction between closely related organisms. However, monoclonal antibodies may overcome this problem and enable the identification of highly specific antigen sites. Tests commonly in use include the complement fixation test (CFT), immunodiffusion (ID), indirect haemagglutination (IHA), indirect immunofluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). Less frequently used tests include latex agglutination, capillary agglutination and card agglutination. Most of these tests are based on the reaction of antibodies with antigenic parasite components (whole or soluble) resulting in antigen-antibody complexes. These complexes are detected by the addition of antiglobulins coupled to fluorescein and rhodamine dyes, radioisotopes or enzymes. Because antibodies can persist for a long time after elimination of the parasites, another drawback of serology is that the demonstration of a specific antibody does not indicate the present parasitological status of the host. The results of a serological test are therefore retrospective. Serodiagnosis of helminth infections is even more difficult because cross-reactivity is more the rule than the exception. Only highly purified, defined antigens allow serodiagnosis to the genus level; species-specific serodiagnosis is unusual. Antigen-capturing ELISA can be used for demonstrating infection with trypanosomes for instance.

The application and reliability of serology is in many cases dependent on the availability of sufficient amounts of high quality antigen. The inability to culture many parasites in vitro and the lack of suitable animal models often hamper antigen preparation.

The use of nucleic acid probes in the diagnosis of parasitic infections is based on the premise that every organism carries unique DNA sequences which differentiate it from other organisms. A diagnostic probe is developed by identifying and isolating these sequences. Two issues are of major importance in nucleic acid diagnostics: the specificity and sensitivity of the probe, i.e. can the probe differentiate between species of parasite and what is the lowest level of infection that can be detected? Sensitivity: Today's technology makes it possible to detect even a single base change in complex genomes. Sensitivity of detection depends on the abundance of unique sequences in the parasite genome. The most sensitive DNA probe is one originating from total genomic DNA since all the nucleic acid sequences of the organisms would be present in such a genomic probe. However, since the host cell genome is on average 1,000 times larger than the parasite genome, the major problem associated with genomic probes is that of host cell DNA contamination. It is therefore important to isolate uncontaminated parasite DNA as a prerequisite to the development of this type of probe. This is not always easy to achieve. Yet another problem is that genomic probes may hybridise to DNA from closely related species. For example, *Leishmania major* kinetoplast DNA (kDNA) cross-hybridises with virtually
every other Leishmania spp. kDNA. With few exceptions, biological functions have not been determined for the highly repetitive DNA sequences that constitute a large percentage of parasitic genomes. Such repetitive DNA, however, is excellent for DNA-based diagnostics because of its abundance and rapid evolution that allows sensitive and specific detection. These sequences should be able to detect parasite DNA at high sensitivity. Highly repeated DNA can be detected by standard hybridisation techniques with a sensitivity of only one order of magnitude less than genomic DNA probes. Specificity: The specificity of nucleic acid probes is due to the ability of DNA to denature under certain conditions and to renature in a highly specific manner. DNA can easily be denatured by heat or by increasing the pH. This leads to a reversible separation of the two complementary strands which will reassociate under the appropriate conditions to reform a duplex molecule. Two separated polynucleotide chains will reassociate only if they are complementary. This reaction is termed nucleic acid hybridisation and occurs both with DNA and RNA. Since the requirement for complementarity is not absolute, non-identical but related nucleic acids can participate in the hybridisation reaction. However, these hybrids are less stable when exposed to low ionic strength buffers or heat allowing the distinction between precisely and imprecisely matched duplexes. The specificity of a nucleic acid probe therefore depends on its base sequence. By using the Southern transfer technique it is possible to detect a single-copy gene with a probe of less than 20 nucleotides long.

**Future prospects**

There are several advantages of nucleic acid detection of parasites which make this approach suitable for diagnostic tests. Firstly, these tests can be used to diagnose any specific organism and can differentiate between closely-related species. Secondly, they are sensitive and highly suited to detect carrier animals, making probes ideal for epidemiological studies. Thirdly, these tests are versatile and can be used to detect micro-organisms which cannot replicate in vitro or that lack protein components. Immunoassays preferentially detect surface proteins or antigens which are synthesised during replication of the parasite. Serological tests cannot always distinguish between closely-related organisms and may be unable to detect the low parasitaemia, or low levels of antibodies associated with carrier animals. Perhaps the most serious disadvantage of immunoassays is that the diagnosis is retrospective because of the presence of antibodies for varying periods of time after infection. However, it is unlikely that nucleic acid probes will ever replace serology entirely unless the techniques associated with probe technology are improved. Dependence on radioactive labelling and on short-lived isotopes is major drawbacks to the routine application of probes in the field. PCR has made the amplification of single-copy genes in individual parasites possible, yielding sufficient DNA for identification. This technique may also be applied to the amplification of parasite sequences in infected blood resulting in increased sensitivity of detection. This is especially important for the identification of carrier animals. Though, microscopic tests are having low sensitivity still it is designated as 'gold standard' tests because of its hundred percent specificity, easiness, low cost and widespread availability.

**Reference:**

Epidemiology and diagnosis of bluetongue in small ruminants
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Bluetongue (BT) is an infectious, non-contagious, insect borne disease caused by Bluetongue virus (BTV). It is transmitted by arthropods of the genus Culicoides and mostly prevalent in tropical, semitropical and temperate regions of the world. Natural cycle of BTV infection involves complex interaction between the insect vector, ruminant host and the environment. BTV infects most of the domestic and wild ruminants, causing variable clinical signs depending upon the species and the breed of the animal. However, Sheep are more sensitive to disease than cattle (MacLachlan, 1994). Most common symptoms of BT are high fever with excessive salivation, swollen lips and tongue, petechial haemorrhage, congestion and small ulcers on the mucous membranes of the mouth and conjunctiva, coroniitis and reproductive disorders leading to abortion or congenital deformities. Clinical BT has been observed in sheep, but the symptoms have never been noticed in goats, although a high level of BTV antibody prevalence has been observed in goats and other small ruminants.

Since the domestics ruminants are the part and parcel of the livestock industry, the disease is a major threat to this industry. The economic losses due to BT are attributed to high morbidity, mortality, abortion, foetal death and deformities as well as milk, meat and fleece losses. The economic impact of BT was in the order of 3 billion USD per year worldwide. Therefore, BT has been placed under list A by the World Organization for Animal Health i.e., Office International des Epizooties (OIE) (Alexander et al., 1994 and OIE, 2000) and represents a major barrier to international trade in animals and some animal products. Now, the disease has been included in the multi-species list of notifiable diseases by the OIE because of its substantial economic impact and potential for rapid spread.

Epidemiology
First decade of bluetongue (1964-1974)

After the initial report of BT in Maharashtra, the disease was reported in exotic sheep, namely Southdown, Rambouillet, Russian Merino and Corriedale, between 1967 and 1970. Severe BT was also reported in the Dorset breed in Andhra Pradesh in 1974. However, the native sheep maintained in close proximity did not present any symptoms. However, the disease was subsequently recorded in native sheep and disease outbreaks have been reported annually since 1981.

Endemic phase

During 1981, BT was widely spread in southern India. Initially, the disease was detected in Karnataka and in the adjoining regions of Maharashtra and Andhra Pradesh, with mortality rates ranging from 2% to 50%. Morbidity was as high as 80%. Later, in 1983, BT outbreaks were reported all over Andhra Pradesh with a case fatality rate of 21.9%. From 1985 onwards, outbreaks were recorded regularly in Andhra Pradesh with case fatality rates
ranging from 2.37% to 38.14%. A cyclical pattern of the disease was observed with variations in severity of infection.

The outbreaks of the disease in Maharashtra were characterised with morbidity and mortality rates of 7.66% and 1.11%, respectively. The case fatality rate was 11.82% (Harbola et al., 1982). Later, an increase in the severity of infection was reported with overall morbidity of 32%, mortality of 8% and a case fatality rate of 25% in rural areas. The disease was recorded regularly in Tamil Nadu where a total of 258 outbreaks were reported between 1986 and 1995. Saravanabava (1992) reported morbidity ranging from 3.3% to 22.8% and mortality from 0% to 6.1%.

The pattern of disease was studied in the organised farms and rural flocks of Andhra Pradesh. The study revealed that the pattern of the disease in organized farms and rural flocks is quite different. Morbidity, mortality and case fatality rates of rural and organised farms were 9.34%, 2.69%, 28.84% and 6.22%, 0.47%, 7.63%, respectively. Higher morbidity and mortality in rural areas may be because of stress factors, such as poor nutrition, parasitic burden, fatigue due to long walks and absence of veterinary aid. Investigations in Andhra Pradesh revealed that sheep aged 6 to 12 months were more susceptible than adults. The disease has not been reported in lambs. Similar observations were also reported from Maharashtra and Haryana.

The occurrence of BT varies between parts of India depending on time of rainfall. Maximum numbers of outbreaks were recorded during the north-east monsoon period (October to December) followed by the south-west monsoon period (June to September) in Andhra Pradesh. Similarly, in Tamil Nadu the outbreaks were more frequent during the north-east monsoon period (Saravanabava, 1992). In Rajasthan, the outbreaks occurred mostly in September and November (Mahajan et al., 1991).

**Diagnostic techniques for bluetongue:**

1. **Identification of the agent**
   - **Virus isolation**
     
     The same diagnostic procedures are used for domestic and wild ruminants. A number of virus isolation systems are in common use, but two of the most efficient are embryonated chicken eggs (ECE) and sheep. Identification of BTV following inoculation of sheep may still be a useful approach if the titre of virus in the sample blood is very low, as may be the case several weeks after infection. Attempts to isolate virus in cultured cells in vitro may be more convenient.
   
   **Isolation in cell culture**

     Virus may also be isolated in mouse L, baby hamster kidney (BHK)-21, African green monkey kidney (Vero) or *Aedes albopictus* (AA) cells in culture. The efficiency of isolation is often significantly lower following direct addition to cultured cells compared with that achieved in ECE. Greatest efficiency of isolation in cell culture is achieved by first passaging ECE homogenates in AA cells, followed by either antigen detection procedures or additional passages in mammalian cell lines, such as BHK-21 or Vero. A cytopathic effect (CPE) is not necessarily observed in AA cells. Cell monolayers are monitored for the appearance of a CPE for 5 days at 37°C in 5% CO₂ with humidity. If no CPE appears, a second passage is made in
cell culture. The identity of BTV in the culture medium of cells manifesting a CPE may be confirmed by antigen-capture ELISA, immunofluorescence, immunoperoxidase or virus neutralisation (VN) tests.

2. Immunological methods

Serogrouping of BTV

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP 7, that are conserved within each serogroup. The cross-reactivity between BT and epizootic haemorrhagic disease (EHD) viruses raises the possibility that an isolate of EHD virus could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. For this reason, a BT serogroup-specific monoclonal antibody (MAb) can be used. A number of laboratories have generated such serogroup-specific reagents (OIE, 2000). In contrast to serogrouping, the usual method of serotyping is by VN testing using methods described below.

Commonly used methods for the identification of virus to serogroup level are as follows:

a) immunofluorescence
b) antigen-capture enzyme-linked immunosorbent assay (ELISA)
c) immunospot test
d) indirect peroxidase/antiperoxidase identification.

Serotyping by virus neutralisation

Neutralisation tests are type-specific for the 24 BTV serotypes currently recognised and can be used to serotype a virus isolate, or can be modified to determine serotype of antibody. In the case of an untyped isolate, the characteristic regional localisation of BTV serotypes should generally obviate the need to attempt neutralisation by all 24 antisera, particularly when endemic serotypes have been identified.

There is a variety of tissue-culture-based methods available to detect the presence of neutralising anti-BTV antibody. Cell lines commonly used are BHK, Vero and L 929. BTV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included to ensure that an effective level of standard antiserum is used against comparable and standardised titres of standard and untyped virus. Four methods to serotype BTV are outlined briefly below:

a) plaque reduction
b) plaque inhibition
c) microtitre neutralisation
d) fluorescene inhibition test.

Polymerase chain reaction

Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis. Results to date indicate that PCR techniques may be used, not only to detect the presence of viral nucleic acid, but also to ‘serogroup’ orbiviruses and provide information on the serotype
and possible geographic source (topotype or genotype) of BTV isolates within a few days of receipt of a clinical sample, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification, may require at least three to four weeks to generate information on serogroup and serotype and yield no data on the possible geographic origin of the isolated virus (Murphy et al., 1999).

Oligonucleotide primers used to date have been derived from RNA 7 (VP7 gene), RNA 6 (NS1 gene), RNA 3 (VP3 gene) and RNA 2 (VP2 gene). The size of the amplified transcripts is usually small (in the order of several hundred nucleotides) but can also be a full-length gene. In the procedure, a 101 nucleotide stretch of RNA 6 is amplified. Primers derived from the highly conserved genes, such as VP3, VP6, VP7, NS1 and NS3, may be used for serogrouping (i.e. they will react with all members of the BT serogroup) and topotyping (i.e. they will react with BTV isolates from the same geographic area), while primers whose sequence was determined from VP2 gene sequences provide information on virus serotype.

The capacity of PCR assays to detect single molecules of nucleic acid means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids. The PCR assay involves three separate procedures. In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanates (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available for this purpose and the protocol describes the use of one such kit: IsoQuick (Orca Research, Bothell, Washington, USA). The reagents provided with the kit are numbered and their use is indicated in the protocol. Other kits are available and one, TrizolTM (Life Technologies, Grand Island, New York), is particularly useful for the extraction of viral nucleic acid from spleen or blood clots. Operators should follow the procedures specified in each kit and use reagent solutions either provided or recommended for the kit of their choice. The second procedure is the denaturation of viral double-stranded RNA and reverse transcription (RT) to generate DNA, which is amplified by PCR. In the procedure, the SuperscriptTM Preamplification System (Life Technologies) is used to transcribe viral RNA and reagents from Perkin-Elmer are used for the PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the PCR product by electrophoresis (OIE, 2000).

**References**


Diagnostic Tests for International Trade of Livestock Products
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Junagadh Agricultural University, Junagadh-362001

Abstract
Meat production is estimated at 6.3 million tones. Standing 5th rank of world’s meat production and account for 3% of total meat production. Although India has acquired number one status in the world, contributing 16.43% of the world’s milk production, the meat production which jibes well with dairying, is still lagging behind. India’s international trade in livestock and livestock products is mainly in live animals, meat and meat products, dairy products and eggs. Food recalls are an everyday event. Three million people die each year from food and water borne illness. Each year, in the United States alone 48 million people get sick; 128,000 are hospitalized and 3,000 die from food borne illness. Food safety is an issue of increasing concern worldwide and prioritisation of food safety as an essential public health function was advocated recently by the World Health Assembly. Better monitoring and surveillance demonstrates that the main burden of food-borne disease is due to microbiological pathogens of animal origin and this has important implications for the veterinary profession at both the international and the national level. This article aims to facilitate international trade animal products and to contribute to the improvement of livestock products services world-wide. The principal target readership is laboratories carrying out veterinary diagnostic tests in international trade.

Key words: Food Recalls, Food Borne Diseases, Livestock Products, Diagnostic Test

Introduction
Animal Products plays an important role in the socio-economic life of India. It is a rich source of high quality of animal products such as milk, meat and eggs. India has emerged as the largest producer of milk with 16.43 percent share in total milk production in the world. India accounts for about 4.95 percent of the global egg production and also the largest population of milch animals in the world, with 112.9 million buffaloes, 157 million goat and 74.5 million sheep. Exports of animal products represent an important and significant contribution to the Indian Agriculture sector. The export of Animal Products includes Buffalo meat, Sheep/Goat meat, Poultry products, Animal Casings, Milk and Milk products and Honey etc. India's exports of Animal Products was Rs. 20,130.90 Crores in 2012-13, which include the major products like Buffalo Meat (Rs. 17400.60 Crores), Sheep/Goat Meat (Rs. 425.66 Crores), Poultry Products (Rs. 494.14 Crores), Dairy Products (Rs. 1412.10 Crores), Animal Casing (Rs. 18.37 Crores), Processed Meat (Rs. 21.56 Crores), Swine Meat (Rs. 2.15 Crores), and Natural Honey (Rs. 356.32 Crore). The demand for Indian buffalo meat in international market has sparked a sudden increase in the meat exports. Buffalo meat dominated the exports with a contribution of over 86%. The product registered 27% growth in export during the financial year 2012-13 as compare to the same period of last year. The main markets for Indian buffalo meat and other animal products are Vietnam Social Republic, Malaysia, Thailand, Saudi Arabia, Egypt Arab Republic and UAE.
In term of export from India, Poultry Products, Processed Meat and Natural Honey recorded 7%, -28% and 11% growth respectively, during the financial year 2012-13 over the same period of last year. The major importing countries of these products were USA, Afghanistan, Saudi Arabia, Indonesia, Germany, Netherland and Denmark etc. Whereas, Dairy products sector register a growth of 388%. Skimmed milk in powder became the largest item of export from India, which accounts for nearly 77% of net milk and milk products exports during the year 2012-13, and Bangladesh, Egypt, UAE, Saudi Arabia and Algeria were the major importing countries of Dairy products from the country during the period. (APEDA)

On August 3, 2013, the world's biggest dairy exporter Fonterra said a bacteria, Clostridium botulinum, which can cause botulism and affects muscles, had contaminated 40 tonnes of its whey protein, most of which was sold to manufacturers to make their own products, including milk powder. A day later, China banned all milk powder imports from New Zealand. Hong Kong recalled 80,000 cans of Cow & Gate baby formula. Other companies that were affected include Shanghai Yanjiu; Dumex Baby Food, a Danone brand; Wahaha Health Food and Wahaha Import & Export; Coca-Cola (China) and Abbott. (Asia, 2013,)

At the same time there is requirement of proper diagnostic test at international level to avoid contamination, fraudulent practices and bioterrorism and to provide safe food for human consumption.

**Procedure for import of livestock products**

No live-stock product shall be imported into India without a valid sanitary import permit. The sanitary import permit shall be issued for import of livestock products if, after a detailed import risk analysis, the concerned authorities are satisfied that the import of the consignment will not adversely affect the health of the animal and human populations of this country. The import risk analysis shall be conducted by the concerned officers of the Department on the basis of internationally recognised scientific principles of risk analysis and the analysis shall be conducted with reference to the specific product and the disease situation prevailing in the exporting country vis-a-vis the disease situation in India. The issue of permits shall be refused if the results of the import risk analysis show that there is a risk of the specific product bringing in one or more specific diseases, which are not prevalent in the country and which could adversely affect the health and safety of the human and animal populations of this country. All livestock products shall be imported into India through the seaports or airports located at Delhi, Mumbai, Kolkata and Chennai, where the Animal Quarantine and Certification Services Stations are located (Department of Animal Husbandry, GOI).

**Screening of Diseases during Export and Import of Livestock Products**

**Cattle sheep & goat**

Bovine Spongiform Encephalopathy (BSE) and Scrapie are compulsorily notifiable diseases in exporting country and the country is free from TSE group of diseases. The exporting country is free from Foot and Mouth disease (Type C, SAT 1,2,3), Vesicular stomatitis, Rinderpest, Peste des petits ruminants, Contagious agalactia and Rift valley fever.

Originates from flocks and establishment that are free from clinical or other confirmation of Caprine Brucellosis, Bluetongue, Q fever, Enzootic abortion, Leptospirosis, Salmonellosis (S abortusovis), Contagious CaprinePleuropneumonia, Aujeszky’s disease, Sheep & Goat
Pox, Pulmonary Adenomatosis, Foot & Mouth Disease (Type O, A, Asia I), Paratuberculosis (Johne’s disease) and these diseases have not been reported during the last two years and no animal from a herd of inferior health status was introduced in the flock / establishment during that period. Caprine arthritis/encephalitis was neither clinically nor serologically diagnosed in the goats present in the flocks of origin during the past five years, and that no goats from a flock of inferior health status was introduced into these flock during this period. Examination must be carried out for the following diseases with negative result in each case within the last 45 days prior to entering the pre-export quarantine and/or within the last 30 days prior to exportation while in the pre-export quarantine:

**poultry meat**

Country is free from Avian Influenza (Highly Pathogenic Avian Influenza and Low Pathogenic Avian Influenza). The consignment comes from birds which were kept in an establishment where the incidence of the following diseases has not been reported during the last one year:


**Diagnostic Test for Livestock Products in International Trade**

The table below lists diagnostic tests in two categories: ‘prescribed’ and ‘alternative’. Prescribed tests are required by the OIE Terrestrial Animal Health Code for the international movement of animals and animal products At present it is not possible to have prescribed tests for every listed disease. Alternative tests are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement.

**Table 1: Diagnostic Test for Livestock Products in International Trade**

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Disease name</th>
<th>Prescribe test</th>
<th>Alternative test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Foot and mouth disease</td>
<td>ELISA*, Virus neutralisation</td>
<td>Compliment Fixation</td>
</tr>
<tr>
<td>2</td>
<td>Heartwater</td>
<td>-</td>
<td>ELISA, Indirect fluorescent antibody</td>
</tr>
<tr>
<td>3</td>
<td>Leishmaniosis</td>
<td>-</td>
<td>Agent identification</td>
</tr>
<tr>
<td>4</td>
<td>Paratuberculosis (Johne’s disease)</td>
<td>-</td>
<td>Delayed-type hypersensitivity, ELISA</td>
</tr>
<tr>
<td>5</td>
<td>Q fever</td>
<td>-</td>
<td>Compliment Fixation</td>
</tr>
<tr>
<td>6</td>
<td>Rinderpest</td>
<td>ELISA</td>
<td>Virus neutralisation</td>
</tr>
<tr>
<td>7</td>
<td>Trichinellosis</td>
<td>Agent identification</td>
<td>ELISA</td>
</tr>
<tr>
<td>8</td>
<td>African swine fever</td>
<td>ELISA</td>
<td>Indirect fluorescent antibody</td>
</tr>
<tr>
<td></td>
<td>Disease</td>
<td>Test(s)</td>
<td>Identification Method</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------</td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>10</td>
<td>Cysticercosis</td>
<td>-</td>
<td>Agent identification</td>
</tr>
<tr>
<td>11</td>
<td>Salmonellosis</td>
<td>-</td>
<td>Agent identification</td>
</tr>
<tr>
<td>12</td>
<td>Bovine Spongiform Encephalopathy (BSE)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Scrapie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Vesicular stomatitis</td>
<td>CF, ELISA, VN</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Peste des petits ruminants</td>
<td>VN</td>
<td>ELISA</td>
</tr>
<tr>
<td>16</td>
<td>Contagious agalactia</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Rift valley fever</td>
<td>VN</td>
<td>ELISA, HI</td>
</tr>
<tr>
<td>18</td>
<td>Brucellosis</td>
<td>BBAT, CF, ELISA, FPA</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Bluetongue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Enzootic abortion</td>
<td></td>
<td>CF</td>
</tr>
<tr>
<td>21</td>
<td>Leptospirosis</td>
<td>-</td>
<td>MAT</td>
</tr>
<tr>
<td>22</td>
<td>Contagious Caparine Pneumonia</td>
<td>--</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Aujeszky’s disease, Sheep &amp; Goat Pox</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>Pulmonary Adenomatosis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>Avian Influenza</td>
<td>Virus isolation</td>
<td>AGID, HI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with pathogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>testing</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>New Castle Disease</td>
<td>Virus isolation</td>
<td>HI</td>
</tr>
<tr>
<td>27</td>
<td>Marek’s disease</td>
<td>-</td>
<td>AGID</td>
</tr>
<tr>
<td>28</td>
<td>Avian Mycoplasmosis</td>
<td>-</td>
<td>Agg., HI</td>
</tr>
<tr>
<td>29</td>
<td>Haemorrhagic enteritis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>Infectious synovitis/sinusitis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Avian chlamydiosis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>Avian infectious bronchitis</td>
<td>-</td>
<td>ELISA, HI, VN</td>
</tr>
<tr>
<td>33</td>
<td>Avian infectious laryngotracheitis</td>
<td>-</td>
<td>AGID, ELISA, VN</td>
</tr>
<tr>
<td>34</td>
<td>Avian Leucosis J virus infections</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>Inclusion body hepatitis</td>
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**Table:**

<table>
<thead>
<tr>
<th></th>
<th>Disease</th>
<th>Test</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>36</td>
<td>Infectious bursal disease, AGID, ELISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Fowl typhoid Pullorum disease, Agent id., Agg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Avian tuberculosis, Agent id., Tuberculin test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Fowl pox, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Egg drop syndrome, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Avian encephalomyelitis, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Chicken anaemia virus, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Fowl cholera, -</td>
<td></td>
<td></td>
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</tbody>
</table>

(Source: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013)

**Conclusion**

International animal health standards designed to facilitate safe trade in livestock and livestock products are set by the Office International des Epizooties (OIE) under the Sanitary and Phytosanitary Agreement of the World Trade Organization (WTO) and documented in the OIE's Terrestrial Animal Health Code. A core principle of the Code is the need for countries to eradicate important Transboundary Animal Diseases (TADs) to reduce the risk of exporting disease to trading partners.

**References:**

Agricultural & Processed Food Products Export Development Authority, Ministry of Commerce and Industry, Government of India.

Department of Animal Husbandry, Government of India.

Introduction:

Brucellosis is a highly contagious and economically important bacterial disease of livestock worldwide. It is one of the five common bacterial zoonoses in the developed and developing world and is caused by organisms belonging to the genus *Brucella*: a gram-negative, non-spore-forming, intracellular bacterium. The genus *Brucella* consists of 9 species at present, of which 7, viz. *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae* and *B. microti* are terrestrial (Scholz et al., 2008) while *B. ceti* and *B. pinnipedialis* are marine species (Foster et al., 2007). Recently, a novel species, *B. inopinata*, isolated from a wound associated with a breast implant has also been included in the genus *Brucella* (Scholz et al., 2010).

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo-skeletal, cardiovascular, and central nervous systems. Precautions should be taken to prevent human infection. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public where the disease is endemic.

It is a serious disease affecting livestock worldwide with the rates of infection varying greatly from one country to another and between regions within a country. The highest prevalence is seen in dairy cattle. In India, brucellosis was first recognized in 1942 and is now endemic throughout the country. The disease has been reported in cattle, buffaloes, sheep, goats, pigs, dogs and humans. Despite the advances made in the diagnosis and therapy, brucellosis is still wide spread and its prevalence in many developing countries is increasing. Economic losses by brucellosis in animals owing to abortions, premature births, decreased milk production and repeat breeding and may lead to temporary or permanent infertility in infected livestock. Economic losses due to brucellosis in livestock are considerable in an agrarian country like India.

Epidemiology of Brucellosis in large animals:

The infection in cattle is usually caused by *B. abortus*. However, *B. melitensis* and rarely *B. suis* can also establish themselves in cattle. These infections are particularly dangerous to humans because of the high virulence of most *B. melitensis* and *B. suis* strains and due to the large numbers of bacteria that are excreted. In cattle and other *Bovidae*, *Brucella* is usually transmitted from animal to animal by contact following an abortion. Pasture or hay may be contaminated and the organisms are hence most frequently acquired by ingestion. Other routes like inhalation, conjunctival inoculation, skin contamination and udder inoculation from infected milking cups are possibilities. The use of pooled colostrums for feeding newborn calves may also transmit infection. Sexual transmission usually plays little role in the epidemiology of bovine brucellosis. However, artificial insemination can transmit the disease and semen must only be collected from animals known to be free of infection. Safe and recommended embryo transfer procedures are to be followed. Transmission to people can occur through the usual routes. However, ingestion of raw or undercooked bone marrow has also been implicated as a source of human infection. In cattle,
sheep, goats and swine, susceptibility to brucellosis is greatest in sexually mature animals. Young animals are often resistant, although it should be noted that latent infections can occur and such animals may present a hazard when mature. Breed may also affect susceptibility with the milking breeds seem to be the most susceptible to *B. melitensis*. Breed differences in susceptibility have not been clearly documented in cattle although genetically determined differences in susceptibility of individual animals have been demonstrated. However, management practices are far more important in determining the risk of infection. Latent or inapparent infections can occur in all farm animal species. These usually result from infection in-utero or in the early post-natal period. Such animals can retain the infection for life and may remain serologically negative until after the first abortion or parturition. Latent infection has been estimated to occur in the progeny of about 5% of infected cows. The extent of the problem in other species is not known, but latency has been documented in sheep. Acquired immunity has a substantial effect on susceptibility. In India calf-hood vaccination is practiced using *B. abortus* strain 19. Female calves aged between 6-12 months are subjected to vaccination. The National Control Programme on Brucellosis has been initiated since August, 2010 in India. It aims at mass screening followed by vaccination in areas of high endemicity.

**Diagnosis:**

The information on the international standards of diagnostic tests is available from the Manual of diagnostic tests and vaccines for terrestrial animals, OIE, 2009. The diagnostic tests can be divided into 3 broad types based on the target of detection:

1. **Identification of the agent**

   a) Staining methods

   Brucella are Gram negative coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or small groups. They do not form spores, and lack flagella, pili, or true capsules are not produced. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by Ziehl-Neelsen’s method. This is the usual procedure for the examination of smears of organs or biological fluids that have been fixed with heat or ethanol. Brucella organisms stain red against a blue background. A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used. However, these methods have a low sensitivity in milk and dairy products where Brucella are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules. The results, whether positive or negative, should be confirmed by culture.

   b) Culture

   **Collection of samples for culture**

   For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include aborted fetuses (stomach contents, spleen and lung), fetal membranes, vaginal secretions (swabs), milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the late pregnant or early post-parturient uterus, and the udder.

   Tissues: Samples are removed aseptically with sterile instruments. The tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, and macerated using a ‘Stomacher’ or tissue grinder with a small amount of sterile phosphate buffered saline (PBS), before being inoculated on to solid media.
Vaginal discharge: A vaginal swab taken after abortion or parturition is an excellent source for the recovery of Brucella and far less risky for the personnel than abortion material. The swab is then streaked on to solid media.

Milk: Samples of milk must be collected cleanly after washing and drying the whole udder and disinfecting the teats. It is essential that samples should contain milk from all quarters, and 10–20 ml of milk should be taken from each teat. The first streams are discarded and the sample is milked directly into a sterile vessel.

Dairy products: Dairy products, such as cheeses, should be cultured on the media described above. As these materials are likely to contain small numbers of organisms, enrichment culture is advised. Samples need to be carefully homogenised before culture, after they have been ground in a tissue grinder or macerated and pounded in a ‘Stomacher’ or an electric blender with an appropriate volume of sterile PBS.

Culture of samples

Direct isolation and culture of Brucella are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. A wide range of commercial dehydrated basal media is available, e.g. Brucella medium base, tryptose (or trypticase)–soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as B. abortus biovar 2, and many laboratories systematically add serum to basal media, such as blood agar base or Columbia agar, with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol dextrose agar, can be used. Selective media are prepared using all the basal media mentioned above. Appropriate antibiotics are added to suppress the growth of organisms other than Brucella. The most widely used selective medium is the Farrell’s medium. Another commonly used selective media is Thayer–Martin medium.

Some samples need enrichment owing to lower number of bacteria as in milk, colostrum and some tissue samples. Enrichment can be carried out in liquid medium consisting of serum–dextrose broth, tryptose broth (or trypticase)–soy broth (TSA) or Brucella broth. The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO₂ for up to 6 weeks, with weekly subcultures on to solid selective medium.

On suitable solid media, Brucella colonies can be visible after a 2–3-day incubation period. After 4 days’ incubation, Brucella colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker.

c) Identification and typing

Identification of Brucella organisms can be carried out by a combination of the following tests: organism morphology after Gram staining, colonial morphology, growth characteristics, urease, oxidase and catalase tests, and the slide agglutination test with an anti-Brucella polyvalent serum. The simultaneous use of several phages e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and rough species of Brucella. However, several characteristics, for example added CO₂ requirement for growth, production of H₂S (detected by lead acetate papers), and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, are revealed by routine tests that can be performed in moderately equipped non-specialised laboratories.
d) Nucleic acid recognition methods

The PCR, including the real-time format, provides an additional means of detection and identification of Brucella spp. Despite the high degree of DNA homology within the genus Brucella, several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP) and Southern blot, have been developed that allow differentiation between Brucella species and some of their biovars to a certain extent. Pulse-field gel electrophoresis has been developed that allows the differentiation of several Brucella species. An assay named AMOS-PCR which can distinguish the abortus, melitensis, ovis and canis spp. has been devised. A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of Brucella. The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most Brucella species as well as the vaccine strains B. abortus S19, B. abortus RB51 and B. melitensis Rev.1. In contrast to other PCRs, Bruce-ladder is also able to detect DNA from B. neotomae, B. pinnipedialis and B. ceti.

2. Serology:

No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals. It should be stressed that the serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The complement fixation test (CFT) is diagnostically more specific than the SAT. The diagnostic performance characteristics of the ELISAs are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred. For the control of brucellosis at the national or local level, the buffered Brucella antigen tests, i.e. the Rose Bengal test (RBT) as well as the ELISA are suitable screening tests. Positive reactions should be retested using a suitable confirmatory and/or complementary strategy.

Rose Bengal plate test (RBT): The RBT is one of a group of tests known as the buffered Brucella antigen tests which rely on the principle that the ability of IgM antibodies to bind to antigen is markedly reduced at a low pH. The RBT and other tests such as the buffered plate agglutination tests and the card test play a major role in the serological diagnosis of brucellosis worldwide. The RBT is a simple spot agglutination test where drops of stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction. The test is an excellent screening test but may be oversensitive for diagnosis in individual animals, particularly vaccinated ones.

ELISA tests: These tests offer excellent sensitivity and specificity whilst being robust, fairly simple to perform with a minimum of equipment and readily available from a number of commercial sources in kit form. They are more suitable than the CFT for use in smaller laboratories and ELISA technology is now used for diagnosis of a wide range of animal and human diseases. Although in principle ELISAs can be used for the tests of serum from all species of animal and man, results may vary between laboratories depending on the exact methodology used. Not all standardization issues have yet been fully addressed. For screening, the test is generally carried out at a single dilution. It should be noted, however, that although the ELISAs are more sensitive than the RBT, sometimes they do not detect infected animals which are RBT positive. It is also important to note that ELISAs are only marginally more specific than RBT or CFT.

Serum agglutination test (SAT): The SAT has been used extensively for brucellosis diagnosis and, although simple and cheap to perform, its lack of sensitivity and specificity mean that it should only be used in the absence of alternative techniques.
Complement fixation test (CFT): The sensitivity and specificity of the CFT is good, but it is a complex method to perform requiring good laboratory facilities and trained staff. If these are available and the test is carried out regularly with good attention to quality assurance, then it can be very satisfactory for diagnosis of brucellosis in humans and animals.

3. Other tests:

Many other serological tests have been employed. Some, such as the Rivanol or 2-ME test, are variations of the SAT and, although more specific, share many of its disadvantages. At present, the use of such procedures in the place of the standard test is not advised.

Milk testing: In dairy herds, milk is an ideal medium to test as it is readily and cheaply obtained, tests can be repeated regularly and give a good reflection of serum antibody. Milk from churns or the bulk tank can be screened to detect the presence of infected animals within the herd which can then be identified by blood testing. This method of screening is extremely effective and is usually the method of choice in dairy herds.

Milk ring test: The milk ring test (MRT) is a simple and effective method, but can only be used with cow’s milk. A drop of haematoxylin-stained antigen is mixed with a small volume of milk in a glass or plastic tube. If specific antibody is present in the milk it will bind to the antigen and rise with the cream to form a blue ring at the top of the column of milk. The test is reasonably sensitive but may fail to detect a small number of infected animals within a large herd. Non-specific reactions are common with this test, especially in brucellosis free areas. The milk ELISA is far more specific than the MRT.

Milk ELISA: The ELISA may be used to test bulk milk and is extremely sensitive and specific, enabling the detection of single infected animals in large herds in most circumstances.

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Epidemiological evidence and symptoms based diagnosis of livestock diseases

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Epidemiology helps not only for getting information on diseases in population but also gives information on factors that can lead to occurrence of diseases. Knowledge on different factors causing diseases helps for easy diagnosis of disease. Epidemiology helps for diagnosis as well as control of diseases since it can predict the occurrence of diseases. Along with epidemiology, both subjective and objective symptoms are needed for getting the complete picture of the disease. Important diseases of bacterial, viral and fungal origin have been discussed in this article which will help for a better diagnosis of these diseases.

BACTERIAL DISEASES

Anthrax (Bacillus anthracis):
It affects all animals except birds. Also it can be transmitted to man also. Outbreaks originating from a soil-borne infection always occur after a major climate change, for example heavy rain after a prolonged drought, or dry summer months after prolonged rain, and always in warm weather when the environmental temperature is over 15°C. Spores remain in soil for 20 or more years and becomes serious threat to livestock. (Thappa and Karthikeyan, 2001)

Symptoms:
- Per acute - Sudden death without clinical symptoms.

Black quarter (Clostridium chauvoei)
It occurs in cattle, buffaloe, sheep (Rarely in other animals). 6 months - 2-year-old animals in good condition are affected. When the disease occurs it is usual for a number of animals to be affected within the space of a few days. The disease is enzootic in particular areas, especially when they are subject to flooding. Typical blackleg of cattle has a seasonal incidence, with most cases occurring in the warm months of the year. The highest incidence may vary from spring to autumn. In some areas there is an increased prevalence in years of high rainfall. The case fatality rate in blackleg approaches 100% (Radostits, 2007).

Symptoms:
- Animals sometimes die without any premonitory symptoms. Fevered animals show lameness/stiffness. Affected region will show trembling and violent twitching. Crepitating swelling in hind or fore quarters. In recently lambed ewes, lesions develop at perineum, which becomes swollen and dark red. In sheep, acute febrile condition develops following injury.

Haemorrhagic septicaemia (Pasteurella multocida)
Hemorrhagic septicemia occurs in cattle, camels, and water buffalo and, to a much smaller extent, pigs and horses. Animals of all ages are susceptible but the most susceptible age group is 6 months to 2 years of age. The incidence of disease is reduced significantly in areas where the vaccine is used. Both morbidity and case-fatality rates vary between 50% and 100%. The overall mean case fatality rate for buffaloes is nearly three times as high as in cattle. Outbreaks of the disease are often associated with wet humid weather during the rainy season. In a study conducted by Dutta et al. (1990) in India, haemorrhagic septicaemia was responsible for the highest mortality and the second highest morbidity rate, as compared to foot and mouth disease, rinderpest, anthrax and blackquarter (blackleg, Clostridium chauvoei infection).

**Symptoms:**

High temperature, drop in milk yield, abdominal pain, diarrhoea or dysentery, rapid respiration, mucous membrane cyanotic, oedema on neck or brisket. Bronchopneumonia accompanied by fever.

**Mastitis** (Staphylococcus aureus, Streptococcus agalactiae, S. dysgalactia, S. uberis, Enterococcus faecalis. E.coli, Coliforms, Arcanobacterium pyogenus, Pseudomonas aeruginosa, Mycobacterium, Nocardia, Mycoplasma, Brucella, Pasteurella, Listeria, Candida, Cryptococcus, Aspergillus).

The prevalence of infected quarters increases with age, peaking at 7 years. Most new infections occur during the early part of the dry period and in the first 2 months of lactation. The highest average incidence of clinical mastitis due to environmental bacteria may occur in herds with the lowest bulk tank milk somatic cell count. High milking rate and large teat canal diameter have been associated with risk of intramammary infection. Peri parturient udder edema may also be a risk factor for clinical mastitis. Dirty udders are associated with increased SCC and an increased prevalence of intramammary infection due to contagious pathogens. Supplementation with antioxidants such as selenium and vitamin E had a beneficial effect on udder health in dairy cattle by decreasing the incidence and duration of clinical mastitis. Animals genetically above average for milk yield are more susceptible to mastitis and that low-yielding cows tend to be more resistant.

**Symptoms:**

1) **Per acute** - Animal febrile, off feed. e.g. 
   S. agalactiae, S. aureus, A. pyogenes, E.coli, P. multocida (In sheep Nocardia, coliform).
2) **Acute form** - Inflammation of gland severe but no systemic reaction, e.g. S. agalactiae, S. dysgalactiae, S aureus, Nocardia, Coliform
3) **Chronic:** when inflammation is mild, gland not swollen, pain and heat absent. (In general high fever, swelling of udder and straw coloured milk for coliform mastitis and thick yellowish milk with clot for staphylococcal and streptococcal mastitis). Presence of clot in watery fore milk is the only abnormality, induration is most readily palpable in the cistern and lower part of udder in S.aureus and coliforms.
4) **Sub acute** - S. zooepidemicus, coliform.
5) Sub clinical: Reduction in milk yield without any apparent changes in udder and milk.
6) Gangrenous mastitis. Skin of the quarter and teat semi- cold and bluish and slough off. e.g. S. aureus

**Brucellosis** (Brucella abortus, B melitensis, B.suis, B.canis)
Cattle, goat, pig and dog can be affected with this disease. It is transmissible to man also. Infection occurs in cattle of all ages but is most common in sexually mature animals, particularly dairy cattle. Abortions occur most commonly in outbreaks in unvaccinated heifers after the fifth month of pregnancy. Movement of an infected animal from an infected herd to a susceptible noninfected herd is a common method of transmission. A cow’s tail heavily contaminated with infected uterine discharges may be a source of infection if it comes in contact with the conjunctiva or the intact skin of other animals. The risk of spread from the infected bull is much higher, if the semen is used for artificial insemination.

**Symptoms:**
In cattle abortion can occur at 7-8 months of pregnancy. In chronic case abortion is less common but retained placenta is seen. In bull unilateral orchitis, synovitis and hygroma of knee are noted (Radostits, 2007). In sheep and goat abortion at 3-4 months of pregnancy - sometimes at full term, lameness, mastitis with discoloration of milk with clots can occur. In acute form loss of weight, pyrexia, diarrhoea, infertility also seen.

**Tuberculosis** (*Mycobacterium bovis*)
Cattle, goats, and pigs most susceptible and sheep and horses showing a high natural resistance. It is transmissible to man also. All age groups and species are susceptible but infection is predominantly in cattle and pigs. Infected cattle are the main source of infection but wildlife reservoirs are important in some regions and preclude the eradication of bovine tuberculosis in some countries. Inhalation is the major method of transmission. Organisms are excreted in the exhaled air, in sputum, feces, milk, urine, vaginal and uterine discharges and discharges from open peripheral lymph nodes.

**Symptoms:**
Symptoms depend on the organ involved. Pulmonary infection gives rise to dry cough, which increases in pregnancy. Animal will have loss of weight. In TB, mastitis milk is almost normal in the beginning and finally whey like and then milk production stops. In chronic productive type, infection is limited to one quarter, gland being enlarged and indurated, normal symmetry of udder will be lost. Supra mammary lymphnode will be enlarged.

**Para-tuberculosis (Johne’s disease)** (*M. avium subsp paratuberculosis*)
The disease occurs most commonly in cattle and to a lesser extent in sheep and goats. Incidence of clinical disease in herds is about 1% annually. Transmitted by fecal-oral route. Prenatal infection occurs. Infection occurs soon after birth. Long incubation period is the peculiarity of this disease. A distinguishing characteristic of Johne's disease is that infection occurs in animals at an early age, usually under 30 days of age, and clinical disease does not occur until 3-5 years of age.

**Colibacillosis (White scour in calves)** (*E. coli*)
Newborn calves, piglets, lambs, goat kids, foals. Risk factors include colostrum deprivation, overcrowding, adverse climatic conditions, inferior milk replacers. The prevalence can be as high as 50-60% in diarrheic calves under 3 days of age and only 5-10% in diarrheic calves 8 days of age.

**Symptoms:**
Scouring, weakness, and prostration. In less acute case, calf is listless, fails to suckle and develop diarrhoea. Swelling at joints and pneumonia in a few cases. High temperature initially, shows abdominal pain, faeces loose in consistency containing mucous and blood, weakness, fall in temperature, coma and death.

**Tetanus (Clostridium tetani)**

It occurs in all farm animals, mainly as sporadic cases, although outbreaks are occasionally observed in young cattle, young pigs, and lambs following wounding management procedures. History of a wound or tissue trauma. Occurs as isolated cases but also as outbreaks in young ruminants following castration and docking. The portal of entry is usually through deep puncture wounds. In young ruminants the case fatality rate is over 80%, but the recovery rate is high in adult cattle.

**Symptoms:**

Symptoms are similar in all animals: mild stiffness, unwillingness to move, last for 12-24 hours. General stiffness of limbs, head, neck and tail becomes rigid, tremor with restriction of jaw movement (locked jaw), anxious and alert expression, erect carriage of ears, absence of movement of eye lid, staring look, dilatation of nostril, drooling of saliva, constipation, retention of urine, bloat is an early sign in cattle, sweating may be profuse. Prolapse of 3rd eye lid occurs in horses.

**Leptospirosis**

*(L.interrogans serovar Icterohaemorrhagia, pomona, Canicola, Hardjo, Grippotyphosa etc)*

It is a major zoonosis. Disease has worldwide distribution. Most commonly in warm and wet climates. Occurs in cattle, sheep and goats, pigs, and horses. Prevalence of infection greater than incidence of clinical disease. Transmission is by urine of infected animals. Some wildlife species may transmit this disease to cattle. Ground surface moisture is most important factor for persistence of organism. Even after clinical recovery, animal may shed leptospirae in the urine for long periods.

**Symptoms:**

In acute form in cattle, high temperature, depression, petechiae of mucous membrane, diarrhoea, severe jaundice, dark coloured urine, dyspnoea and mortality can occur. Calves are more susceptible. In chronic form, stoppage of rumination, milk reduced or ceases, red in colour and may contain blood clot and abortion.

**Infectious foot rot (Dichelobacter nodusus)**

Sheep are the species principally affected but goats are also susceptible. Source of infection is lesion discharge from other infected sheep. Highly contagious disease with high attack rate in warm wet conditions. Moistness of the pasture and environmental temperature are major determinants for the transmission of foot rot.

**Symptoms:**

A sudden onset of lameness of several sheep is the usual presenting sign of foot rot. On close examination the earliest sign of virulent foot rot is swelling and moistness of the skin of the interdigital cleft and a parboiled and pitted appearance at the skin-horn junction in the cleft. There is a distinctive, foul-smelling exudate, which is always small in amount. Lesions are present on both claws of the foot and commonly in more than one foot. Significant effect on productivity.
Contagious bovine pleuropneumonia (Mycoplasma Mycoides subsp. Mycoides)

CBPP occurs only in cattle; rare natural cases have been observed in buffalo. In outbreak of susceptible cattle-morbidity can be up to 100%, mortality up to 50% if cattle is stressed. The principal route of infection is by the inhalation of infective droplets from active or carrier cases of the disease. Outbreaks due to introduction of inapparent 'carriers'. Incubation period is 3-6 weeks. The focus of infection is often provided by recovered 'carrier' animals in which a pulmonary sequestrum reserves a potential source of organisms for periods as long as 3 years.

Symptoms:
- Fever, agalactia, anorexia, depression, coughing, thoracic pain, back arched, dyspnea, expiratory grunting, pleuritic friction rubs, dull areas of lung, edema of throat and dewlap.

Contagious caprine pleuropneumonia (CCPP) (Mycoplasma capricolum subsp Capripneumonia)

Disease of goat and sheep. Infectivity is high with a morbidity of 100%. And the illness is acute and severe with a case mortality rate of 60-100%. The disease is readily transmitted by inhalation. Roy et al. (2010) have reported a high prevalence of CCPP infection in goats of Gujarat.

Symptoms:
- Rise in body temperature (104.5°F – 106°F) mucopurulent nasal discharge, coughing, laboured respiration, salivation and loss of weight. Abortion in pregnant animals. Mortality very high. Some cases arthritis with swelling of leg joints, lameness, oedematosus swelling in head, neck and limbs.

Fungal diseases

Dermatophytoses (Species of genera, Microsporum, Trichophyton and Epidermophyton)

All warm-blooded animals are susceptible to this disease. Housed animals are most susceptible. Direct contact with infected animals is the common method of spread of ringworm, but indirect contact with inanimate objects, particularly bedding, harness, grooming kits and horse blankets, is probably more important. Spores can exist on the skin without causing lesions, and up to 20% of normal animals in an infected group will act as 'carrier animals'.

Symptoms:
- Disease may be acute, chronic or sub-clinical, range from slight erythema to highly inflammatory with folliculitis, suppurating body lesions, extensive areas of alopecia and scarring. Circumscribed areas of hairless skin, thick gray crumbly crusts (cattle), or shiny, bald areas (horse), heavy pityriasis; common locations where infection likely to contact e.g. neck, sides.

Viral diseases

Foot and Mouth disease (Picorna virus)

FMD affects all cloven-footed animals. The morbidity rate in outbreaks of FMD in susceptible animals can rapidly approach 100%. However, the case fatality is generally very low, about 2% in adults and 20% in young stock. Spread from one animal to another is by inhalation or by ingestion. Spread between cattle is more likely to be by airborne means. The virus can persist in aerosol form for long periods in temperate or subtropical climates but not in hot and dry climates. The speed and direction of the wind are important factors in
determining the rate of airborne spread. In the most favorable circumstances, it is now estimated that disease can be transmitted between 250 km (156 miles). During an outbreak in UK, FMD was transmitted by mechanical carriage of virus especially humans (Kitching and Hughes, 2002). There are peaks of spread at dawn and dusk. The disease is most important in cattle and pigs but goats, sheep and buffaloes are also affected. It is the most contagious disease of livestock and has a great potential for causing severe economic loss in high producing animals.

Symptoms:

High fever, stringy salivation, smacking of lips, vesicles on the tongue, gums, dental pad, cheeks, around the muzzle, coronary band, interdigital cleft and udder.

**Rinderpest** (Paramyxovirus)

Animals susceptible include cattle, buffalo, sheep, goat, deer and pigs. It is highly contagious disease with high mortality. Presently, India is free of this disease. Natural infection occurs commonly only in domestic cattle and buffalo but in some outbreaks, sheep and goats do become infected and show clinical signs. When epidemics occur in highly susceptible populations, the morbidity and case-fatality rates approximate 100% and 50% (25-90%) respectively, and large numbers of in-contact animals may have to be destroyed. Cattle and buffalo of all ages are susceptible to rinderpest, unless they have been vaccinated or have recovered from a previous infection.

Symptoms:

Sharp rise in temperature, frothy salivation, mucopurulent oculonasal discharge, grayish white ulcers in the mouth, lower lip, gums, cheeks, tongue, foul smelling shooting diarrhoea. In atypical forms the above symptoms may be mild and the diarrhoea may be absent.

**PPR** (*Peste des petits ruminants*) (Goat Plague) (Paramyxovirus)

This contagious disease occurs mostly in goats and sheep. Outbreaks invariably occur when new stock is introduced into a farm. Infection rates in enzootic areas are generally high (above 50%) and can be up to 90% of the flock during outbreaks. Case fatality rates are much higher in goats (55-85%) than in sheep (less than 10%). Close contact with an infected animal or contaminated fomites is required for the disease to spread.

Symptoms:

Fever, dry muzzle and a serous nasal discharge later becoming mucopurulent Marked salivation due to the erosions on the mucous membrane of buccal cavity. Ulcers in the mucosa of alimentary, respiratory and urinary tracts. Profuse diarrhoea resulting in severe dehydration. Conjunctivitis with ocular discharge is a constant feature

**Mucosal disease/ Bovine viral diarrhoea** (Flavi virus)

It is a disease of cattle. In general, young cattle are most susceptible to BVDV infection but adult cattle may develop severe disease if infected with the highly virulent genotypes of the virus. Unvaccinated animals, improperly vaccinated animals, or animals whose immune status has waned are most susceptible to infection and the potential for clinical disease.

Symptoms:
Sharp rise in temperature frothy salivation, mucopurulent oculonasal discharge, greyish white ulcers in the mouth, lower lip, gums, cheeks, tongue, foul smelling shooting diarrhoea. In atypical forms diarrhoea may be absent.

**Viral enteritis** (Rota, Corona and Astro viruses)

Disease of young animals. Calves are most susceptible to rotavirus diarrhea between 1-3 weeks of age. This age occurrence is related in part to the rapid decline in specific colostral antibody. Peak incidence of rotavirus diarrhea which is at 5-7 days of age. Disease is more common during the winter months, which may reflect enhanced survival. The fecal-oral route is the presumed method of transmission but aerosol transmission may also occur.

*Symptoms:*

- Diarrhoea in young animals. Faeces may be watery, sometimes blood and mucus.

**Infectious bovine Rhinotracheitis/ Infectious Pustular vulvo vaginitis** (IBR/IPV) (Bovine Herpes virus)

A disease of cattle. Morbidity and mortality are higher in feedlot cattle than in dairy herds because of the frequent introduction of susceptible animals into an enzootic situation. The case fatality rate in the systemic form of the infection in newborn calves is almost 100%. All ages and breeds of cattle are susceptible. But the disease occurs most commonly in animals over 6 months of age, probably because of their greater exposure.

*Symptoms:*

- Respiratory symptoms; abortions; sometimes genital infections. Vulvo-vaginitis in cows and balanoposthitis in bulls.

**Ephemeral fever** (Three day sickness) (Rhabdo virus)

Cattle are susceptible to this disease. Although the case-fatality rate is very low, considerable loss occurs in dairy herds due to the depression of milk flow- up to 80% in cows in late lactation. Vectors includes the mosquitoes and biting midges. High morbidity but low case fatality. This disease is an enzootic in tropical areas.

*Symptoms:*

- Sudden appearance of high fever, muscular shivering, shifting lameness, stiffness of joints and enlargement of peripheral lymph nodes, reduction in milk yield, recumbency as a later manifestation.

**Cow pox and buffalo pox** (Pox virus)

This disease is trans-missible to man also. It is generally assumed that the virus gains access to tissues through injuries to teat skin, and extensive outbreaks of cowpox are likely to occur when the environment is conducive to teat injuries. Spread is rapid within a herd and immunity is solid, so that the disease tends to occur in sharp outbreaks of several months'duration with subsequent immunity protecting the cattle for at least several years.

*Symptoms:*

- Small red papules on teat and udder, In buffalo the lesions may be seen around the ears, ear flap, eyes, neck region and sometimes throughout the body.

**Sheep pox and Goat pox**

Both sheep pox and goat pox affect sheep and goats of all ages, breeds and sex but young and old animals and lactating females are more severely affected. Sheeppox and goaptox are highly contagious. The virus enters via the respiratory tract and transmission commonly is by aerosol infection associated with close contact with infected animals.
Symptoms:

Sheep:

High rise of temperature, increased respiratory rate, swollen eye lids, dermal oedema, with marked raised circular thickened plaques with congested borders. Lesions in wool free areas. Generalisation may occur - lesions in the oral, intestinal and respiratory tracts. Nodules in internal organs.

Goat:

Pocks on mucous membrane and skin, teat and udder. Pox like lesions on the lips - proliferative type of lesions in sheep and goats.

Blue tongue (Reovirus)

A disease seen in Sheep, Goat & cattle. Infection occurs in a number of animals but significant disease occurs only in sheep. Cattle are the reservoir and amplifying host and have a high titer viremia. Under natural conditions infection occurs in sheep and cattle. The disease is not contagious and is transmitted biologically by certain species of Culicoides. When the disease occurs in a flock for the first time the incidence of clinical disease may reach 50-75% and the mortality 20-50%. In a study conducted in Gujarat (Patel et. al., 2007), overall rate of seroprevalence of blue tongue in buffaloes was 35.26, 39.88 and 60.12 % using different tests such as BT-AGID, CCIE and c-ELISA, respectively.

Symptoms:

High rise of temperature, copious salivation, oculonasal discharges, swelling and hyperaemia of the mucosa of the mouth, ulceration of the tongue, dental pad and lips. Tongue becomes swollen, cyanotic, difficult to be retracted. Swelling and tenderness of the coronary band may occur.

Rabies (Rhabdo virus)

All warm blooded animals including man and birds are affected with this fatal disease. There is no variation in susceptibility with age. 1-day-old pigs have been found affected. The source of infection is always an infected animal, and the method of spread is almost always by the bite of an infected animal, although contamination of skin wounds by fresh saliva may result in infection. Traditionally, the dog, and to a minor extent the cat, have been the main source animals. Spread of the disease is often seasonal, with the highest incidence in the late summer and autumn because of large scale movements of wild animals at mating time and in pursuit of food.

Symptoms:

Rapidly fatal encephalitis following a somewhat long incubation period. Excitement followed by paralyses and death in 3-6 days following the onset of clinical symptoms.

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Differential Staining Techniques for Bacterial Identification
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Simple staining

Staining is an important technique in microbiology, which enables us to visualize the microorganisms to have the idea of shape and size. Simple staining procedure includes staining of the bacterial smears with a single stain. For example staining using methylene blue or the staining of blood smears from animals with stains to visualize the presence of microorganisms.

- In simple staining basic stains are used to stain the negatively charged particles. Usually the bacterial cell wall components and nucleic acids carry a negative charge which strongly attract the basic stain with the positively charged chromogen.
- The morphology and arrangement of the bacteria are visualized by simple staining. The basic stains commonly used for simple staining are methylene blue, crystal violet and carbol fuchsin.

Procedure:

1. Take a glass slide clean with alcohol and cotton, dry it
2. Make a thin smear from culture/broth/blood and heat fix over the flame.
3. Place the slide on staining rack and flood with the stain (methylene blue/Geimsa stain/ safranin) allow for 30 sec to 1 min
4. Wash the stain under the tap water in a slanting position
5. Air dry, observe under the oil immersion microscope

Differential staining:

These stains show differences among different types of bacteria and therefore are useful in their differential identification. These procedures involve more than one dye. Gram’s and acid fast stains belong to this category.

- Differential staining uses at least three reagents.
  - The first reagent is called primary stain, the second reagent is called a decolourizer and the third one is called counter stain.
  - The primary stain imparts colour to all the cells. The decolourizer is used to establish a colour contrast. The counter stain stains the cells that are decolourised.

Preparation of Smears

- Bacteria differ a slightly in refractive index from the surrounding medium. Hence, it is difficult to see unstained bacteria in ordinary microscope. So staining is the primary requirement to see the bacteria in the light microscope.
- Smear can be prepared from fluid materials like culture, urine, sputum, pus etc by taking a loopful of the material in an inoculating loop and spread it thinly on a clean glass slide. The smear is then allowed air dry.
- The smear can also be dried by holding it high over a bunsen flame.
- The dried smear is then fixed by passing it through the flame slowly three times with the smear upwards.
• Alternatively the fixing can also be done by heating through the slide. In this method the slide is held with the smear on top in the top of the Bunsen flame for a few seconds so that the slide becomes hot.
• The slides are then marked on one end with a diamond or grease pencil on the side having the smear. In slides with a ground matt surface at one end ordinary graphite pencil can be used to mark the slide.
• For preparation of smears from material like cultures on agar first a loopful of water or saline is placed on the slide and then with a sterilized loop a minute quantity of material is taken from the culture and put on the water placed on the slide and emulsified. Then a thin smear is prepared.

**Gram’s staining**

- Gram’s staining is a differential staining method. This method was developed by Christian Gram.
- Based on this staining bacteria are grouped into two categories, gram positive and gram negative.
- In this staining method four different reagents namely
  - primary stain,
  - mordant,
  - decolorizer and
  - counterstain.
- The *primary stain* normally used in Gram’s staining is crystal violet.
- The *mordant* used is Gram’s iodine. The iodine in Gram’s iodine combine with violet and forms a complex. The crystal violet iodine complex binds to magnesium RNA components in the cell. The resultant magnesium - RNA – crystal violet – iodine complex is bigger and difficult to remove from the cell.
- The *decolourising* agent used is ethyl alcohol (95%) which functions as a lipid solvent and dehydrating agent.
- Safranin or dilute carbol fuchsin is used as the *counter stain*.

**Materials Required**

- Primary stain – Crystal violet
- Mordant – Gram’s iodine
- Decolorizer – 95% ethanol
- Counter stain – Safranin
- Glass slide with smear
- Immersion oil
- Blotting paper
- Microscope

**Procedure:**

1. Keep the slide over a staining rack and flood the smear with crystal violet and allow it to react for 1-2 minutes.
2. Wash the smear with running tap water.
3. Flood the smear with Gram’s iodine and allow to react for one minute.
4. Wash the smear with running tap water.
5. Decolorize the smear using 95% ethanol for 10-20 seconds.
6. Wash the smear with running tap water.
7. Counter stain with safranin for 1-2 minutes.
8. Wash the smear with running tap water.
9. Blot dry and focus the smear under low power, high dry and oil immersion.

**Inference:**
- Gram positive bacteria stain deep violet.
- Gram negative bacteria stain red.
- By microscopic observation we can describe the staining, morphology and arrangement.

**Acid Fast Staining (Ziehl-Neelsen’s Staining)**

**Principle**
- Acid fast staining is an important differential staining technique.
- The primary stain referred to as acid fast stain binds strongly to only bacteria having waxy material on the cell wall. This method is used to identify organisms under the genus Mycobacterium.
- The important pathogenic species under this genus are *M.tuberculosis*, *M.bovis*, *M.avium*, *M.partuberculosis* and *M.leprae*. In this method strong carbol fuchsin is used as the primary stain, acid alcohol mixture is used as decoloriser and methylene is used as counter stain.
- The acid fast organisms retain the colour of the primary stain i.e., red, whereas the non-acid fast organisms take the color of methylene blue.
- The acid fastness is due to presence of waxes (mycolic acid) in the cell wall of the organisms.
- The acid fast organisms can withstand the decolorising action of acid alcohol mixture whereas the non-acid fast organisms cannot.

**Procedure**
- Flood the smear with carbol fuchsin and heat the stain using spirit lamp for 5 minutes. Do not allow the stain to evaporate. When fumes start emanate the stain stop heating. Once fumes subside start heating again. The purpose is to keep the stain under hot condition. Never allow the stain to dry over the smear.
- Wash the smear in running tap water after cooling.
- Decolorize with acid alcohol (until the smear appears colourless).
- Wash the smear in running tap water.
- Counter stain with methylene blue for two minutes.
- Wash the smear in running tap water.
- Blot dry and focus under oil immersion.

**Inference:**
If positive, acid fast (red) organisms are observed in the blue background. We can describe staining character and morphology.

**Staining Of Bacterial Capsule**

**Principle**
- The capsule is gelatinous in nature. Because of this it is difficult to stain the capsule, hence special staining methods are used.
The bacterial capsule is demonstrated by negative staining method. In negative staining the object is not stained but the background is stained. Hence object appears as colourless particle in a dark background.

Normally the objects that are difficult to stain or that won't take any stain are stained by negative staining method.

One of the best reagents to produce dark background is the India ink used for drawing. Since the bacterial capsules are gelatinous nature they won't take any stain. In a black background the capsules appear as halos.

The organisms are stained by simple stain like crystal violet.

**Procedure**

- Place a drop of 6% glucose solution at one corner of the slide.
- Add a drop of bacterial culture to the glucose solution on the slide and mix well.
- Add a drop of India ink to the mixture and mix well again.
- Using another slide prepare a thin smear.
- Air dry the smear thoroughly and then fix for 15 seconds in methanol.
- Drain away the methanol and remove the residual methanol by passing the slide through a gentle flame.
- Stain the smear with crystal violet for two minutes.
- Pour off the stain and wash the smear in running tap water.
- Air dry and examine under oil immersion.

**Inference**

If capsules are present they appear as unstained areas in the dark background. Inside the capsule the stained bacterium is seen.

**Staining Of Bacterial Spore**

**Principle**

- Certain genera under gram positive bacteria form spore during adverse conditions. These spores are referred to as endospores when they remain within the cell.
- The spores may either bulge the bacteria or not. Depending up on the position of the spore inside the cell, they are referred as terminal, subterminal or central.
- Spores are special structures consisting of a very thick cell wall, nucleic acid and a few ribosomes.
- The spore wall is rich in dipicolinic acid. Under favourable condition a spore may germinate into a vegetative bacterium.
- The process by which spores are formed within the cell is called sporogenesis.
- The process by which a spore turns into a vegetative bacterium is called germination.
- Spores are stained by special staining methods.
- The primary stain used is malachite green. Since spores have an impermeable coat the primary stain has to be applied as hot stain. The decolorizer used is tap water.
- Tap water can not remove the stain that has entered the spore. Safranin is used as counter stain. In this method the spores appear as green colour dots inside cells which are stained pink.

**Procedure**

- Prepare a smear from the suspected culture in a glass slide. Air dry it and fix with heat.
- Allow the water to boil in beaker and place the smear over the beaker on a staining rack.
- When water droplets are noticed below the slide, flood the smear with Schaeffer and Fulton stain solution A and allow to react for one minute.
- Wash the smear in cold tap water after cooling.
- Counter stain the smear with Schaeffer and Fulton solution B for 30 seconds.
- Wash the smear with tap water, blot dry and view under oil immersion.

**Inference**
If spores are present they are stained green and the vegetative part of the bacteria are stained red. We can describe the location of the spore - terminal, subterminal or central and whether it is bulging the bacterium or not.
Regulatory bodies and their role in animal product safety in context of infectious diseases

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Animal products such as meat, milk, and eggs are significant sources of high-quality food for humans and represent approximately one-sixth of their food energy and one-third of their food protein on a global basis. The microbiological safety of these animal products is of paramount importance to protect human health. Microbial food-borne illnesses are the largest class of emerging infectious diseases. Infectious diseases caused by the consumption of animal products is an important food safety issue worldwide and have also become an important cause of decreased economic productivity in both developed as well as developing countries.

Rapid industrialization, change in food preferences and food habits, mass food processing and lack of effective food quality control system has led to the emergence of many food-borne pathogens. More than 250 known diseases are transmitted to humans through food and most of these diseases are infections caused by a variety of bacteria, parasites, and viruses that can be food-borne. Food products of animal origin are the major sources for many of infectious organisms, which include Salmonella spp., Campylobacter spp., Listeria monocytogenes, Escherichia coli, Yersinia enterocolitica, Clostridium spp., Staphylococcus aureus, Vibrio spp., Mycobacterium tuberculosis etc. In addition to these, a number of viruses and parasites also get transmitted through the consumption of animal products. Most of these foods borne pathogens cause serious and some time fatal diseases in human being. Hence keeping in the view the impact of these food-borne pathogens, it is imperative to check their entry into the animal products to provide safe and wholesome food for human consumption. This control on the animal products is practiced by various agencies at national and international level.

Regulatory bodies for control of infectious diseases in animal products

There are various bodies at national and international level which have the responsibility to ensure the infection free milk, meat, egg and other animal products.

Regulatory bodies at national level

Various agencies at national level govern the microbiological safety of the animal products. These agencies have the responsibility to establish microbiological standards for different animal products along with the formulation of standard detection procedures. Agencies such as BIS, FSSAI, EIC, APEDA, AGMARK etc. are involved in regulating the indigenous, imported and exported animal products in India.
Bureau of Indian Standards (BIS)

National standardization activity started in India in 1947 with the establishment of the Indian Standards Institution (ISI) as a society under the Societies Registration Act 1860, to prepare and promote the adoption of national standards. In 1986 the national authorities made a review of the structure and status of ISI and assessed the impact made by it on the national economic development and the technological growth of various sectors of Indian industry. The Government of India felt that a new thrust had to be given to standardization and quality control activities, and that a national strategy had to be evolved for giving appropriate recognition and importance to standards and for integrating them with the growth and development of production and exports in different sectors. The Government of India therefore decided to create a statutory organization as the national standards body which was named as the Bureau of Indian Standards (BIS), with adequate autonomy as well as flexibility in its operations to achieve harmonious development of the activities of standardization, certification marking and connected matters. The Bureau of Indian Standards Act was passed by the Parliament in 1986 and BIS came into being on 1st April 1987. BIS has its Headquarters at New Delhi and its 05 Regional Offices (ROs) are at Kolkata (Eastern), Chennai (Southern), Mumbai (Western), Chandigarh (Northern) and Delhi (Central). BIS represent India in ISO. Microbiological criteria for various foods including those of animal origin are established by BIS through following standards.

<table>
<thead>
<tr>
<th>IS No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2491 : 1998</td>
<td>Food hygiene—General principles—Code of practice</td>
</tr>
<tr>
<td>15000 : 1998</td>
<td>Food hygiene—Hazard analysis and critical control point (HACCP)—System and guidelines for its application</td>
</tr>
</tbody>
</table>

Acceptability of a product is based on the absence or presence, or number of micro-organisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot.

Food Safety and Standards Authority of India (FSSAI)

The Food Safety and Standards Authority of India has been established under the Food Safety and Standards Act, 2006 as a statutory body for laying down science based standards for articles of food and regulating manufacturing, processing, distribution, sale and import of food so as to ensure safe and wholesome food for human consumption. The Act aims to establish a single reference point for all matters relating to food safety and standards, by moving from multi-level, multi-departmental control to a single line of command. The Act incorporates the salient provisions of the Prevention of Food Adulteration Act, 1954 and is based on international legislations, instrumentalities and codex Alimentarius Commission. In a nutshell, the Act takes care of International practices and envisages an over-reaching policy frame work and provision of single window to guide and regulate persons engaged in
manufacture, marketing, processing, handling, transportation, import and sale of food including that of animal origin.

The Agricultural and Processed Food products Export Development Authority (APEDA)

The Agricultural and Processed Food Products Export Development Authority (APEDA) was established by the Government of India under the Agricultural and Processed Food Products Export Development Authority Act passed by the Parliament in December, 1985. The Act came into effect from 13th February, 1986. In accordance with the Agricultural and Processed Food Products Export Development Authority Act, 1985, APEDA functions for fixing of standards and specifications for the scheduled products for the purpose of exports and also carry out inspection of meat and meat products in slaughter houses, processing plants, storage premises, conveyances or other places where such products are kept or handled for the purpose of ensuring the quality of such products.

AGMARK

Agmark is an acronym for agricultural marketing. Agmark is a quality certification mark provided by the Government of India. This certification confirms that the product or commodity in better term is scientifically laid down. It confirms the quality control and the best hygienic condition of the food including that of animal origin. The certification also marks the food standards keeping in mind the requirements of WTO (World Trade Organization).

Regulatory bodies at international level

Various regulatory bodies function at international level to harmonize the trade of animal products between different countries. These agencies are involved in establishing science based standards including microbiological standards to protect the importing countries from biological hazard and at the same time promoting the trade between the countries.

Codex alimentarius

The Codex Alimentarius Commission, established by FAO and WHO in 1963 develops harmonized international food standards, guidelines and codes of practice to protect the health of the consumers and ensure fair practices in the food trade. The Commission also promotes coordination of all food standards work undertaken by international governmental and non-governmental organizations. The Codex Alimentarius international food standards, guidelines and codes of practice contribute to the safety, quality and fairness of the international food trade. Consumers can trust the safety and quality of the food products they buy and importers can trust that the food they ordered will be in accordance with their specifications. The reference made to Codex food safety standards in the World Trade Organizations' Agreement on Sanitary and Phytosanitary measures (SPS Agreement) means that Codex has far reaching implications for resolving trade disputes. WTO members that wish to apply stricter food safety measures than those set by Codex may be required to justify...
these measures scientifically. Codex members cover 99% of the world’s population. More and more developing countries are taking an active part in the Codex process.

**Sanitary and phytosanitary (SPS) measures**

The Agreement on the Application of Sanitary and Phytosanitary Measures, also known as the SPS Agreement, is an international treaty of the World Trade Organization. It was negotiated during the Uruguay Round of the General Agreement on Tariffs and Trade, and entered into force with the establishment of the WTO at the beginning of 1995. Under the SPS agreement, the WTO sets constraints on member-states’ policies relating to food safety (bacterial contaminants, pesticides, inspection and labelling) as well as animal and plant health (phytosanitation) with respect to imported pests and diseases. There are 3 standards organizations who set standards that WTO members should base their SPS methodologies on. They are the Codex Alimentarius Commission (Codex), World Organization for Animal Health (OIE) and the Secretariat of the International Plant Protection Convention (IPPC).

**International Organization for Standardization**

The International Organization for Standardization known as ISO is an international standard-setting body composed of representatives from various national standards organizations. Founded on 23 February 1947, the organization promotes worldwide proprietary, industrial and commercial standards. It is headquartered in Geneva, Switzerland. ISO 22000 is a standard developed by the International Organization for Standardization dealing with food safety. It is a general derivative of ISO 9000. Food safety is linked to the presence of food-borne hazards such as infectious organisms in food at the point of consumption. Since food safety hazards can occur at any stage in the food chain it is essential that adequate control be in place. Therefore, a combined effort of all parties through the food chain is required. ISO 22000 integrates the principles of the Hazard Analysis and Critical Control Point (HACCP) system and application steps developed by the Codex Alimentarius Commission.

**OIE**

The World Organization for Animal Health is the intergovernmental organization responsible for improving animal health worldwide. It was created by an international agreement as the International Office of Epizootics (still known by its French acronym Office International des Epizooties – OIE) on 25 January 1924. It is recognized as a reference organization by the World Trade Organization (WTO) and maintains permanent relations with 35 other international and regional organizations. Its headquarters are in Paris, France. The organization was created following the rinderpest epizootic in Belgium in 1920. The disease had originated in India and concern over the spread led to an international conference in Paris in March 1921. An agreement was signed on January 25, 1924 by 28 countries. OIE has a total of 178 members.

Each Member Country undertakes to report the animal diseases that it detects on its territory. The OIE then disseminates the information to other countries, which can take the necessary
preventive action. This information also includes diseases transmissible to humans and intentional introduction of pathogens. Information is sent out immediately or periodically depending on the seriousness of the disease. OIE safeguard world trade by publishing health standards for international trade in animals and animal products. The OIE develops normative documents relating to rules that Member Countries can use to protect themselves from the introduction of diseases and pathogens, without setting up unjustified sanitary barriers. OIE standards are recognized by the World Trade Organization as reference international sanitary rules. To provide a better guarantee of food of animal origin and to promote animal welfare through a science-based approach OIE Member Countries have decided to provide a better guarantee of the safety of food of animal origin by creating greater synergy between the activities of the OIE and those of the Codex Alimentarius Commission. The OIE’s standard-setting activities in this field focus on eliminating potential hazards existing prior to the slaughter of animals or the primary processing of their products (meat, milk, eggs, etc.) that could be a source of risk for consumers. Among the Specialist Commissions, the one most closely connected with standardization is the Biological Standards Commission. This Commission establishes standards for diagnostic methods (including diagnostic preparations) and for vaccines. Its terms of reference reflect the Commission’s obligation to participate in the standardization of biological products, including vaccines used for prophylactic purposes. The Biological Standards Commission is responsible for the preparation of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, and the organization of Reference Laboratories for many of the diseases on the OIE List.

ICMSF

The International Commission on Microbiological Specifications for Foods (ICMSF, the Commission) was formed in 1962 through the action of the International Committee on Food Microbiology and Hygiene, a committee of the International Union of Microbiological Societies (IUMS). Through the IUMS, the ICMSF is linked to the International Union of Biological Societies (IUBS) and to the World Health Organization (WHO) of the United Nations. The primary goal is to provide timely, science-based guidance to government and industry on appraising and controlling the microbiological safety of foods including foods of animal origin.

Conclusion

Animal Products plays an important role in the socio-economic life of each and every country. The importance of the microbiological safety of these products cannot be ignored in this era of emerging and reemerging animal food borne infections. An infectious disease caused by the consumption of animal products is an important food safety issue and various national and international agencies viz. BIS, FSSAI, APEDA, AGMARK, CAC, ISO, OIE, ICMSF etc. are working continuously to protect human health from these infections. These agencies are involved in laying down the standards regarding infectious diseases in the products of animal origin to protect the human health within the country and harmonizing the trade between the countries.
References:


http://www.fssai.gov.in/

http://www.bis.org.in/

http://www.oie.int/
RT- PCR: Real Time Polymerase Chain Reaction

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Introduction: The advent of Polymerase Chain Reaction (PCR) by Kary B. Mullis in the mid-1980s revolutionized molecular biology. PCR is a fairly standard procedure now, and its use is extremely wide-ranging. At its most basic application, PCR can amplify a small amount of template DNA (or RNA) into large quantities in a few hours. This is performed by mixing the DNA with primers on either side of the DNA (forward and reverse), Taq polymerase (of the species *Thermus aquaticus*, a thermophile whose polymerase is able to withstand extremely high temperatures), free nucleotides (dNTPs for DNA, NTPs for RNA), and buffer. The temperature is then alternated between hot and cold to denature and reanneal the DNA, with the polymerase adding new complementary strands each time. In addition to the basic use of PCR, specially designed primers can be made to ligate two different pieces of DNA together or add a restriction site, in addition to many other creative uses. Clearly, PCR is a procedure that is an integral addition to the molecular biologist’s toolbox, and the method has been continually improved upon over the years. Several different types of real-time PCR are being marketed at this time, each with their advantages.

Fairly recently, a new method of PCR quantification has been invented. This is called “Real-time PCR” because it allows to actually view the increase in the amount of DNA as it is amplified. It is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, quantitative PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

Basic principle: Real-time PCR

It is able to detect sequence-specific PCR products as they accumulate in "real-time" during the PCR amplification process. As the PCR product of interest is produced, real-time PCR can detect their accumulation and quantify the number of substrates present in the initial PCR mixture before amplification began. Quantitative PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorochrome. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and DNA polymerase.

The PCR process generally consists of a series of temperature changes that are repeated 25 – 40 times, these cycles normally consist of three stages: the first, at around
95 °C, allows the separation of the nucleic acid’s double chain; the second, at a temperature of around 50-60 °C, allows the binding of the primers with the DNA template;[10] the third at between 68 - 72 °C, facilitates the polymerization carried out by the DNA polymerase. Due to the small size of the fragments the last step is usually omitted in this type of PCR as the enzyme is able to increase their number during the change between the alignment stage and the denaturing stage. In addition, some thermal cyclers add another short temperature phase lasting only a few seconds to each cycle, with a temperature of, for example, 80 °C, in order to reduce the noise caused by the presence of primer dimers when a non-specific dye is used. the temperatures and the timings used for each cycle depend on a wide variety of parameters, such as: the enzyme used to synthesize the DNA, the concentration of divalent ions and deoxyribonucleotides (dNTPs) in the reaction and the bonding temperature of the primers. There are several Alternative forms of Real Time PCR viz.qPCR (Quantitative polymerase chain reaction),QRT-PCR (Quantitative reverse- transcriptase PCR),RT-qPCR (Reverse- transcriptase quantitative PCR).

Real-time PCR

1. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/µl.

2. Set up the experiment and the following PCR program on ABI Prism SDS 7000. Do not click on the dissociation protocol if you want to check the PCR result by agarose gel. Save a copy of the setup file and delete all PCR cycles (used for later dissociation curve analysis). Please note the extension steps are slightly different from described in our paper.
   1. 50°C 2 min, 1 cycle
   2. 95°C 10 min, 1 cycle
   3. 95 °C 15 s -> 60 °C 30 s -> 72 °C 30 s, 40 cycles
   4. 72°C 10 min, 1 cycle

3. A real-time PCR reaction mixture can be either 50 µl or 25 µl. Prepare the following mixture in each optical tube.

<table>
<thead>
<tr>
<th>Mixture 1</th>
<th>Mixture 2</th>
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</thead>
<tbody>
<tr>
<td>25 µl SYBR Green Mix (2x)</td>
<td>12.5 µl SYBR Green Mix (2x)</td>
</tr>
<tr>
<td>0.5 µl liver cDNA</td>
<td>0.2 µl liver cDNA</td>
</tr>
<tr>
<td>2 µl primer pair mix (5 pmol/µl each primer)</td>
<td>OR 1 µl primer pair mix (5 pmol/µl each primer)</td>
</tr>
<tr>
<td>22.5 µl H₂O</td>
<td>11.3 µl H₂O</td>
</tr>
</tbody>
</table>

4. After PCR is finished, remove the tubes from the machine. The PCR specificity is examined by 3% agarose gel using 5 µl from each reaction.

5. Put the tubes back in SDS 7000 and perform dissociation curve analysis with the saved copy of the setup file.
6. Analyze the real-time PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

Dyes & Fluorescence detection chemistry in PCR

- SYBR Green I
- TaqMan Probes
- Molecular bacon
- Scorpion
- Light cycler probe

**SYBR Green:** It binds the Minor groove of double stranded DNA only and emits a strong fluorescent signal upon binding to double stranded DNA. Longer amplicons create a stronger signal.

Mechanism: At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation. During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.
Advantages

a. Flexible, compatible with existing RT-PCR primers.
b. Relatively low costs.

Disadvantages

a. Non specific binding (primer dimers, mismatching)
b. Dissociation curve analysis necessary
c. Multiplexing not possible
d. A standard curve is required for absolute quantification
e. Requires extensive optimisation.

**TaqMan Probes** are oligonucleotides that contain a fluorescent dye, typically on the 5' base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.

Advantages:

a. Sequence-specific detection
b. Multiplex PCR
c. No melting curve needed (faster)
d. Can be used for allelic discrimination

Disadvantages:

a. Probe design may be challenging
b. Probes are expensive

**Molecular beacons** also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement.
Applications:

There are numerous applications for quantitative polymerase chain reaction in the laboratory. It is commonly used for both diagnostic and basic research. Uses of the technique in industry include the quantification of microbial load in foods or on vegetable matter, the detection of GMOs (Genetically modified organisms) and the quantification and genotyping of human viral pathogens.

Diagnostic uses

Diagnostic quantitative PCR is applied to rapidly detect nucleic acids that are diagnostic of, for example, infectious diseases, cancer and genetic abnormalities. The introduction of quantitative PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases, and is deployed as a tool to detect newly emerging diseases, such as new strains of flu, in diagnostic tests.\(^{[30]}\)

Microbiological uses

Quantitative PCR is also used by microbiologists working in the fields of food safety, food spoilage and fermentation and for the microbial risk assessment of water quality (drinking and recreational waters) and in public health protection.

The antibacterial assay Virtual Colony Count\(^{[32]}\) utilizes a data quantification technique mathematically identical to real-time PCR, except bacterial cells, rather than copies of a PCR

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**Figure 1.** Diagram of molecular beacon. This beacon is 33 nucleotides long with a reporter dye attached to the 5' end and a quencher attached to the 3' end. The nine 5' bases are able to form base pairs with the nine 3' bases which brings the reporter and quencher in very close proximity. Therefore, when the reporter is excited by the appropriate light, its emission is absorbed by the quencher and no fluorescence is detected. The pink lines represent nucleotides that can form base pairs with the PCR product under investigation.
product, increase exponentially. The Virtual Colony Count equivalent of the threshold cycle is referred to as the "threshold time".

**Uses in research**

In research settings, quantitative PCR is mainly used to provide quantitative measurements of gene transcription. The technology may be used in determining how the genetic expression of a particular gene changes over time, such as in the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in environmental conditions. It is also used for the determination of zygosity of transgenic animals used in research.

**Detection of phytopathogens**

The agricultural industrial is constantly striving to produce plant propagules or seedlings that are free of pathogens in order to prevent economic losses and safeguard health. Systems have been developed that allow detection of small amounts of the DNA of *Phytophthora ramorum*, a fungus that kills Oaks and other species, mixed in with the DNA of the host plant. Discrimination between the DNA of the pathogen and the plant is based on the amplification of ITS sequences, spacers located in ribosomal RNA gene’s coding area, which are characteristic for each taxon. Field-based versions of this technique have also been developed for identifying the same pathogen.

**Detection of genetically modified organisms**

Q-PCR (using reverse transcription) can be used to detect GMOs given its sensitivity and dynamic range in detecting DNA. Alternatives such as DNA or protein analysis are usually less sensitive. Specific primers are used that amplify not the transgene but the promoter, terminator or even intermediate sequences used during the process of engineering the vector. As the process of creating a transgenic plant normally leads to the insertion of more than one copy of the transgene its quantity is also commonly assessed. This is often carried out by relative quantification using a control gene from the treated species that is only present as a single copy.

**Clinical quantification and genotyping**

Viruses can be present in humans due to direct infection or co-infections. This makes diagnosis difficult using classical techniques and can result in an incorrect prognosis and treatment. The use of Q-PCR allows both the quantification and genotyping of a virus such as the Hepatitis B virus. The degree of infection, quantified as the copies of the viral genome per unit of the patient’s tissue, is relevant in many cases; for example, the probability that the type 1 herpes simplex virus reactivates is related to the number of infected neurons in the ganglia. This quantification is carried out either with reverse transcription or without it, as occurs if the virus becomes integrated in the human genome at any point in its cycle, such as happens in the case of HPV (human papillomavirus), where some of its variants are associated with the appearance of cervical cancer.

**Conclusion:** PCR has proved to be a useful tool in research and diagnosis. However, its use has also brought new challenges to research. The sensitivity found in PCR technology and the availability of quantitative results will bring new problems to the interpretation of these
results. A great deal of work is needed to generate a basis of knowledge for correct interpretation of these tests. In medicine, PCR-based diagnostics are just becoming widely used and because of the increased cost-effectiveness of the newer assays, knowledge for their interpretation will soon become available. Real-time technology has significantly extended the use and scope of RT-PCR assays, with the potential for quantification of mRNA targets a particular advantage. However, considerable doubts remain about the biological validity of quantitative data. There is a particular requirement for rules concerning the information relating to experimental and analytical procedures that should be made publicly available with any publication involving this technology. Until this is implemented, real-time RT-PCR will not be able to make the most of its potential beyond its current role as a research tool.
Recent Approaches in Diagnosis of Mycobacterial Infections in Animals
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Mycobacterial organisms are intracellular acid fast bacilli which causes many important diseases such as tuberculosis in animals and human being. This genus consists of *Mycobacterium tuberculosis* (*M. tuberculosis*) complex, *M. avium* complex and other non-tuberculous mycobacteria. Main types of *M. tuberculosis* complex (mammalian tubercle bacilli) recognized are *M. tuberculosis*, *M. bovis*, *M. caprae*, *M. pinnipedii*, *M. africanum*, *M. microti* and the newly described *M. canetti* (Bercovier and Vincent, 2001). *M. avium avium* is the only species of consequence in birds. Paratuberculosis or Johne's disease is caused by the *Mycobacterium avium* subspecies *paratuberculosis*. Non-tuberculous mycobacteria includes *M. scrofulaceum* (scrofula), *M. genavense* (avian mycobacteriosis) and *M. marinum* (water related occupational granuloma) etc. Common and important mycobacterial diseases are Tuberculosis (TB) and Paratuberculosis (JD). It is estimated that more than 50 million cattle are infected with *Mycobacterium bovis* worldwide, resulting in severe economic losses (Fend *et al.*, 2005). *M. bovis* can cause progressive disease in most warm-blooded vertebrates, including humans. In a study conducted in Gujarat, true prevalence of JD was calculated to be 15.68% (Trangadia *et al.*, 2012). Diagnosis of mycobacterial infections especially TB and JD has to be performed as early as possible since both can cause severe economic loss. Zoonotic nature of Tuberculosis is another factor necessitating the correct diagnosis. Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same diagnostic tests of *M. bovis* can be used for its diagnosis.

The three basic purposes of diagnostic tests are infection discovery, confirmation and exclusion. Discovery tests are used for screening apparently healthy livestock populations to detect *M. bovis*-infected animals. A high sensitivity is desired in such assays and modest false-positive rates can be tolerated, since the discovery test is usually followed by a confirmatory test. Confirmatory tests are used when *M. bovis* infection is strongly suspected. Confirmatory tests must have a specificity approaching 100%, but lower sensitivity is acceptable. Exclusionary tests are used to rule out the presence of *M. bovis* infection when suspected. Thus, exclusionary tests must have a sensitivity approaching 100%. Exclusionary tests are usually too expensive for discovery purposes. Some diagnostic tests may be useful for more than one of these purposes. During the past century, the tuberculin skin lest has been the most frequently used diagnostic test world-wide, approved by OIE for use as the primary screening test in most countries for on-the-farm diagnosis of *M. bovis* infection of cattle. Recent advances in technology have allowed the development of new in vitro techniques, such as antibody-based, cell-mediated immunity-based and nucleic acid-based diagnostics, which allow more rapid diagnosis than bacteriological culture (Adams, 2001).
In vivo diagnosis

Tuberculin test

The tuberculin test is a surrogate test that is entirely dependent upon the appropriate cell-mediated immunological response of the host during the various phases of disease pathogenesis. Tuberculins are complex mixtures of soluble antigens produced by mycobacteria (M. bovis or M. avium) grown as floating cultures in synthetic liquid media, harvested after the mycobacteria are heat killed and removed by nitration followed by heat concentration (old tuberculin) (Koch, 1890) or chemical fractionation (purified protein derivative [PPD]).

Single intradermal (SID) test:

This test is applied by the intradermal injection of bovine tuberculin PPD (0.1 ml-unknown status, 0.2 ml - infected herd) into a skin fold and the subsequent detection of swelling as a result of delayed hypersensitivity. The reaction is read between 48 and 96 hours after injection with a preference for 48-72 hours for maximum sensitivity and at 96 hours for maximum specificity, and a positive reaction constitutes a diffuse swelling at the injection site. The cervical fold test is thought to provide greater sensitivity, the caudal fold providing the greater specificity (Radostits et al., 2007).

Stormont test:

This test is performed similarly to the single intradermal test in the neck with a further injection at the same site 7 days later. An increase in skin thickness of 5 mm or more, 24 hours after this second injection, is a positive result.

Comparative test:

Avian and bovine tuberculin are injected simultaneously into two separate sites on the same side of the neck, 12 cm apart and one above the other, and the test is read 72 hours later. The greater of the two reactions indicates the organism responsible for the sensitization.

Animals that are inconclusive by the single intradermal test should be subjected to another test after an interval of 42 days to allow desensitisation to wane (in some areas 60 days for cattle and 120 days for deer are used). Animals that are not negative to this second test should be deemed to be positive to the test. Animals that are positive to the single intradermal test may be subjected to a comparative intradermal test or blood test. The caudal fold test was reported to have a sensitivity of 72% with a specificity of up to 98.8%; the single intradermal cervical test has a sensitivity of 91.2% with a specificity of 75.5%. The comparative cervical test with PPD-B and PPD-A had a sensitivity of 68.6%-95% with a specificity of 88.8%-99.9% depending upon the circumstances in each country, the interpretation parameters, quality and dose of tuberculins, timing of prior tuberculin test, cross-reacting environmental mycobacteria and other organisms, temporal variations and chronicity of infections in populations, timing of testing relative to calving, and the skill of the applicator. Although the intradermal skin test can be useful for the detection of infected herds of cattle, the problem of false-positive test results in individual cattle associated with exposure to atypical mycobacteria highlights the need for tests with higher specificity.
In vitro diagnosis

It includes antibody-based, cell-mediated immunity-based and nucleic acid-based diagnostics, which allow more rapid diagnosis than bacteriological culture. Presumptive diagnosis of bovine tuberculosis via DTH response to PPD, clinical history, herd history, clinical findings and gross necropsy findings is significantly reinforced and/or confirmed by histopathological results, histochemical and immunohistochemical staining and especially by in situ hybridisation with *M. bovis*-specific probes. The gross and histopathological findings and the distribution of tuberculous lesions are directly influenced by the route, dose and virulence of the tubercle bacillus, the susceptibility of the host animal and the time after challenge. Gross lesion inspections not infrequently fail to detect minor or invisible lesions (particularly in the upper respiratory tract and lymph nodes of the head) in skin-test negative or positive animals that are subsequently found to be *M. bovis* culture-positive, causing concern regarding the efficiency of accurate diagnoses and the associated implications for tuberculosis control programmes.

Thorough gross and histological examination of six pairs of lymph nodes (mediastinal, medial retropharyngeal, bronchial, parotid, prescapular and prefemoral) together with the mesenteric nodes and lungs will detect 95% of cattle with gross tuberculous lesions. Following the detection of granulomatous lesions compatible with tuberculosis, the Ziehl-Neelsen or the auramine O acid-fast stains may be applied to impression smears or histosections to identify acid-fast bacilli. If the bacilli are confirmed to be acid fast, then immunohistochemical staining (Cassidy et al., 1999; Gutierrez et al., 1995) with antibodies specific to *M. tuberculosis* complex and/or polymerase chain reaction (PCR) (Miller et al., 1997), using either a 248 base-pair (bp) segment of IS1081 or a 123 bp segment of IS6110, may be performed to specifically identify *M. tuberculosis* complex organisms in tissues or paraffin-embedded histological sections. Immunohistochemical staining of tissue sections had a sensitivity equal to or greater than Ziehl-Neelsen staining on tuberculous tissues and provided a more specific *M. bovis* diagnosis. Tests based on PCR performed on formalin-fixed paraffin-embedded sections with typical tuberculous lesions and acid-fast bacilli were 93% positive (Miller et al., 1997), whereas PCR-based tests on fresh tissues were 91% positive (Wards et al., 1995). However, the PCR results were highly specific for *M. tuberculosis* complex mycobacteria and were obtained more rapidly than bacteriological culture (three to four weeks faster). Lastly, *M. bovis* has been detected by a deoxyribonucleic acid (DNA) oligonucleotide probe/PCR assay directly from bovine blood using general Al/Bl primers for spacer regions of the 16S and 23S ribosomal RNA genes specific for the *M. tuberculosis* complex and hybridised with an oligonucleotide probe specific to *M. tuberculosis* complex with a claimed analytical sensitivity of ten organisms in 100 μl of blood (Barry et al., 1993).

Bacteriological culture

The success of primary isolation of *M. bovis* from clinical specimens is influenced by the culture medium employed, the decontamination procedure and incubation conditions. Decontamination procedures for the primary tissues may affect the recovery of the mycobacteria significantly, thus these procedures must be followed carefully.
Decontamination has been achieved without adverse effects on recovery using 0.075% hexadecylpyridinium chloride. Most laboratories recommend the use of both an agar-based medium (Middlebrook 7H11) and an egg-based medium (Stonebrink or Löwenstein-Jensen with pyruvate) for primary isolation. Modified Middlebrook 7H11 medium (Veereman et al., 1986), B83 medium (Cousins et al., 1989) and radiometric detection (Neill et al., 1986) improved the recovery rate and speed of culture when used in conjunction with standard decontamination and concentration procedures. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used. Identification of mycobacterial isolates may be obtained by standard procedures including acid-fast staining, colony morphology, and a panel of biochemical tests, such as susceptibility to 2-thiophene carboxylic acid hydrazine (TCH) and isoniazid. These procedures require an additional three to four weeks. The use of monoclonal antibodies or molecular biology techniques reviewed below allow definitive identification of *M. bovis* and DNA fingerprinting of the isolate to be performed in one day.

**Antibody-based diagnostics**

A sensitive and specific serological test to detect antibodies to *M. bovis* antigens in domestic livestock and sylvatic populations exposed to tuberculosis would be very useful to replace or supplement the intradermal test. In general, antibody production by infected individuals is variable, largely undetectable during the early subclinical stages of tuberculosis, and usually occurs during the advanced stages of the disease when cell-mediated immunity tests may be negative due to anergy. The spectrum of purified and complex crude antigens identified and the use of these antigens in a variety of antibody detection procedures, mostly based on the enzyme-linked immunosorbent assay (ELISA). The sensitivities and specificities of virtually all the tuberculosis serological tests, including the indirect and competitive ELISA and Western blot, are relatively poor. This is probably due to the high degree of polymorphism in the antigen recognition, variable kinetics of the antibody response, possible *M. bovis* heterogeneity, and variable rate of disease progression of tuberculosis from animal to animal. Additionally, high rates of false positive serological tests are observed due to extensive cross-reactivity among mycobacterial species. But the exception, memory immunoglobulin (Ig) G1 recombinant MPB70 ELISA (Lightbody et al., 1998) before and after skin testing, serological tests are best used to complement cell-mediated immunity-based diagnostic tests.

**Cell-mediated immunity-based diagnostics**

Predominant immune response to mycobacterial infections in cattle is cellular rather than humoral. The developments range from the early lymphocyte blastogenic assays to the detection of specific lymphokines from specific T cells stimulated with crude, purified and recombinant antigens, or synthetic peptides. Gamma-interferon assay is an alternative test for early detection of tuberculosis. In this test, the release of a lymphokine gamma interferon (IFN-γ) is measured in a whole-blood culture system. The assay is based on the release of IFN-γ from sensitized lymphocytes during a 16–24-hour incubation period with specific antigen (PPD-tuberculin) (Wood et al., 1990). The test makes use of the comparison of IFN-γ production following stimulation with avian and bovine PPD. The detection of bovine IFN-γ
is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon. The use of defined mycobacterial antigens such as ESAT 6 and CFP-10 shows promise for improved specificity of this test. The IFN-γ ELISA has been evaluated worldwide and found to be almost always useful as a complement to skin-testing in most countries and, under some conditions, suitable for use as a primary tuberculosis test.

**Nucleic acid-based diagnostics**

Since *M. bovis* has the widest host range of the mycobacteria in the *M. tuberculosis* complex, it is one of the important obstacles to differentiate strains. From the early DNA fingerprint analysis by restriction endonuclease procedures by Collins and de Lisle (1985), to the restriction fragment length polymorphism (RFLP) analysis by Thierry *et al.* (1990), followed later by the spoligotyping methods of Kamerbeck *et al.* (1997), and multiplex PCR procedures of Sreevatsan *et al.* (2000), the detection and differentiation of *Mycobacterium* spp. by nucleic techniques clearly hold great promise for epidemiological investigations at local, national and international population levels. Nucleic acid-based procedures help not only to detect *M. bovis*, but to differentiate among members of the *M. tuberculosis* complex and identify strains infecting different populations of animals. This area of tuberculosis investigation has experienced a tremendous growth in productive research with direct application in laboratories on a world-wide basis. These techniques serve as the basis for improved tuberculosis control and eradication programmes at farm, regional, national and international levels. Combinations of multiple RFLP analysis and spoligotyping were found to yield the best differentiation among field strains of *M. bovis*. Further refinements for molecular fingerprinting of *Mycobacterium* spp. are underway and include genome-based fluorescent amplified-fragment length polymorphism (FAFLP) using two restriction endonucleases (Goulding *et al.*, 2000) and analysis of whole genomic relatedness through high-density DNA microarrays (Behr *et al.*, 2000). Both these procedures offer an enhanced ability to differentiate among strains without having to use technically demanding and laborious procedures.

The diagnosis of bovine tuberculosis by clinical examination is of very limited value given that most animals infected with the bacterium do not show clinical signs of the disease and that there are no pathognomonic signs of bovine tuberculosis in cattle. So it is important to diagnose the disease as early as possible to avoid spread of infection to healthy animals. Suspected cases can be confirmed using advanced diagnostic techniques. Recent diagnostic techniques can replace the time consuming procedures of culturing also.

**References**


Antibiotic Resistance: Practices and Management

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Introduction

In the past 50 years, antibiotics have been critical in the fight against many diseases and infections. Their discovery was one of the leading causes for the dramatic rise of average life expectancy in the 20th century and their significance to public health would be impossible to overstate. Antibiotics are defined as any compound which either kills or severely impedes the growth of bacteria. Upon the introduction of penicillin into general clinical practice in 1944, formerly deadly illnesses such as Strep throat and tuberculosis became instantly curable. Today, our dependence on antibiotics is absolute. In 1998, in the United States, it was estimated that there were 80 million prescriptions of antibiotics for human use, the equivalent of about 12,500 tons in one year. When animal and agricultural uses of antibiotics are added to human use, it is estimated that in the past 50 years, more than 1 million tons have been produced and disseminated.

Almost as soon as antibiotics were introduced into clinical circulation, cases where their ability to effectively stop infection were observed. As the use of antibiotics became more widespread, the prevalence of antibiotic resistant bacteria increased. In a recent study in Atlanta, 25% of bacterial pneumonia cases were shown to be resistant to penicillin, while a further 25% of cases were resistant to more than one antibiotic. Resistance development has resulted in perpetual research and development in the search of new antibiotics in order to maintain a pool of effective drugs at all times. While the development of resistant strains is inevitable, the speed and scale of development has been exacerbated by the practices through which we use and disseminate antibiotics.

Resistance to antimicrobials is a natural biological phenomenon. The introduction of every antimicrobial agent into clinical practice has been followed by the detection in the laboratory of strains of microorganisms that are resistant, i.e. able to multiply in the presence of drug concentrations higher than the concentrations in animals receiving therapeutic doses. Such resistance may either be a characteristic associated with the entire species or emerge in strains of a normally susceptible species through mutation or gene transfer. Resistance genes encode various mechanisms which allow microorganisms to resist the inhibitory effects of specific antimicrobials. These mechanisms offer resistance to other antimicrobials of the same class and sometimes to several different antimicrobial classes.

Types of resistance

1. Natural Resistance: Inherently or genetically resistant due to lack of penetration of drug into bacterial cell, absence of metabolic pathway or target site or rapid inactivation of drug in bacterial cell.
2. **Acquired resistance**: Resistance against drug to which bacteria was previously sensitive. It is due to inappropriate use of antimicrobials. It is done by mutation or gene transfer.

**Development and acquisition of resistance**

Many infectious diseases have been brought under control around the world yet this remains the leading cause of death in the world. Furthermore, previously controlled infections are becoming increasingly common in patients with diseases like AIDS where the immune system is compromised. The microbes responsible for these infections are often antibiotic resistant pathogens. The ability for the pathogens to grow despite the presence of antibiotics, through the development of antibiotic resistance, has rendered victims as vulnerable as patients from the pre-antibiotic era. The development of resistance is inevitable following the introduction of a new antibiotic. In fact initial rates of resistance to new drugs are normally on the order of 1%. However modern uses of antibiotics have caused a huge increase in the number of resistance bacteria. In fact within eight to twelve years, after wide spread use, strains resistant to multiple drugs become widespread.

How do bacteria become resistant to antibiotics and what are the biochemical mechanisms that they use? Several mechanisms have been developed by bacteria in order to deal with antibiotics but all require either the modification of existing genetic material or the acquisition of new genetic material.

Originally it was believed that all resistance was acquired through spontaneous mutation. Development of resistance through this method is called primary resistance. Errors in DNA synthesis during replication and occasional failures in the DNA repair systems result in a spontaneous mutation frequency for an individual base pair of about 10^-7-10^-8. This means that for every 10^7-10^8 bacteria, we would expect one single base pair to be changed. Mutation is a very rare event. However, the spontaneous mutation rate to acquire a mutation that causes resistance is often even lower since multiple mutations must take place before primary antibiotic resistance can be acquired. In *E. coli*, it has been estimated that primary streptomycin resistance is acquired at a rate of approximately 10^-9 when exposed to high concentrations of streptomycin. While this is an extremely rare event, the very fast growth rate of bacteria means that it doesn’t take long before resistance is developed in a population. Once the resistance genes are acquired, the genes can be transferred directly to all the bacteria’s progeny. This is known as vertical gene transfer.

**Mechanism of resistance**

1. **Reduced drug accumulation**: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface
2. **Drug inactivation or modification**: e.g., enzymatic deactivation of *Penicillin* in some penicillin-resistant bacteria through the production of β-lactamases.
3. **Alteration of target site**: e.g., alteration of PBP — the binding target site of penicillins — in MRSA and other penicillin-resistant bacteria.
4. Alteration of metabolic pathway: e.g., some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides. Instead, like mammalian cells, they turn to utilizing preformed folic acid.

Table 1: Reports of resistance developed in bacteria from India

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Resistance to</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae</td>
<td>Penicillin, cotrimoxazole, tetracycline, erythromycin, ciprofloxacin</td>
<td>Goyal et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chawala et al., 2010</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>Penicillin, erythromycin, trimethoprim</td>
<td>Capoor et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bergmann et al., 2012</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Clindamycin, Vancomycin</td>
<td>Gupta et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thati et al., 2011</td>
</tr>
<tr>
<td>E. Coli</td>
<td>Ampicillin, tetracycline, co-trimazole, trimethoprim, carbenicillin</td>
<td>Sukumaran et al., 2012</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>Nalidixic acid, ciprofloxacin, ampicillin, chloramphenicol, ampicillin and trimethoprim</td>
<td>Rowe et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nagshetty et al., 2010</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Ceftizoxime, cefotaxime, carbenicillin</td>
<td>Sikarwar &amp; Batra, 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nagaraj et al., 2012</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>Newer gen. fluoroquinolones, 3rd gen. Cephalosporins</td>
<td>Bhattacharya et al., 2012</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>Ciprofloxacin, ceftazidime, cefepime, gentamicin, amikacin</td>
<td>Chaudhary et al., 2013</td>
</tr>
</tbody>
</table>

Consequences of resistance

The emergence of antimicrobial resistance has an impact on the cost of animal and human health care worldwide. Ineffective therapy due to antimicrobial resistance is associated with increased animal and human suffering, loss of productivity and often death (WHO, 2001). Resistant strains of bacteria are found around the world. Organisms that are resistant to one drug are more likely to become resistant to others. The bacteria Streptococcus pneumonia has become resistant to penicillin and now demonstrates some resistance to several other antibacterials. Resistant pathogens are expensive to control and extremely difficult to eradicate. Ineffective therapy can seriously affect the progress and outcome of disease. It has significant impact the cost of treating disease. Limited clinical effectiveness of readily available cheap antimicrobials in many regions which results in difficult to choice and to use more effective but more expensive drugs to treat. Resistant animal pathogens in food products may cause infections in humans that are difficult to treat. Loss of public confidence in the safety of food which affects the demand for products, with potentially serious economic effects on the farming sector.

Practices responsible for failure of antimicrobials in disease state:

- Improper diagnosis (Viral not Bacterial infection)
- Improper selection of drug (Causative organisms are not sensitive to drug).
- The microorganisms have developed resistance to drug.
Mixed infection & narrow spectrum drug
Penetration of drug into site infection is not proper due to pus, debris, exudates etc.
The host defense mechanism is impaired
Improper route of administration with inadequate duration of treatment
Interaction of drug with other administered drugs.
Late administration of antimicrobial drug
Use of expired drug
The owner or attendant of animal does not comply with therapeutic regimen
Improper nursing and feeding

Management of the Resistance Problem
How can we select antibiotics?
Requires clinical judgment and detailed knowledge of pharmacological and microbiological factors.
Antibacterials : empirical therapy, definitive therapy, and prophylactic therapy.
Empirical therapy: infecting organism has not been identified - Combination therapy/broad-spectrum agent
Infecting microorganism is identified : Narrow-spectrum AB
Select an antibacterial based on indication
The diagnosis may be masked if therapy is started before cultures are obtained.
Antibacterials may be used immediately if disease is severe
Initiation of optimal empiric antibacterial therapy: knowledge of most likely infecting organisms and their antibacterial susceptibilities.
Simple and rapid laboratory tests may permit more rational selection of initial antibacterial therapy.
Blood should be taken prior to the institution of drug therapy.
For definitive therapy, Use specific & narrow-spectrum antibacterial once an organism has been identified & its susceptibility is known.

Successful Antimicrobial Therapy depends on:
For definitive therapy, recommend a narrow-spectrum drug
Keep the broad spectrum drug reserve for life threatening infection
Prefer bactericidal over bacteriostatic drug with less toxicity
Prefer drug requires administration at long interval
For less severe infections prefer an oral administration in small animals
For severe infection ➔ parenteral administration
Always use antimicrobial agent in proper dose
Proper duration of time
Do not combine antimicrobials without valid cause
Do not use antimicrobial indiscriminately
Avoid overuse of newer agent if older is effective
Use drug manufactured by reliable pharmaceutical firm.
Do not use antimicrobials to treat slight, self-limiting or unbeatable infections.

How can we fight back?

ASCAD Training on “Advanced approaches for diagnosis of livestock diseases” (25-30 Nov., 2013)
Maintain good hygiene and infection control measures – particularly hand washing.
Strict infection control measures should be monitor in hospitals
Don’t use antibacterial in minor or self limiting viral infections
Farmers should not use antibacterials of previous prescription
Educate farmers: help them to understand about cost of unnecessary use of antibacterials
Communicate with farmers about progression of disease after initiation of therapy
Use laboratory tests to support your diagnosis & select the right antibacterial.
Record of vaccinations must be generated.
Develop and implement guidelines, protocols and drug utilization reviews to ensure that use of antibacterial drug is optimized
Ensure surveillance for changes in the occurrence and pattern of antimicrobial resistance in different bacteria.
Emphasise good animal husbandry practices (adequate and clean quarters)
Work with governments to move away from using antibacterials as growth promoters.
Collaborate in monitoring of antibacterial use and resistant pattern with institutes
Educate the public and health professionals about the antibacterial resistance
Coordinate the development and implementation of regional programs to optimize antibacterial use and to prevent the spread of resistant organisms.
Develop the rapid affordable systems for diagnosis and susceptibility testing.
Ensure that antibacterials remain available through prescription only, rather than as over-the-counter medications.

Table 1: Type, mode and spectrum of activity of different antimicrobial agents

<table>
<thead>
<tr>
<th>Group of antimicrobials</th>
<th>Type of action</th>
<th>Mode of action</th>
<th>Spectrum of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>Bactericidal</td>
<td>Inhibition of cell wall synthesis</td>
<td>Narrow</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Bactericidal</td>
<td>Inhibition of cell wall synthesis</td>
<td>Broad</td>
</tr>
<tr>
<td>Carbapenems</td>
<td>Bactericidal</td>
<td>Inhibition of cell wall synthesis</td>
<td>Broad</td>
</tr>
<tr>
<td>Polypeptide Antibacterials</td>
<td>Bactericidal</td>
<td>Inhibition of cell wall synthesis</td>
<td>Narrow</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Bactericidal</td>
<td>Inhibits DNA synthesis</td>
<td>Broad</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>Bactericidal</td>
<td>Inhibits DNA synthesis</td>
<td>Narrow</td>
</tr>
<tr>
<td>Rifamycins</td>
<td>Bactericidal</td>
<td>Inhibitions of RNA transcription</td>
<td>Narrow</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Bacteriostatic/Bactericidal</td>
<td>Inhibition of protein synthesis</td>
<td>Narrow</td>
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<tr>
<td>Lincosamides</td>
<td>Bacteriostatic</td>
<td>Inhibition of protein synthesis</td>
<td>Narrow</td>
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<td>Macrolides</td>
<td>Bacteriostatic</td>
<td>Inhibition of protein synthesis</td>
<td>Narrow</td>
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<tr>
<td>Tetracyclines</td>
<td>Bacteriostatic</td>
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<td>Broad</td>
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<td>Chloramphenicol</td>
<td>Bacteriostatic</td>
<td>Inhibition of protein synthesis</td>
<td>Broad</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Bacteriostatic</td>
<td>Competitive inhibition</td>
<td>Broad</td>
</tr>
</tbody>
</table>

Table 2: Spectrum of activity of commonly used antimicrobials

<table>
<thead>
<tr>
<th>G+ve activity</th>
<th>G-ve activity</th>
<th>Anaerobic activity</th>
</tr>
</thead>
</table>

ASCAD Training on "Advanced approaches for diagnosis of livestock diseases" (25-30 Nov., 2013)
<table>
<thead>
<tr>
<th>Penicillins</th>
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<th>Penicillins</th>
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<tr>
<td>Macrolides</td>
<td>Aminoglycosides</td>
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<td>Cephalosporins</td>
<td>Cephalosporins</td>
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<tr>
<td>Sulpha + TMP</td>
<td>Sulpha + TMP</td>
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<td>OTC</td>
<td>OTC</td>
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<tr>
<td>Imipenem</td>
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<tr>
<td>Chloramphenicol</td>
<td>Chloramphenicol</td>
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</tr>
<tr>
<td>Rifampin</td>
<td>Clindamycin</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Last-resort antibacterials

<table>
<thead>
<tr>
<th>Drug</th>
<th>Why last resort?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Meropenem and other carbapenem</td>
<td>Potency &amp; lack of resistance</td>
</tr>
<tr>
<td>2) Vancomycin</td>
<td>Anti-MRSA</td>
</tr>
<tr>
<td>3) Co-trimoxazole</td>
<td>Powerfull</td>
</tr>
<tr>
<td>4) Piperacillin/Tazobactam</td>
<td>Broad coverage</td>
</tr>
<tr>
<td>5) Levofoxacin</td>
<td>Broad spectrum &amp; PO</td>
</tr>
<tr>
<td>6) Linezolid</td>
<td>Anti-MRSA</td>
</tr>
<tr>
<td>7) Cefepime</td>
<td>Broad spectrum</td>
</tr>
<tr>
<td>8) Polymyxin B (Colistin)</td>
<td>Potent</td>
</tr>
<tr>
<td>9) Tigecycline</td>
<td>Anti-MRSA</td>
</tr>
<tr>
<td>10) Aztreonam</td>
<td>Anti-pseudomonal</td>
</tr>
</tbody>
</table>

Reference:
Introduction:

It is extremely important to apply a systematic approach when conducting field investigations and an autopsy (necropsy) is essential for avian veterinarians or technical services personnel seeking to establish a preliminary diagnosis. But now a days there is increase numbers of emerging and re emerging diseases that make havoc in poultry industry. Therefore there is prime demanding in the development of diagnostic techniques to overcome problems. Here some important recent diagnostic pathological techniques are discussed which are now days used to prevent or control as well as confirmative of poultry disease diagnosis.

Immunohistochemistry (IHC):

Immunohistochemistry or IHC refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry). The procedure was conceptualized and first implemented by Dr. Albert Coons in 1941. Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors like as Lymphoid leukosis and Marek’s Disease. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death. IHC is also widely used in basic research to understand the distribution and localization of infectious agenets like as bacteria , virus or parasite, abrrenet proteins; e.g. alphafoetoprotein in liver tumours, identification of cell types, cytokeratin in epithelial cells, detection of tumour antigen.

Immunoperoxidase Technique

This is one of the EIA which has been used for the detection of several infectious agents. Conjugation of antibodies with certain enzymes has been widely used in research for diagnostic purpose. The main advantage of this technique is that the reaction is read under light microscope. The fact that the enzyme is not altered during the reaction with its substrate and that each antibody molecule is labeled with several enzyme molecules, will help in the proper enzyme substrate reaction and gives accurate diagnosis. Among the enzymes most commonly used is HRPO. Because of its low molecular weight (40,000Da) the penetration of labeled antibodies into the cell is very satisfactory and better than those antibodies labeled with other enzymes like acid phosphatase, alkaline phosphatase, etc and resistant to various histological operations, heat, solvents etc. Intracellular antigens can be detected by this method.

There are two methods of immunoperoxidase technique.
(a) Direct (b) Indirect as in the case of IFA.

**Direct Method**

In this method specific antibody conjugates added to the monolayer of the infected cells on the glass slide. After incubation, a substrate which is reactive to the enzyme is added and then examined under ordinary light microscope. The cells with viral particles will take brown or blue color depending upon the substrate used.

**Indirect Method**

Specific antibody is added on the monolayer of the cells on the glass slide and incubated. Then the anti-antibody conjugate (anti—antibodies conjugated with HRPO enzyme) is added on to the slide and incubated. After adding substrate, the slides are washed, dried and can be examined under light microscope for the color reaction of either brown or blue.

**Advantages**
- Sensitive, specific and rapid.
- Visualization under light microscope.
- Permanent preparation.

**Immunofluorescence:**

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualisation of the distribution of the target molecule through the sample. Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyse the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluorescence are used to differentiate and confirmatory diagnosis of Infectious larynotrachitis virus and other upper respiratory bacterial infection. Still it has limited use in poultry disease diagnosis and as it is very costly technique and required skilled experience persons to perform the test.

**Enzyme Linked Immunosorbant assay (ELISA):**

ELISA is a test that can be used to detect either antibody (Ab) or antigen such as viral proteins. There are numerous methods for developing ELISA tests. Use of ELISA serology can be very practical tool to determine the nature and timing of infection under field conditions. Diagnosis can made accurate when one combines serology with clinical symptoms and isolation of the pathogen. Now a days most of important viral poultry diseases like Avian Influenza, New castle disease, IBD, Avian encephalomyelitis, are diagnosed by different types of ELISA test.
Polymerase chain Reaction (PCR):

Polymerase chain Reaction (PCR) is an in-vitro technique that enables the amplification of defined (target) DNA sequences so as to increase the amount of DNA exponentially. The technique was originally described by Saiki et al., (1985) and later invented by Karry Mullis (1986). By this technique even a single gene copy can be amplified to a million copies within a few hours. It has received widespread application in diverse areas such as forensic science, microbiology, Pathology, Prenatal diagnostics etc. It has also get attention in poultry disease diagnosis. It is very useful for confirmation as well as differentiation of diseases with similar showing Post mortem lesions.

Real Time PCR (RT-PCR):

A quantitative polymerase chain reaction (qPCR), also called real-time polymerase chain reaction, is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, quantitative PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in "real time". This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for the detection of products in quantitative PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence to quantify messenger RNA (mRNA) and non-coding RNA in cells or tissues.

There are numerous applications for quantitative polymerase chain reaction in the laboratory. It is commonly used for both diagnostic and basic research. Quantitative PCR is applied to rapidly detect nucleic acids that are diagnostic of, for example, infectious diseases, cancer and genetic abnormalities. The introduction of quantitative PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases and is deployed as a tool to detect newly emerging diseases, such as new strains of bird flu, New castle disease, Infectious bursal disease, Infectious bronchitis etc. in diagnostic tests.

References:


Tripathi, B. N. (2007), Immunopathological and molecular techniques in Diagnostic Pathology, First edition, Indian Veterinary research Institute, Izatnagar, India
Dairy animals play an important role in the economy of our nation. Many feed stuffs are made from dairy products and many farmers are earning livelihood through dairy farming. For profitable dairy farming, the dairy animals should come in to heat at right age, inseminated at right time, there is no difficulty during pregnancy and at the time of delivery. The inter-calving interval should be as small as possible. The main hinderance in achieving these targets is abortion. Whenever an animal aborts, there is loss of future progeny, loss due to expenditure on treatment and sometimes animals does not come into milk or come in milk after long treatment. Abortion is a significant cause of reproductive wastage and is of economic importance especially when there is large scale abortion in a herd. So, it is worthwhile to say that economical well being of any country depends upon a healthy productive livestock.

Some of the infectious causes of abortion in dairy animals represent an important zoonotic risk to humans. Abortion is the termination of pregnancy after organogenesis is complete but before the expelled fetus can survive. If pregnancy ends before organogenesis, it is called early embryonic death. Preterm birth of immature viable fetuses is called premature birth. A dead full-term fetus is a stillbirth (its lungs are not inflated). Abortion in dairy cattle is commonly defined as a loss of the fetus between the age of 42 days and approximately 260 days. Pregnancies lost before 42 days are usually referred to as early embryonic deaths, whereas a calf that is born dead between 260 days and full term is defined a stillbirth. The etiological agents associated with the abortion are enormous. So, diagnosis of causative agent is a difficult and often frustrating task. The diagnostic success rate is relatively low: 30-40% for bovine cases submitted to diagnostic laboratories. This can be due to:

1. Sometimes, initial infection occurs by weeks or months before the causative agent is apparent at the time abortion occurs.
2. Expulsion may follow fetal death by hours or days, with lesions obscured by autolysis.
3. Fetal membranes and the aborted fetus.

**Infectious causes:**
Infectious causes of abortion in cattle include a wide range of bacterial, viral and protozoan microorganisms (Anderson et. al. 2007).

**(I) Bacterial causes:**

**(a) Brucellosis** (Bang’s disease):
Among bacterial causes in our country Brucella abortus causes maximum abortions. So, whenever there is abortion during later part of gestation followed by retention of placenta then one must think of brucellosis. Brucellosis causes abortions in the second half of gestation (usually ~7 mo), and ~80% of unvaccinated cows in later gestation will abort if exposed to Brucella abortus. During pregnancy, the bacteria localize in the superficial inguinal lymph nodes and udder and then infect the placenta and fetal fluids. Bacteria localize in the epithelial cells of the chorion, where they cause necrosis, eventual death of fetus and subsequent abortion. After abortion, bacteria do not persist in uterus and disappear in about
four weeks, as it grows well only in fetal placenta. The sugar alcohol, erythritol present in the placenta and male genital tract is strong growth stimulant for *Br. abortus*, accounting for its localization in these tissues. The placenta appears leather like, thickened and brownish in color with marked necrosis of cotyledons. The fetus may be normal or autolytic with bronchopneumonia. Diagnosis can be made by maternal serology combined with fluorescent antibody staining of placenta and fetus or isolation of *Br abortus* from placenta, fetus, or uterine discharge. Prevention is by calf hood vaccination of heifers.

2. *Vibriosis/ Campylobacteriosis:*
   *Campylobacter fetus venerealis* causes venereal disease that usually results in infertility but occasionally causes abortion between 5 and 8 mo of gestation. The fetus can be fresh with partially expanded lungs or severely autolyzed. Mild fibrinous pleuritis and peritonitis may be noted, as well as bronchopneumonia. Placentitis is mild with hemorrhagic cotyledons and an edematous intercotyledonary area. Diagnosis is by examination of preputial washing from suspected infected bulls. Repeated cultures are often needed for a certain diagnosis. Three cultures can be assured to diagnosis 1 infected bull from a group of 1000 bulls. *Campylobacter* spp can be identified by darkfield examination of abomasal contents or culture of placenta or abomasal contents. Venereal campylobacteriosis can be controlled by artificial insemination and vaccination. To be effective, vaccinations should be given prior to breeding each year. If bulls are to be used, they should be young, virgin bulls.

3. *Leptospirosis:*
   Leptospira causes abortions in the last trimester, 2-6 wk after maternal infection. There is diffuse placentitis with avascular, light tan cotyledons and edematous, yellowish intercotyledonary areas. The fetus usually dies 1-2 days before expulsion and therefore is autolyzed. Occasionally calves are born alive but weak. There are no specific lesions, but placenta and fetus should be submitted to the laboratory for fluorescent antibody staining or PCR testing for *Leptospira*. For control, sources of infection (such as feed or water contaminated by dogs, rats, or wildlife) should be identified and eliminated. Leptospirosis is zoonotic, and urine and milk of dams may be infective for up to 3 months.

4. *Actinomyces pyogenes, Bacillus, Streptococcus spp.* and other common bacteria found in the environment can be the cause of sporadic abortions in a dairy herd. These organisms usually get to the placenta and fetus by way of the cow’s circulatory system.

5. *Listeriosis:*
   *Listeria monocytogenes* can cause placentitis and fetal septicemia. Abortions are usually sporadic but may affect 10-20% of a herd. Abortion is at any stage of gestation, and the dam may have fever and anorexia before the abortion; retained placenta is common. The fetus is retained for 2-3 days after death, so autolysis may be extensive. Fibrinous polyserositis and white necrotic foci in the liver and/or cotyledons are common. Diagnosis is by culture of *Listeria* from fetus or placenta. It is a reportable disease in many areas and is a serious zoonosis with spread possible through improperly pasteurized milk.

(II) Viral causes:

(a) *Bovine Viral Diarrhea virus* (BVD). Infection of the fetus before 125 days of gestation can cause fetal death and abortion, resorption, mummification, developmental abnormalities (often involve the brain i.e. hydrocephalus or water head, cerebellar hypoplasia leading neurological signs at birth and may result in cataracts), or fetal immunotolerance and persistent infection. After 125 days of gestation, BVD may cause abortion, or the fetal immune response may clear the virus. Diagnosis is by identification of BVD virus by
isolation, immunologic staining, PCR, or detection of precolostral antibodies in aborted calves. If a calf is exposed in the uterus during the last trimester, the virus will have no effect on the calf, except that it will be born with antibodies to BVD in its blood. The use of an effective BVD vaccine and increased attention to biosecurity should be a routine part of a herd disease prevention program. The closed herd concept or quarantine of newly purchased animals can prevent infections. To control abortions, vaccinations are given to both heifers prior to their first breedings and the milking cows during the dry period. This provides maximum protection during these highly vulnerable periods.

(b) Infectious Bovine Rhinotracheitis virus:
(IBR, BHV-1) is a serious contagious herpes virus disease of cattle that can cause a variety of different disease syndromes, the most common of which is respiratory disease (pneumonia, “red nose”). It remains the most commonly diagnosed viral cause of abortions in cattle. Abortions most commonly occur from 4 months to term, and may occur weeks after the disease has gone through the herd. Autolysis is consistently present. Occasionally there are small foci of necrosis in the liver, but in a large majority of cases there are no gross lesions in the placenta or fetus. The use of effective IBR vaccines and biosecurity measures should be a routine part of a herd disease prevention program. Many vaccination options are available for IBR. MLV intramuscular vaccines provide lifetime immunity when given after 6-7 months of age. These vaccines can not be used in pregnant cows especially after the 5th month of pregnancy. When given close to heat periods, there may be a reduction in pregnancy rates. Nasal vaccination sprays are also used and can be used in pregnant cows to provide quick immunity. Killed IBR vaccines can be used in pregnant cows. These vaccines require two doses given 14-28 days apart. Annual vaccines are suggested to provide long term immunity. Chemically altered IBR vaccines can also be used in pregnant cows.

(III) Fungal causes:
Fungal placentitis due to Aspergillus, Mucor, Absidia or Rhizopus is an important cause of bovine sporadic abortion. Abortions occur from 4 month to term and are most common in winter and spring months, since this is when cows are often kept in total confinement and can be exposed to moldy hay or silage. There is severe and necrotizing placentitis. Cotyledons are enlarged and necrotic with turned-in margins. The intercotyledonary area is thickened and leathery. Adventitious placentation is common. The fetus seldom is autolyzed, although it may be dehydrated; ~30% have gray ringworm-like skin lesions principally involving the head and shoulders. The diagnosis is based on the presence of fungal hyphae associated with necrotizing placentitis, dermatitis, or pneumonia. Fungi can also be isolated from the stomach contents, placenta, and skin lesions.

(IV) Protozoal causes:-

(a) Trichomoniasis: Tritrichomonas (Trichomonas) foetus infection causes a venereal disease that usually results in infertility but occasionally causes abortion in the first half of gestation. Placentitis is relatively mild with hemorrhagic cotyledons and thickened intercotyledonary areas covered with flocculent exudate. The placenta is often retained, and there may be pyometra. The fetus has no specific lesions, although T foetus can be found in abomasal contents, placental fluids, and uterine discharges. Diagnosis is made by examination of preputial washing from the sheath of infected bulls using special isolation media. Cervical mucus or pyometrial fluid from cows can also be checked for infectious organisms. Control of trichomoniasis within known infected herd is to begin artificial insemination using noninfected bulls. When AI is not possible, vaccination can be used to
control infections. Even a switch in bull breeding to AI for 6 months will greatly reduce the effects of trichomoniasis.

(b) Neosporosis: Neospora caninum is a protozoal parasite that does not appear to cause any disease in mature cattle, except for abortions. Abortions due to Neospora usually occur sporadically in a herd in the middle of gestation (4-5 months), although they can occur anywhere from about 3 months onward. Dogs are the definitive host for Neospora and can be the source of infection. The dogs are thought to transmit the protozoan eggs or oocysts in their feces. Eating the dog feces with the oocysts infects cows. Once a cow becomes infected, they pass the infections on to the fetuses they are carrying through the placenta. Cows are not clinically ill, and placental retention is not common. The fetus is usually autolyzed and rarely has gross lesions. Diagnosis can be made from distinctive brain and heart lesions in aborted fetus or blood titers in cows. Prevention of abortions can be aided by restriction of dogs on dairies from the stored feed and feeding areas. Sero-positive, infected cows will continue to have seropositive, infected calves. These seropositive cows have also been found to have reduced milk production and earlier culling than seronegative cows. Some consideration should be given to culling seropositive cows.

(c) Sarcocystis and Toxoplasma gondii has also been reported for causing abortion.

(V) Miscellaneous causes:

Insemination of pregnant animal, corticosteroid or prostaglandin therapy to pregnant animal, Allergy, dehydration and stress has been associated with abortion.

Conclusions:

All abortion diseases can be controlled by vaccination and biosecurity to the point that no serious economic effect should occur in a dairy herd. It is vitally important to follow the vaccine manufacturer’s instructions to insure maximum protection against clinical disease and abortions. For control of abortion diseases, vaccinations should be given prebreeding. Surveillance is based primarily on submission of aborted fetuses, tissue samples and paired blood samples from suspect cows to a veterinary diagnostic laboratory. In bull bred herds, bulls should be examined when poor reproductive performance happens. Assistance in developing a comprehensive vaccination and prevention program by the attending herd veterinarian can be a great asset for the dairymen.

Further Reading:
Merck Veterinary manual 9th edition
## Differential Diagnosis:

<table>
<thead>
<tr>
<th>Infectious factor</th>
<th>Infectious causes of abortion</th>
<th>Abortion rate</th>
<th>Abortion timing</th>
<th>Recurrence of abortion</th>
<th>Foetal lesions</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial</strong></td>
<td></td>
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<tr>
<td><em>Brucella abortus</em></td>
<td>Brucellosis</td>
<td>Up to 80% of unvaccinated animals infected in 1st or 2nd trimester</td>
<td>6-9 months. Abortion or stillbirth 2 wk to 5 mo after infection</td>
<td>Majority abort only once</td>
<td>Placenta: retained, cotyledons necrotic, red-yellow.; area between thickened</td>
<td>placenta, foetus, or uterine discharge</td>
</tr>
<tr>
<td><em>Campylobacter fetus venerealis</em></td>
<td>Vibriosis</td>
<td>&gt;10%</td>
<td>5-8 months</td>
<td>Uncommon, convalescent cows resistant to infection</td>
<td>Placenta: mild placentitis, hemorrhagic cotyledons and an edematous intercotyledonary area. Foetus: fresh or autolysed; mild fibrinous pleuritis, peritonitis, bronchopneumonia.</td>
<td>Placenta, foetal abomasal contents, vaginal flushing</td>
</tr>
<tr>
<td><em>C fetus fetus</em></td>
<td>Sporadic</td>
<td>4-9 months</td>
<td>Uncommon, convalescent cows resistant to infection</td>
<td>See above</td>
<td>See above</td>
<td>See above</td>
</tr>
<tr>
<td><em>C jejuni</em></td>
<td></td>
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<tr>
<td><em>Leptospira interrogans</em>, serovars <em>grippotyphosa</em>, <em>pomona</em>, <em>hardjo</em>, <em>canicola</em>, <em>icterohaemorrhagiae</em></td>
<td><em>Zoonosis</em></td>
<td>5-40%</td>
<td>Last trimester Abortion 2-5 weeks after infection</td>
<td>Immunity to the serotype causing abortion but sensitive to other types</td>
<td>Placenta: diffuse placentitis with avascular, light tan cotyledons and edematous, yellowish intercotyledonary areas Foetus: autolysed</td>
<td>Placenta, foetus</td>
</tr>
<tr>
<td><em>Arcanobacterium (Actinomyces)</em></td>
<td>Sporadic</td>
<td>Sporadic</td>
<td>Any stage</td>
<td>Not known</td>
<td>Placenta: endometritis and diffuse placentitis, reddish</td>
<td>Placenta, foetus</td>
</tr>
<tr>
<td><strong>Aspergillus sp</strong> (60-80%)</td>
<td>Usually sporadic but can reach 5-10%</td>
<td>4 months to term</td>
<td>May recur</td>
<td>Placenta: severe, necrotising placentitis. Cotyledons enlarged, necrotic, intercotyledonary area is thickened and leathery. Foetus: autolysed. 30% have gray ringworm-like skin lesions.</td>
<td>Foetus, placenta</td>
<td><strong>Diagnosis:</strong> isolation from stomach contents, placenta, and skin lesions.</td>
</tr>
<tr>
<td><strong>Mucor sp, Absidia, or Rhizopus sp</strong></td>
<td>Usually sporadic but can reach 5-10%</td>
<td>Most common in winter</td>
<td>May recur</td>
<td>Placenta: severe, necrotising placentitis. Cotyledons enlarged, necrotic, intercotyledonary area is thickened and leathery. Foetus: autolysed. 30% have gray ringworm-like skin lesions.</td>
<td>Foetus, placenta</td>
<td></td>
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<tr>
<td><strong>Protozoan</strong></td>
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<tr>
<td><strong>Tritrichomonas foetus</strong> (Trichomonas foetus)</td>
<td>Sporadic</td>
<td>First half of gestation</td>
<td>Animal gains immunity but probably not life-long</td>
<td>Placenta: retained, mild placentitis with hemorrhagic cotyledons and thickened intercotyledonary areas covered with flocculent exudates. Foetus: no specific lesions</td>
<td>Placenta, foetus, vaginal/uterine discharge</td>
<td><strong>Diagnosis:</strong> detection in abomasal contents, placental fluids, and uterine discharges</td>
</tr>
<tr>
<td><strong>Trichomoniasis</strong></td>
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<tr>
<td><strong>Neospora caninum</strong></td>
<td>High in first gestation and when infection enters the naïve herd</td>
<td>Any stage, but most often 5-6 months</td>
<td>Decreases with parity but always possible</td>
<td>Placenta, foetus: no specific gross lesions, autolysed. Microscopic: focal encephalitis with necrosis and nonsuppurative inflammation, hepatitis in</td>
<td>Placenta, foetus (brain, heart, liver, body fluids), serum samples from the dam</td>
<td><strong>Diagnosis:</strong> detection of antigen in brain histology samples. Immunochemistry in tissue samples</td>
</tr>
<tr>
<td><strong>Neosporosis</strong></td>
<td>Up to 30% first outbreak</td>
<td></td>
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<tr>
<td>Viral</td>
<td>Enzootic: 5-10%</td>
<td>Complex pathology</td>
<td>Uncommon, immunity develops</td>
<td>Placenta: retained, no specific lesions</td>
<td>Abs - PCR, ELISA</td>
<td></td>
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<tr>
<td>BVD-MD</td>
<td>Usually low</td>
<td>Abortion usually up to 4 months</td>
<td>Placenta: retained, no specific lesions</td>
<td>Foetus: no specific lesions, autolysed, mummified</td>
<td>Placenta, foetus (preferred - spleen), dam and herdmates serum</td>
<td></td>
</tr>
<tr>
<td>Bovine Viral Diarrhoea Virus</td>
<td>5-60% in non vaccinated herds</td>
<td>Possibly any stage but most common from 4 months to term</td>
<td>In the majority of cases there are no gross lesions in the placenta or foetus</td>
<td>Foetus: necrotizing vasculitis</td>
<td>Diagnosis: isolation, immunologic staining, PCR, or detection of precolostral antibodies in aborted calves</td>
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<tr>
<td>Bovine Herpesvirus type I (BHV I)</td>
<td>Usually low</td>
<td>Variable</td>
<td>Unlikely</td>
<td>No specific</td>
<td>Placenta, foetus, serum samples from the dam</td>
<td></td>
</tr>
<tr>
<td>Infectious Bovine rhinotracheitis virus (IBRV)</td>
<td>Can reach 75%</td>
<td>Usually in the last trimester</td>
<td>Placenta: No specific</td>
<td>Foetus: hepateomegaly, splenomegaly, and generalized lymphomegaly. Microscopically - marked lymphoid hyperplasia in the spleen and lymph nodes and granulomatous inflammation in most organs.</td>
<td>Diagnosis: elevated foetal Ig-G</td>
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<tr>
<td>IBR</td>
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<td>Placenta, foetus, serum samples from the dam</td>
<td>Diagnosis: virus isolation</td>
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<tr>
<td>IBR-IPV</td>
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<td>Blue tongue virus</td>
<td>Usually low</td>
<td>Variable</td>
<td>Unlikely</td>
<td>Foetus: autolysed</td>
<td>Placenta, foetus, serum samples from the dam</td>
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<td>Blue tongue</td>
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<td>Epizootic Bovine Abortion Foothill Abortion etiologic agent has not been definitively determined, vector tick</td>
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ASCAD Training on “Advanced approaches for diagnosis of livestock diseases” (25-30 Nov., 2013)
Advances in Animal Cell Culture and Its Utility in Diagnosis of Viral Diseases of Livestock  
B.S.Mathapati, D.B.Barad and B.B.Javia  
Department of Veterinary Microbiology  
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Cell culture is the complex process by which cells are grown under controlled conditions, generally outside of their natural environment. In practice, the term "cell culture" now refers to the culturing of cells derived from multi-cellular eukaryotes, especially animal cells. However, there are also cultures of plants, fungi and microbes, including viruses, bacteria and protists. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. The 19th-century English physiologist Sydney Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for maintaining the beating of an isolated animal heart outside of the body. In 1885, Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture. Ross Granville Harrison, working at Johns Hopkins Medical School and then at Yale University, published results of his experiments from 1907 to 1910, establishing the methodology of tissue culture. Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The injectable polio vaccine developed by Jonas Salk was one of the first products mass-produced using cell culture techniques. This vaccine was made possible by the cell culture research of John Franklin Enders, Thomas Huckle Weller, and Frederick Chapman Robbins, who were awarded a Nobel Prize for their discovery of a method of growing the virus in monkey kidney cell cultures.

Cell cultures are separated into 3 types:

**Primary cells** - prepared directly from animal or human tissues and can be subcultured only once or twice e.g. primary monkey or baboon kidney

**Semi-continuous diploid cells** - which are derived from human fetal tissue and can be subcultured 20 to 50 times e.g. human diploid fibroblasts such as MRC-5

**Continuous cells** - derived from tumours of human or animal tissue e.g. Vero, Hep2

Primary cultures are maintained by changing the fluid 2 or 3 times a week. When the cultures become too crowded, the cells are detached from the vessel wall by either trypsin or EDTA, and portions are used to initiate secondary cultures. In both primary and secondary cultures, the cells retain some of the characteristics of the tissue from which they are derived. Cells from primary cultures can often be transferred serially a number of times. The cells may then continue to multiply at a constant rate over many successive transfers. Eventually, after a number of transfers, the cells undergo culture senescence and cannot be transferred any longer. For human diploid cell cultures, the growth rate declines after about 50 duplications. During the multiplication of the cell strain, some cells become altered in that they acquire a different morphology, grow faster, and become able to start a cell culture from a smaller number of cells. These cells are immortalized and have an unlimited life-span. However, they retain contact inhibition. Cell cultures vary greatly in their susceptibility to different viruses. It is of utmost importance that the most sensitive cell cultures are used for a particular suspected virus. Specimens for cell culture should be
transported to the laboratory as soon as possible upon being taken. Swabs should be put in a vial containing virus transport medium. Bodily fluids and tissues should be placed in a sterile container.

Concepts in mammalian cell culture

Isolation of cells

Cells can be isolated from tissues for ex vivo culture in several ways. Cells can be easily purified from blood; however, only the white cells are capable of growth in culture. Mononuclear cells can be released from soft tissues by enzymatic digestion with enzymes such as collagenase, trypsin, or pronase, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture. This method is known as explant culture. Cells that are cultured directly from a subject are known as primary cells. With the exception of some derived from tumors, most primary cell cultures have limited lifespan. After a certain number of population doublings (called the Hayflick limit), cells undergo the process of senescence and stop dividing, while generally retaining viability. An established or immortalized cell line has acquired the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. Numerous cell lines are well established as representative of particular cell types.

Maintaining cells in culture

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37 °C, 5% CO2 for mammalian cells) in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes. Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the cell growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from the serum of animal blood, such as fetal bovine serum, bovine calf serum, equine serum, and porcine serum. One complication of these blood-derived ingredients is the potential for contamination of the culture with viruses or prions, particularly in medical biotechnology applications. Current practice is to minimize or eliminate the use of these ingredients wherever possible and use chemically defined media, but this cannot always be accomplished. Alternative strategies involve sourcing the animal blood from countries with minimum BSE/TSE risk, such as Australia and New Zealand, and using purified nutrient concentrates derived from serum in place of whole animal serum for cell culture. Also the use of recently developed universal, fully defined and animal free alternatives like Serum-Free avoids these complications. Plating density (number of cells per volume of culture medium) plays a critical role for some cell types. For example, a lower plating density makes granulosa cells exhibit estrogen production, while a higher plating density makes them appear as progesterone-producing theca lutein cells.

Cells can be grown either in suspension or adherent cultures. Some cells naturally live in suspension, without being attached to a surface, such as cells that exist in the bloodstream. There are also cell lines that have been modified to be able to survive in suspension cultures so they can be grown to a higher density than adherent conditions would allow. Adherent cells require a surface, such as tissue culture plastic or microcarrier, which may be coated with extracellular matrix (such as collagen and laminin) components to increase adhesion properties and provide other signals needed for growth and differentiation. Most cells derived from solid tissues are adherent. Another type of adherent culture is organotypic culture, which involves growing cells in a three-dimensional (3-D) environment as opposed to two-dimensional culture dishes. This 3D culture system is biochemically and physiologically more similar to in vivo tissue, but is technically challenging to maintain because of many factors (e.g. diffusion). Cell line cross-contamination can be a problem for scientists working with cultured cells. Studies suggest
anywhere from 15–20% of the time, cells used in experiments have been misidentified or contaminated with another cell line. Problems with cell line cross-contamination have even been detected in lines from the NCI-60 panel, which are used routinely for drug-screening studies. Major cell line repositories, including the American Type Culture Collection (ATCC), the European Collection of Cell Cultures (ECACC) and the German Collection of Microorganisms and Cell Cultures (DSMZ), have received cell line submissions from researchers that were misidentified by them. Such contamination poses a problem for the quality of research produced using cell culture lines, and the major repositories are now authenticating all cell line submissions. ATCC uses short tandem repeat (STR) DNA fingerprinting to authenticate its cell lines. To address this problem of cell line cross-contamination, researchers are encouraged to authenticate their cell lines at an early passage to establish the identity of the cell line. Authentication should be repeated before freezing cell line stocks, every two months during active culturing and before any publication of research data generated using the cell lines. Many methods are used to identify cell lines, including isoenzyme analysis, human lymphocyte antigen (HLA) typing, chromosomal analysis, karyotyping, morphology and STR analysis. One significant cell-line cross contaminant is the immortal HeLa cell line.

Manipulation of cultured cells

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells. These are generally performed using tissue culture methods that rely on aseptic technique. Aseptic technique aims to avoid contamination with bacteria, yeast, or other cell lines. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics (e.g. penicillin and streptomycin) and antifungals (e.g.amphotericin B) can also be added to the growth media. As cells undergo metabolic processes, acid is produced and the pH decreases. Often, a pH indicator is added to the medium to measure nutrient depletion. In the case of adherent cultures, the media can be removed directly by aspiration, and then is replaced. Media changes in non-adherent cultures involve centrifuging the culture and resuspending the cells in fresh media.

Another common method for manipulating cells involves the introduction of foreign DNA by transfection. This is often performed to cause cells to express a protein of interest. More recently, the transfection of RNAi constructs have been realized as a convenient mechanism for suppressing the expression of a particular gene/protein. DNA can also be inserted into cells using viruses, in methods referred to as transduction, infection or transformation. Viruses, as parasitic agents, are well suited to introducing DNA into cells, as this is a part of their normal course of reproduction.

Passaging of cells

Passaging (also known as subculture or splitting cells) involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached; this is commonly done with a mixture of trypsin-EDTA; however, other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture.

Hybridomas and cell strains

It is possible to fuse normal cells with an immortalised cell line. This method is used to produce monoclonal antibodies. In brief, lymphocytes isolated from the spleen (or possibly blood) of an immunised
animal are combined with an immortal myeloma cell line (B cell lineage) to produce a hybridoma which has the antibody specificity of the primary lymphocyte and the immortality of the myeloma. Selective growth medium (HA or HAT) is used to select against unfused myeloma cells; primary lymphocytes die quickly in culture and only the fused cells survive. These are screened for production of the required antibody, generally in pools to start with and then after single cloning. A cell strain is derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or characteristics which must be defined. Cell strains are cells that have been adapted to culture but, unlike cell lines, have a finite division potential. Non-immortalized cells stop dividing after 40 to 60 population doublings and, after this, they lose their ability to proliferate (a genetically determined event known as senescence).

Applications of cell culture

Mass culture of animal cell lines is fundamental to the manufacture of viral vaccines and other products of biotechnology. Biological products produced by recombinant DNA (rDNA) technology in animal cell cultures include enzymes, synthetic hormones, immunobiologics (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells. An important example of such a complex protein is the hormone erythropoietin. The cost of growing mammalian cell cultures is high, so research is underway to produce such complex proteins in insect cells or in higher plants, use of single embryonic cell and somatic embryos as a source for direct gene transfer via particle bombardment, transit gene expression and confocal microscopy observation is one of its applications. It also offers to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process.

Rapid Culture Techniques

One of the most significant contributions to rapid diagnosis has been the application of centrifugation cultures to viral diagnosis. For a number of years, it has been recognized that low-speed centrifugation of specimens onto cell monolayers enhances the infectivity of certain viruses as well as chlamydia. The cell culture is stained by monoclonal antibodies for the presence of specific viral antigens 24-48 hours later. The best known example of this technique is the DEAFF test used for the early diagnosis of CMV infection. In the DEAFF test, the specimen is inoculated onto human embryonic fibroblasts and then spun at a low speed. After a period of 24-48 hours, the cells are then stained by monoclonal antibodies against CMV early antigen. Therefore a rapid diagnosis of CMV infection can be made without having to wait 1-3 weeks for the CPE to appear.

Advances in cell culture

Cell culture in two dimension and three dimension

Research in tissue engineering, stem cells and molecular biology primarily involves cultures of cells on flat plastic dishes. This technique is known as two-dimensional (2D) cell culture, and was first developed by Wilhelm Roux who, in 1885, removed a portion of the medullary plate of an embryonic chicken and maintained it in warm saline for several days on a flat glass plate. From the advance of polymer technology arose today's standard plastic dish for 2D cell culture, commonly known as the Petri dish. Julius Richard Petri, a German bacteriologist, is generally credited with this invention while working as an assistant to Robert Koch. Various researchers today also utilize culturing laboratory flasks, conicals, and even disposable bags like those used in single-use bioreactors. Aside from Petri dishes, scientists have long been growing cells within biologically derived matrices such as collagen or fibrin, and more recently,
on synthetic hydrogels such as polyacrylamide or PEG. They do this in order to elicit phenotypes that are not expressed on conventionally rigid substrates. Cell culture in three dimensions has been touted as "Biology's New Dimension". Nevertheless, the practice of cell culture remains based on varying combinations of single or multiple cell structures in 2D. That being said, there is an increase in use of 3D cell cultures in research areas including drug discovery, cancer biology, regenerative medicine and basic life science research. There are a variety of platforms used to facilitate the growth of 3 dimensional cellular structures such as nanoparticle facilitated magnetic levitation, gel matrices scaffolds, and hanging drop plates.

3D Cell Culturing by Magnetic Levitation method (MLM) is the application of growing 3D tissue by inducing cells treated with magnetic nanoparticle assemblies in spatially varying magnetic fields using neodymium magnetic drivers and promoting cell to cell interactions by levitating the cells up to the air/liquid interface of a standard petri dish. The magnetic nanoparticle assemblies consist of magnetic iron oxide nanoparticles, gold nanoparticles, and the polymer polylysine. 3D cell culturing is scalable, with the capability for culturing 500 cells to millions of cells or from single dish to high-throughput low volume systems.

**Culture of non-mammalian cells**

**Insect cell culture**

Cells derived from Drosophila melanogaster (most prominently, Schneider 2 cells) can be used for experiments which may be hard to do on live flies or larvae, such as biochemical studies or studies using siRNA. Cell lines derived from the army worm Spodoptera frugiperda, including Sf9 and Sf21, and from the cabbage looper Trichoplusia ni, High Five cells, are commonly used for expression of recombinant proteins using baculovirus.

**Commonly used cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Tissue origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>Baby hamster</td>
<td>kidney fibroblast cells</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster</td>
<td>ovary</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human</td>
<td>Cervical cancer</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human</td>
<td>Embryonic kidney</td>
</tr>
<tr>
<td>MDCK (Madin Darby canine kidney)</td>
<td>Canine</td>
<td>Kidney</td>
</tr>
<tr>
<td>Vero cells</td>
<td>African green monkey</td>
<td>Kidney epithelium</td>
</tr>
<tr>
<td>DuCaP</td>
<td>Human</td>
<td>Metastatic prostate cancer epithelium</td>
</tr>
</tbody>
</table>

**Reference:**

1. Animal cell culture and virology by Robert Joseph Kuchler
Rabies is an enzootic disease in India and has a serious public health and economic implication in our country. With the exception of Andaman and Nicobar and Lakshadweep and to some extent in Nagaland, the disease is present throughout the country. Rabies continues to be endemic in Asia for various reasons. The large population of stray dogs together with a lack of effective control strategies has been responsible for increase in number of deaths from rabies. A recent national survey by the Association of the Prevention and Control of Rabies in India (APCRI) estimated that in India a total of 20,000 human deaths occur as a result of rabies each year (Sudarshan et al., 2006). In India, rabies occurs mainly in the urban form, although the existence of a sylvatic cycle cannot be ruled out. In the urban form, dogs play an important role as the reservoir and transmitter of the disease to humans and domestic animals, while jackals and foxes maintain the virus in sylvatic form. Worldwide, transmission from dogs accounts for more than 90% of human cases. In developed countries, bats, foxes, coyotes, raccoons, and skunks are major reservoirs.

Rabies is caused by classical rabies virus (CRV) belong to genotype-1, since the other member of genus lyssavirus have been found to cause illness indistinguishable from classical rabies (Smith, 1996). These facts indicate a great need to strengthen accurate and rapid laboratory diagnostic capabilities, which involves virus detection and genotyping. For suspected human cases, rapid ante-mortem diagnosis ensures appropriate patient management. Postmortem diagnosis enables the rapid administration of post-exposure treatment to all human and animal contacts.

Genome: Rabies virus (Genus Lyssavirus, family Rhabdoviridae) possesses a single-stranded, linear non-segmented, negative-sense RNA approximately 12 kb in length with helical symmetry. The principal genotype of lyssaviruses is the classical RV, genotype-1, which is present throughout the world. The remaining six members of the genus form a group of viruses termed as rabies related viruses (RRV) that show a more restricted geographical distribution and are more commonly associated with the specific host. RRV includes, Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat Lyssavirus 1 and 2 (genotypes 5 and 6 respectively) and Australian bat Lyssavirus (ABLV) (genotype 7). The RRVs have been found to cause illness indistinguishable from classical rabies (Smith, 1996).

**Diagnostic Techniques**

1. Identification of the agent

   Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another. The only way to perform a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests. As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the ‘rabies diagnostic chain’.
Several laboratory techniques may be used and the methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied, though less effectively, to other organs (e.g. salivary glands). In the brain, rabies virus is particularly abundant in the thalamus, pons and medulla. The structure of choice is the thalamus. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested. To reach these parts of the brain, it is necessary to remove the entire organ after having opened the skull in a necropsy room. Under some conditions (e.g. in the field or when sampling for large epidemiological studies), a simplified method of sampling through the occipital foramen, or through the orbital cavity, can be used.

a) Shipment of samples: During the shipment of suspect material for diagnosis (animal heads, brain or other tissue samples), no risk of human contamination should arise: brains must be placed in a leak-proof rigid container (animal heads will be wrapped in absorbent material) as prescribed in the International Air Transport Association (IATA) Dangerous Goods Regulations must be followed. When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is closely linked to the tests to be used for diagnosis: Formalin inactivates the virus, thus the isolation tests cannot be used and diagnosis depends on using a modified and less sensitive direct fluorescent antibody test (FAT), immunohistochemistry or histology. Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS). It does not protect against titre decline due to thermal conditions and therefore, because rabies is thermolabile, the virus titre will decline during glycerol/PBS storage. Under normal transport conditions in the tropics, this protection may only be effective for a matter of several days. Therefore, whenever possible samples in glycerol/saline should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples.

b) Collection of samples: Usually the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected. This step may be hazardous if laboratory technicians are not fully trained, or under field conditions. In such cases, there are two possible methods of collecting some brain samples without opening the skull:

**Occipital foramen route for brain sampling:** A 5 mm drinking straw or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidial bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata.

**Retro-orbital route for brain sampling:** In this technique, a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

c) Routine laboratory tests: Laboratory diagnosis can be performed by using three kinds of procedure. Histological identification of characteristic cell lesions: Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Immunohistochemical tests are the only histological test specific to rabies. An unfixed tissue smear may be stained by the Seller’s method; diagnosis is then obtained in under 1 hour. Generally, histological tests, such as Mann’s test, are performed on fixed material after a paraffin embedding step, and the result of the test is obtained within 3 days.
These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Whichever staining method is used, the evidence of infection is provided by intracytoplasmic acidophilic bodies. These histological methods, especially the Seller’s method, can no longer be recommended because they have very low sensitivity and should be abandoned.

**Immunochemical identification of rabies virus antigen:**

i) Fluorescent antibody test: The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. This test may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The FAT gives reliable results on fresh specimens within a few hours in more than 95-99% of cases. The sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled, on the type of lyssavirus and on the proficiency of the diagnostic staff. Sensitivity may be lower in samples from vaccinated animals due to localization of antigen, which is confined to the brainstem. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue that includes the brain stem, are fixed in high-grade cold acetone and then stained with a drop of specific conjugate. Anti-rabies fluorescent conjugates may be prepared in the laboratory. Those available commercially are either polyclonal conjugates specific to the entire virus or specific to the rabies nucleocapsid protein, or they may be prepared from a mix of different MAbs. In the FAT, the specific aggregates of nucleocapsid protein are identified by their fluorescence. The specificity and sensitivity of these anti-rabies fluorescent conjugates for locally predominant virus variants should be checked before use. The FAT may be applied to glycerol-preserved specimens. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme. However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue.

ii) Immunochemical tests: The antibody may be conjugated to an enzyme such as peroxidase instead of fluorescein isothiocyanate (FITC). This conjugate may be used for direct diagnosis with the same sensitivity as FAT, but attention should be paid to the risk of nonspecific false-positive results. This risk is considerably reduced by the thorough training of the technicians. It must also be emphasised that this technique needs one incubation step more than the FAT. Peroxidase conjugate may be used on sections of formalin-fixed tissue for immunohistochemical tests. An enzyme-linked immunosorbent assay (ELISA) that detects rabies antigen is one variation of the immunochemical test. This rapid rabies enzyme immunodiagnosis test (RREID) is available commercially. The correlation between the FAT and the RREID ranges between 96% and 99%. The ‘routine’ version of this test is not sensitive to rabies-related viruses as RREID only detects genotype 1 lyssaviruses.

**Detection of the replication of rabies virus after inoculation:**

These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known human exposure.

i) Mouse inoculation test: Five-to-ten mice, 3-4 weeks old (12-14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. It is recommended, though not strictly essential, to
use specific pathogen free (SPF) mice. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon’s horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetised when inoculated. The young adult mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For street fox rabies strains, deaths due to rabies generally begin 9 days post-inoculation. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation. This in-vivo test is quite expensive, particularly if SPF mice are used, and should be avoided where possible. It does not give rapid results (compared with in-vitro inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this low-tech test is that it can be easily and practicably be applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

ii) Cell culture test: Neuroblastoma cell lines, e.g. CCL-131 in the American Type Culture Collection are used for routine diagnosis of rabies. The cells are grown in Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO₂. Its sensitivity has been compared with that of baby hamster kidney (BHK-21) cells. This cell line is sensitive to street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 hours (one replication cycle of virus in the cells); generally incubation continues for 48 hours or in some laboratories up to 4 days. This test is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results. It is often advisable to carry out more than one type of test on each sample, at least when there has been human exposure.

d) Other identification tests: The tests above may be completed in specialized laboratories using MAbs, nucleic acid probes, or the polymerase chain reaction (PCR), followed by DNA sequencing of genomic areas for typing the virus. This enables a distinction to be made between vaccine virus and a field strain of virus, and possibly the geographical origin of the latter.

2. Serological tests

Serological tests are rarely used in epidemiological surveys, due to late seroconversion and the low percentage of animals surviving the disease and therefore having post-infection antibodies. Oral immunization of rabies reservoirs is the method of choice for wildlife rabies control. For follow-up investigations in oral vaccination campaigns, virus neutralization (VN) tests in cell culture are preferred. However, if poor quality sera are submitted, the VN tests in cell culture are sensitive to cytotoxicity, which could lead to false-positive results. For such samples, the use of an indirect ELISA with rabies glycoprotein-coated plates has been shown to be as sensitive and specific as the VN test on cells. a) Virus neutralization test in cell culture: fluorescent antibody virus neutralization test (a prescribed test for international trade): The principle of the fluorescent antibody virus neutralization (FAVN) test is the neutralization in vitro of a constant amount of rabies virus (‘challenge virus standard’ [CVS] strain adapted to cell culture) before inoculating cells susceptible to rabies virus: BHK-21 C13 cells. The serum titre is the dilution at which 100% of the virus is neutralized in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralizing dilution of a standard serum under the same experimental conditions (OIE serum of dog origin or WHO standard for rabies immunoglobulin
human]. Several publications have shown that the FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results.

b) The rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody (a prescribed test for international trade): Each well of an eight-well tissue-culture chamber slide contains 25-50 distinct microscopic fields when observed at x160-200 magnification. One unit of virus for the RFFIT is determined as the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells (the focus-forming dose, FFD₅₀). The stock virus suspension should contain at least 1 x 10⁴ FFD₅₀ per 0.1 ml (i.e. the well with cells infected with the 10⁻⁴ dilution of the virus should contain at least one focus of infected cells in 50% of the observed microscopic fields). A stock virus suspension of this titre can then be diluted to 10⁻²⁻³ to obtain a challenge virus containing 50 FFD₅₀.

Reference sera: A national or international reference serum standard diluted to a potency of 2.0 IU/ml should be included in each test. The reference serum used at the Centres for Disease Control and Prevention is the first international standard for rabies immunoglobulin, which may be obtained from the NIBSC. The reference serum should be maintained as frozen aliquots in amounts sufficient for 1 week of tests. A positive serum control standard diluted to a potency of 0.5 IU/ml and a negative serum control standard with a potency of <0.1 IU/ml should also be prepared by the laboratory and included in each test. Test sera: Serum samples should be heated at 56°C for 30 minutes before testing in order to inactivate complement. If sera are frozen, they should be reheated after thawing. Serial dilutions of test sera may be prepared in an eight-well tissue-culture chamber slide. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy. Calculation of virus-neutralising antibody titres: Residual virus is detected using a standard fluorescence microscope. The serum neutralization end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralisation titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralized and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the national reference standard included in each test. It should be noted that it is also valid to perform the RFFIT using BHK-21 cells instead of neuroblastoma cells. A modified protocol for this has been published. c) Virus neutralisation in mice: This method is no longer recommended by either OIE or WHO and should be discontinued.

d) Enzyme-linked immunosorbent assay: Commercial kits are available for indirect ELISA that allow a qualitative detection of rabies antibodies in individual dog and cat serum samples following vaccination. In accordance with the WHO recommendations, 0.5 IU per ml rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity that correlates with the ability to protect against rabies infection. The ELISA provides a rapid (~ 4 hours) test that does not require handling of live rabies virus, to determine if vaccinated dogs and cats have sero-converted. The sensitivity and specificity of any kit used should be determined by comparison with virus neutralization methods. The ELISA is acceptable as a Prescribed Test for international movement of dogs or cats provided that a kit is used that has been validated and adopted on the OIE Register as fit for such purposes. Virus neutralization methods may be used as confirmatory tests if desired.
ELISA methods are also useful for monitoring of vaccination campaigns in wildlife populations, provided the kit used has been validated for the wildlife species under study.

References


Demonstration of Bright field, Phase contrast and Fluorescent Microscopy for Identification of Disease Causing Agents
D. B. Barad, B. B. Javia and B. S. Mathapati
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Bright field Microscopy
Bright field microscopy is best suited to viewing stained or naturally pigmented specimens such as stained prepared slides of tissue sections or living photosynthetic organisms. It is not much useful for living specimens of bacteria, and inferior for non-photosynthetic protists or metazoans, or unstained cell suspensions or tissue sections.

Preparation of Smears
- Bacteria differ a slightly in refractive index from the surrounding medium. Hence, it is difficult to see unstained bacteria in ordinary microscope. So staining is the primary requirement to see the bacteria in the light microscope.
- Smear can be prepared from fluid materials like culture, urine, sputum, pus etc by taking a loopful of the material in an inoculating loop and spread it thinly on a clean glass slide. The smear is then allowed air dry.
- The smear can also be dried by holding it high over a bunsen flame.
- The dried smear is then fixed by passing it through the flame slowly three times with the smear upwards.
- Alternatively the fixing can also be done by heating through the slide. In this method the slide is held with the smear on top in the top of the Bunsen flame for a few seconds so that the slide becomes hot.
- The slides are then marked on one end with a diamond or grease pencil on the side having the smear. In slides with a ground matt surface at one end ordinary graphite pencil can be used to mark the slide.
- For preparation of smears from material like cultures on agar first a loopful of water or saline is placed on the slide and then with a sterilized loop a minute quantity of material is taken from the culture and put on the water placed on the slide and emulsified. Then a thin smear is prepared.
• Smears are then stained with appropriate stains such as gram’s, acid fast, giemsa etc. for viewing bacteria

**Procedure to handle bright field microscope**

• Mount the specimen on the stage  
• Optimize the lighting  
• Adjust the condenser  
• Focus, locate, and center the specimen  
• Adjust eyepiece separation focus  
• Select an objective lens for viewing  
• Adjust illumination for the selected objective lens

**Phase contrast Microscopy**

Before the introduction of the phase contrast microscope it was difficult to observe living cells. As living cells are translucent they must be stained to be visible in a traditional light microscope. Unfortunately, the process of staining cells generally kill the cells. With the invention of the phase contrast microscopy it became possible to observe unstained living cells in detail. After its introduction in the 1940s, live cell imaging rapidly became popular using phase contrast microscopy. Its inventor, Fritz Zernike, was awarded the Nobel Prize in 1953.

**Procedure for Phase contrast Microscopy**

• Put a drop of broth culture of organism to be examined  
• Then put coversilp on the drop and observe under microscope  
• Turn on the microscope.  
• Rotate the condenser turret  
• Place the specimen on the stage and focus on the specimen with the 4X and then 10X Phase objective  
• Adjust the diopter and interpupillary distance.  
• Center and focus the condenser.  
• Place the green interference filter (GIF) on the filter holder around the field lens.  
• Center the phase annular diaphragm
• Adjust the field diaphragm so that it is just inside or outside of the view field.
• If you switch the objective, also switch the condenser turret so that it matches the phase code of the objective. At this time, check that the phase plate of the objective and the image of the phase annular diaphragm are aligned. Also readjust the size of the field diaphragm.
• If using an oil immersion type objective, apply immersion oil between the specimen and the objective.

Fluorescent Microscopy
Fluorescent Antibody Test
Principle
Fluorescent dyes are commonly used as labels in primary binding tests. The most important and commonly used dye is FITC (fluorescein isothiocyanate). FITC is a yellow compound that can be bound to antibodies without affecting their reactivity. When irradiated with UV light, FITC re-emits visible green/or yellow green light at 525 nm. Immunoglobulin, separated from hyper immune serum and concentrated, is conjugated with a stable fluorescent dye (fluorescein isothiocyanate) to produce a labeled antibody. The labeled antibody is used to detect the corresponding specific antigen (bacteria, virus) in tissues or specimens.

There are two methods of fluorescent antibody technique
Direct method
In the direct method specific antibacterial or antiviral serum is first conjugated with fluorescein dye and then treated with the antigen before it is examined under ultra violet microscope.

Indirect method
In the indirect method unlabelled known antibacterial or antiviral serum is first incubated with a test antigen. If the test antigen is specific for the known antibody, the antigen-antibody complex is formed. Labeled antiglobulin (Antibody against immuno globulin of the species from which antibacterial or antiviral serum is obtained) serum is then added. It combines with the antigen-antibody complex and, under the fluorescent microscope, the reaction is seen as a yellowish green light.
Demonstration of Immunological Techniques  
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PRECISION TESTS

Precipitation and agglutination reactions are similar except in that for precipitation reaction antigen must be in soluble form. The precipitation reaction is highly specific and very sensitive for the detection of antigen being capable of demonstrating antigens in dilutions of 1:100,000 to 1:1,000,000 or more. The essential ingredients for precipitation test are soluble antigen (precipitinogen), antibody (precipitin) and an electrolyte (normal saline).

Precipitation ring test

The antigen is carefully layered over the antiserum, without mixing so that an interface is formed. Diffusion of each reagent will then occur into each other. If the system is homologous, precipitation will occur at the point where the proper ratio of antigen to antibody is reached.

Ascoli’s thermo precipitation test

This test is used for diagnosis of Anthrax. In advanced decomposition of carcass and when microscopical examination is almost impossible, the precipitation method of Ascoli is used for diagnosis of Anthrax. This method is a mixing of immunized rabbit serum with an extract of organs under examination for the production of a precipitate.

Procedure

- Grind up the organs (e.g. muzzle) of suspected animals.
- Suspend in saline.
- Boil for five minutes
- Filter through filter paper or muslin cloth and allow to cool
- Carefully add 0.5 ml of this extract in a small test tube and 0.5 ml hyper immune serum is poured into gently through the wall of the test tube.
- Allow to stand for 15 minutes at room temperature.
- Definite ring like precipitate forms at junction of two fluids in positive cases.
- Control tests should be run with known anthrax positive extract and with normal saline (negative control).

Agar gel precipitation test (AGPT) or Ouchterlony's test

Antigen and antibody placed in wells cut in agar gels diffuse and form an opaque band of precipitate of the antigen-antibody complex in the zone of optimal proportions. Since each antigen combines with its own specific antibody, an antigenic mixture will give multiple bands. Setting up the reaction in adjacent wells assesses the immunological relationship between two antigens.

- When the lines are confluent it indicates immunological identity in terms of antiserum used.
- A spur develops with partially related antigens.
The lines cross indicate unrelated antigens.

**Materials**
- Antigen
- Antiserum
- Glass slides or Petridish.
- 1% agarose in normal saline. In case of poultry pathogens the composition is Agarose 1g; sodium chloride 8g, Distilled water – 100ml (To avoid contamination during storage and incubation add sodium azide 0.02g or merthiolate 1 in 10,000 concentration).
- Template
- Gel cutter
- Needle
- 50 µl micropipette & pipette tips
- Normal saline
- Staining solution.
  - Coomassie brilliant blue (R 250) 0.5 g.
  - Ethanol (96%) 45.0 ml
  - Glacial acetic acid 10.0 ml
  - Distilled water 45.0 ml
- Destaining solution
  - Staining solution without Coomassie brilliant blue.

**Procedure**
- Select a clean, scratch free, flat bottom Petridish and pour the melted agarose.
- After the agarose has solidified, with the help of the template and gel cutter, pattern of wells are cut. The inter-well distance can be varied from 4 to 6mm. The agarose from the wells can be removed with the help of needle. Seal the bottom of wells with the help of 0.5% melted agarose gel.
- Add hyper serum in the central well.
- Add test samples (antigen) in the peripheral wells with known positive and negative control antigen.
- Cover the plate with the lid and incubate at 37 °C in the box under humid atmosphere (to prevent drying to the gel) for 24 to 72 hours.
- Results can be read periodically at every 24 hours.
- A single antigen will give rise to a single precipitation line in presence of homologous antibody.

**Staining of precipitation bands**
- After optimal development of precipitation patterns, the plates can be stained for considerable improvement in sensitivity.
- The plates are submerged in normal saline and change saline in hourly intervals several times to remove non-precipitated proteins.
• Cracking of gel because of air bubbles can be avoided by filing the wells with a drop of agarose solution; Salt is removed by keeping the plates in distilled water for one hour.
• Gels are finally dried at 37°C overnight by keeping a wet filter paper over them. After drying, filter paper may be removed by slight wetting.
• The gels are then stained for 15-30 minutes by using the Coomassie brilliant blue stain followed by destaining.

AGGLUTINATION TEST

The principle of the test is that antibodies possess two antigen binding sites that will attach specifically with the antigenic determinants on the surfaces of the bacteria, cell or particles. Under suitable conditions one antibody molecule will combine with the determinant group on the surfaces of two bacteria and in this way a crystal lattice is formed. These lattices are generally visible with the naked eye as clumps and they sediment readily due to the large size of the clump.

Prerequisites for agglutination reaction

• The antigen (agglutinogen) should be in particulate form and remain in the suspension for sufficiently long time.
• The antibody (agglutinin) should be directed to the target surface epitope.
• Antigen – antibody should be present in optimal proportions.
• An electrolyte is necessary for the reaction to take place and usually the reaction is carried out in physiological saline solutions (0.85% NaCl). Because of the overall negative charge on red cells or bacteria at neutral pH it is difficult for them to come close enough, so that for molecule such as IgG antibodies are able to form a bridge between epitope of two different cells or bacteria. The presence of salt tends to neutralize the charge effects to allow agglutination.

Plate agglutination test

• This is employed to screen sera within a short period for preliminary testing. This test is commonly used for screening of antibody against *Mycoplasma gallisepticum*, *Salmonella Pullorum*, *Salmonella Gallinarum* and *Brucella abortus*.

Materials

• *Brucella abortus* colored antigen
• Suspected serum
• Glass plate

Procedure

• Place one drop of the suspected serum and one drop of Brucella abortus antigen adjacent to each other on a glass plate.
• Mix the reagents with an inoculation loop and rotate the plate while tilting the sides slowly. Watch for the agglutination reaction
• Check the antigen for auto agglutination by replacing the serum with a loopful of saline.
• Known negative and positive control serum should used simultaneously.
Abortus bang ring test
This test is also known as Milk Ring Test (MRT). This test is employed to test milk from brucella infected cow/buffalo herd.

Materials
- Milk from brucella infected and non-infected herd
- Tetrazolium stained *B. abortus* antigen
- Test tubes for conducting the test

Procedure
- Pipette 2 ml of milk in 2 different tubes from each sample. Add 1 drop of brucella ring test antigen and mix thoroughly.
- Incubate the milk for 1 hour at room temp, or for 30 minutes at 37°C in a water bath.
  - Observe the result.

Interpretation
- The clumps of agglutinated organisms are carried to the surface of the milk along with fat globules.
- A positive test is indicated by a column of discolored milk with a red cream layer on the top.
- A negative test will show red colour of the milk throughout with an uncolored cream layer on tap.

Tube agglutination test for Brucella
- The tube agglutination tests are more accurate than the plate test, but require relatively long incubation time.
- Set up six agglutination tubes in a row and add 0.8 ml of saline to tube No. 1 and 0.5 ml to the other five tubes.
- Add 0.2 ml of test serum to tube No.1. Mix well and then transfer 0.5 ml to tube No.2 Repeat two fold dilutions up to tube No.6 and then discard 0.5 ml (dilution 1:5, 1:10 and so on in each tube).
- Add 0.5 ml of plain antigen to each tube and mix well (final dilution of serum resulted will be 1:10, 1:20 and so on).
- Control tubes are also set in the test.
- Positive control with 0.5 ml of known positive serum and 0.5 ml plain antigen.
- Negative control with 0.5 ml saline and 0.5ml plain antigen.
- Tubes 6,7,8,9, and 10 serve as antigen control as well as 0.25,50 and 100 % agglutination standard respectively. (Opacity of the suspension to be compared with the opacity of the suspension in tubes 1 to 5).
- Because of the special significance of the 50% end point, control tube should be set up with 0.75 ml saline and 0.25 ml plain antigen.
- All tubes are then incubated at 37°C for 18-20 hour before the results are read.
Result
- The degree of agglutination is determined by reading the degree of clearing before shaking the tubes.
- The highest serum dilution showing 50% agglutination (i.e. 50% clear) or more is taken as the end point or titre in the serum.

Interpretation

<table>
<thead>
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<th>%</th>
<th>Agglutination</th>
<th>(Comparable with tube)</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>Agglutination</td>
<td>(Comparable with tube 6)</td>
<td>-</td>
</tr>
<tr>
<td>25%</td>
<td>Agglutination</td>
<td>(Comparable with tube 7)</td>
<td>+</td>
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<td>Agglutination</td>
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</tr>
<tr>
<td>75%</td>
<td>Agglutination</td>
<td>(Comparable with tube 9)</td>
<td>+++</td>
</tr>
<tr>
<td>100%</td>
<td>Agglutination</td>
<td>(Comparable with tube 10)</td>
<td>++++</td>
</tr>
</tbody>
</table>

- The highest serum dilution showing 50% agglutination is taken as the end titre of the serum.
- A titre of 1:40 or above indicates an infected animal.

Latex Agglutination Test

Latex beads are coated with either antigen or antibody, depending upon what we want to detect. Sensitized latex beads are added to the clinical samples. In positive cases, antigen and antibody combines usually in clumping of latex beads leading to agglutination.

Materials
- Saturated ammonium sulphate solution
  - Ammonium sulphate
  - Distilled water
  - The salt was dissolved at 50\(^{0}\)C and was allowed to stand at room temperature overnight.
  - The pH was adjusted to 7.2 with dilute ammonia or sulphuric acid.
- Latex beads – Latex beads (0.8 um) was used.
- Carbonate bicarbonate buffer:
  - Sodium carbonate - 1.5 g
  - Sodium bicarbonate - 2.93 g
  - Distilled water - 1000 ml
  - The pH of the solution was adjusted at 9.6 before making up to 1000 ml.

Procedure
The serum is precipitated by ammonium sulphate precipitation for isolation and concentration of Ig as per the protocol given in exercise-1.
- Coating of beads
100 µl of latex beads (10% suspension) is mixed with 900 µl of carbonate bicarbonate buffer. To this equal volume of immunoglobulin suspension in carbonate bicarbonate buffer is added. Mix the content well and keep at 4°C overnight for coating. The coated beads are centrifuged at 10,000 rpm for 15 min and the supernatant discarded and the nonspecific sites of the beads are blocked with 5mg/ml of bovine serum albumin and incubated at 37°C for 1 hour. After 1 h, the mixture is centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant is discarded and the beads are washed thrice in carbonate bicarbonate buffer.

One drop of the coated beads suspension is mixed with one drop of clarified supernatant of the suspected sample on a cavity slide.

Positive and negative controls are also used in the test for comparison. Agglutination of coated beads indicate positivity of the sample

**Interpretation**

Agglutination of coated latex beads indicate positivity reaction (sample is positive), No agglutination of coated latex beads indicate negative reaction (sample is negative)

**ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)**

The ELISA is extremely sensitive and can be designed in different formats to detect antigen or antibody. A variety of direct, indirect and reversed assays have been described, most involve a liquid phase ELISA. These systems depend on the capacity of antigen or antibody coated in a plate, which form an immnosorbant for subsequent attachment of antibody or antigen as the case may be.

**Components of Indirect ELISA**

The most successful and simple technique for measurement of antibodies in serum is indirect ELISA.

- Adsorption (coating) of antigen into an ELISA plate and washing off the excess unattached antigen.
- Blocking of unabsorbed areas with bovine serum albumin (BSA).
- Addition of serum, followed by incubation and then washing off the excess unattached antibody.
- Addition of enzyme labeled anti immunoglobulin conjugate, again followed by incubation and washing.
- Addition of enzyme – specific soluble substrate which produces a colour change. This is measured spectrophotometrically.
- In most cases, the enzyme used is horse radish peroxidase. Several soluble substrates have been used for peroxidase enzyme {(Ortho phenylene diamine dihydrochloride (OPD), or Tetra methyl benzidine (TMB), or 2, 2'- azino di (3-ethylbenothiaoline-6-sulfonic acid) ABTS)}. Different substrates give different colors after enzyme reaction.
Materials
Coating buffer (Carbonate-bicarbonate buffer)
  o Disodium Carbonate - 1.59 g
  o Sodium bi carbonate - 2.93 g
  o Distilled water 1000 ml
  o Adjust the pH to 9.6 and autoclave at 15 lb for 15 mts
  o Store at 4 °C
Blocking buffer
  o BSA - 0.5 g
  o PBS - 100 ml
  o Store at 4 °C
Washing buffer
  o Tween 20 - 5 ml
  o Distilled water - 1000 ml
Conjugate buffer
  o BSA - 0.8 g.
  o PBST - 100 ml
Substrate buffer (pH 4.5 – 5.5)
  o Di sodium hydrogen phosphate - 7.4 g.
  o Citric acid - 5.0 g.
  o Distilled water - 1000 ml
Stopping reagent (1N HCl)
  o Conc. HCl - 100 ml
  o Distilled water - 900 ml
Preparation of working conjugate
  Pre-assessed working dilution of peroxides conjugated anti immunoglobulin antibody is made using conjugate buffer (say 1:2000)
Preparation of substrate (To be prepared just before use)
  o Substrate buffer - 10 ml
  o H₂O₂ - 5 ml
  o TMB - 100 ml
Commercially available TMB H₂O₂ substrate diluted before use as per manufacturer’s instruction.
  • 96 well microtitre ELISA plate.
  • Micro pipette with tips
  • ELISA plate washer
  • ELISA reader
  • Pipette - 1 ml
  • Measuring cylinder - 1000 ml

Procedure
Establish the working dilution of the antigen and conjugate by checker board titration.
Dilute the antigen in coating buffer to its optimal dilution and add 100\(\mu l\) of diluted antigen in each well of a polystyrene microtitre plate.
Incubate the plate over night at 4\(^0\) C in a humid chamber.
Wash the plate thrice using washing buffer.
Add 100 \(\mu l\) of the blocking buffer to each well and incubate at 37\(^0\) C for 30 minutes
Wash the plate thrice using washing buffer.
Make 2 fold dilutions (1:100, 1:200, 1:400 and so on) of the test and reference sera. Add 100 \(\mu l\) to each well. Include a negative control.
Incubate the plate for 1 h at 37 \(\O C\) in humid chamber.
Wash the plate thrice using washing buffer
Add 100 \(\mu l\) of the prepared conjugate to each well. Incubate at 37 \(\O C\) for 1 h.
Wash the plate thrice using washing buffer.
Add 100 \(\mu l\) of the working substrate (OPD) to each well. Leave for 15 minutes for the reaction to take place in a dark chamber.
Stop the reaction by adding 100ml of stopping solution to each well.
Take the OD values (optical density) at 490 nm in an ELISA reader.

**Antibody Detection**
- Antibody can be detected using indirect ELISA.
- Serum or some other test sample containing primary antibody is added to an antigen coated microtitre plate well.
- After an incubation period free primary antibody is removed by washing, the presence of antibody bound to the antigen is detected by adding an enzyme tagged secondary antibody or antispecies antibody or antiglobulin which binds to the primary antibody.
- After an incubation period any free secondary antibody is washed away and a substrate for the enzyme is added.
- The amount of colored reaction product formed is measured by specialized ELISA readers.

**Interpretation**
- The highest dilution of serum showing OD value that is twice and above the negative control OD is taken as the end point.
- The reciprocal of the dilution is taken as the serum titre.
Demonstration of PM-PCR for diagnosis of Hemorrhagic Septicemia

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Introduction

*Pasteurella multocida* is a Gram-negative, facultative anaerobic, coccobacilli. It occurs as a commensal in the nasopharynx of apparently healthy animals. It can also be a primary or secondary pathogen in disease processes of domestic animals and birds (Rimler and Rhoades, 1989). It causes a number of diseases in various domestic and wild animals. The most important diseases are, hemorrhagic septicemia (HS) in cattles & buffaloes and fowl cholera (FC) or avian cholera in poultry/turkey resulting in heavy economic losses to livestock owners. In India, during the past four decades, HS has accounted for 46-55 per cent of all bovine mortality resulting in heavy economic losses (Dutta et al., 1990).

Laboratory diagnosis of *P. multocida* infection mainly based on isolation and identification by cultural and biochemical methods, this procedure has its own limitation. The development of *P. multocida* species specific polymerase chain reaction (PM-PCR) assay by Townsend et al. (1998) has provided a rapid and specific detection of *P. multocida*.

DNA Extraction

The genomic DNA of *P. multocida* isolates can be isolated according to Wilson (1987) with minor modification.

The culture should be prepare by inoculating the isolate in Luria broth and incubating at 37°C for 24 h in a shaker waterbath. About 50 ml of broth culture require to centrifuge at 10,000 rpm for 10 min at 4°C. The supernant should be discarded and the pellet will be used for extraction of nucleic acid extraction.

Isolation of genomic DNA by Proteinase-K-SDS method

Pellet containing bacterial cells should be suspended in 2 ml Tris-EDTA (pH-8.0), 250 µl SDS (10% w/v) and10µl of proteinase K solution (20mg/ml, w/v) and incubated for 1 h at 37°C. Subsequently, 500 µl of 5M NaCl followed by 100 µl CTAB (hexadecyl trimethyl ammonium bromide, 10% solution in 0.7M NaCl) should be added and incubated in water bath for 10 min at 65°C. The solution should be spun at 8,000 rpm for 10 min after mixing with equal volume of chloroform : isoamyl alcohol (24:1) and upper phase require to transfer to clean microfuge tube. Add Equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), mixed well by inverting, spun for 10 min at 10,000 rpm and upper aqueous phase should be transfer again to a clean microfuge tube. DNA should be precipitated in the collected supernant with one-tenth volumes
with ammonium acetate (7.5M) and double the volume with chilled absolute ethanol. Centrifuge the tube for 10 min at 11,000 rpm and discard the ethanol. Washed the pellet with 70% ethanol and again spun for 5 min at 11,000 rpm. Discard the Ethanol and pellet should be dried. DNA will be resuspended in 200 µl sterile distilled water and kept in water bath at 65°C for one hour and stored at -20°C till use.

**Quality checking and quantization of DNA**

**Quality** and purity of DNA should be checked by agarose gel electrophoresis. Use 0.8 percent agarose in 0.5X TBE (PH 8.0) buffer (Sambrook *et al.*, 1989) for submarine gel electrophoresis. Add Ethidium bromide (1 %) @ 0.5µl /100ml. Charged he wells with 5µl of DNA preparations mixed with BPB dye. Carry out Electrophoresis at voltage 5V/cm for 20 min at room temperature. DNA should visualize under UV transilluminator.

**Quantity** of DNA should be calculated by spectrophotometric method. OD at 260 and 280 should be taken in UV spectrophotometer with distilled water as reference.

Concentration (µg/ml) = OD at 260 x dilution factor x 50

Where 50 is concentration of dsDNA at OD of 1

**PCR reaction for PM-PCR**

The following components should be used in sequence in PCR mixture.

i. 10 X PCR buffer

ii. MgCl₂ 25mM

iii. dATP 10mM

iv. dCTP 10mM

v. dGTP 10mM

vi. dTTP 10mM

vii. *Taq* DNA polymerase (5U/µl)

viii. A pair of eubacterial *P. multocida* specific primer used by Townsend *et al.* (1998) should be used at a final concentration of 10 pmol/µl.

Primer KMT1SP6 5’- GCT GTA AAC GAA CTC GCC AC - 3’

Primer KMT1T7 5’- ATC CGC TAT TTA CCC AGT GG – 3’

*ASCAD Training on “Advanced approaches for diagnosis of livestock diseases” (25-30 Nov., 2013)*
ix. DNA samples should be diluted to final concentration of 30ng/µl and 3µl of this preparation (containing 90ng DNA) can be use as template for PCR reaction

**PM-PCR Conditions**

PCR should be carry out in final reaction volume of 25 µl. Each reaction contained 2.5µl of 10X PCR buffer, 1.5 µl of 25mM MgCl₂, 0.5 µl of 10mM of each dNTPs, 0.5 µl of 10pmol/µl of each primer, 0.2µl of Taq DNA polymerase, 3µl of template DNA and 16.30 µl of distilled water in 200µl thin walled PCR tubes. PCR should be perform with a standard thermal under the following conditions: Initial denaturation at 95°C for 5 min, then 32 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min. and extension at 72°C for 2 min followed by final extension at 72°C for 6 min. PCR products should be electrophorese in 2 per cent agarose gel along with standard marker. Visualize the amplicons as a single band under UV light.

**Result**

*P. multocida* species specific PCR (PM-PCR) performed using primer pair KMT1SP6 and KMT1T7 (Townsend et al., 1998). will produced an amplified product of approximately 460 bp size. It is evident from various results and related reports, that PM-PCR provides rapid identification of all serotype of *P. multocida* isolates and can be used as rapid method specific for detection of *P. multocida* infection in animal and poultry.

**References**


Demonstration of diagnostic methods for parasitic infections/infestations of animals
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Introduction
Parasitic infections are responsible for substantial morbidity and mortality worldwide. The diagnosis of parasitic infections in animals has great importance due to the major threats created by this group of diseases on the welfare and productivity of domesticated animals and wildlife. A range of methods and procedures aiming at the direct or indirect detection of parasite infections through laboratory examinations have been described in the last decade. Sensitive, reliable and cost-effective laboratory test systems are desirable for conducting epidemiological surveys, as well as a prerequisite for routine on farm parasite monitoring. Various host material matrices are being used to test for parasite infections, e.g., blood, serum, faeces, liquor, skin scrapings or saliva, depending on the parasite under detection and its localisation within the host. A large range of different techniques, including basic microscopy based-methods, molecular, immunological or recent nanotechnological tools, have been adopted to provide specific diagnostic solutions for the detection of parasite infections in animals.

Macroscopic Examination

Endoparasites
Clinical samples should be looked over for any intact specimens, which may have been passed. The following are the most commonly encountered and most likely to be visible on cursory examination. Many others may well be found by a more diligent examination. Like presence of *Entrobius vermiculus* adults, occasionally hookworms in human stool, either as whole specimens of *Anoplocephala perfoliata* or as segments of *A. perfoliata* and *A. magna*, L4 and effete adult trichonemes; *Oxyuris equi* females in horse, *Moniezia* spp. segments; *Oesophagostomum* adults; *Chabertia* adults; occasionally *Toxocara vitulorum* adults in cattle faeces, *Moniezia* spp. *Thysanosoma*, *Stilesia*, and *Avitellina* spp. segments; *Oesophagostomum* adults; *Chabertia ovina* adults may be present in faeces of sheep and goat, *Oesophagostomum* adults; occasionally *Ascaris* adults in pigs, *Taenia* spp. and *Dipylidium* spp. segments in dogs and cats faeces. *Heterakis* adults; lengths of strobila of *Hymenolepis* spp. in poultry birds.

Ectoparasites
Several parasitic flies and its larva, lice, fleas, ticks etc are required to be identified for the control of this menace. For this purpose ectoparasites life stages were collected from the animal body or its habitats. Different ectoparasites were identified based upon its shape, size, form, presence or absence of various external structures like adult flies having wings (with few exceptions such as *Melophagus ovinus*) and lice, fleas and ticks are wingless. Similarly, flies, lice and fleas are possessing antennae but it is absent in ticks. Within species the identification is based upon differences in wing venation, number of segment of antennae, body segments, presence or absence of eye if present simple eye or compound eye etc. In case of fly larvae, identification is done by microscopic examination of posterior spiracle, number of spine on body surface etc.
Microscopic examination

Endoparasites
Parasites having many life stages like eggs, cyst, oocyst, larvae etc which is microscopic. These small forms of helminth parasite and unicellular protozoan parasites develops in different part of body and released time to time through excretion and secretion in external environment. The identification of these life stages has great importance in diagnosis of parasitic diseases. For the microscopic examination of faeces or other body secretion /excretion for parasitic infection, there is no all-purpose technique. Nor is there any "best" technique for any one purpose. For reliable diagnosis of intestinal parasites, a combination of several techniques is required. Reliability of the results of an examination depends less on the technique selected than on how well the procedures of the selected technique are carried out. Investigator’s experience and technician training are factors of great importance. Some form of quality control should be a part of the routine of every diagnostic laboratory. Some of the common technique which is used in laboratory for the routine diagnosis of endoparasites is direct smear examination, sedimentation technique, floatation technique, and special staining technique such as Modified Ziehl-Neelsen's technique for Cryptosporidium, Leishman/Giemsa/Wright stain for haemoprotozoan etc.

Ectoparasites
Ectoparasite like mites is usually microscopic. Most common mite infestations are diagnosed by deep or superficial skin scarping. For a deep skin scraping, a dulled, rounded scalpel blade and the area of skin to be scraped are coated with mineral oil. The site selected for scraping should be at the periphery of a lesion or the predilection site of the suspected parasite. The blade should be scraped back and forth over the skin until capillary bleeding is evident (a shallower scraping is done for surface-dwelling mites). For collection of Demodex, the follicle mite, a skin fold should be squeezed between the fingers to express the mites before the scraping is done. The debris collected on the scalpel blade is then placed on a microscope slide, coverslipped, and examined using the 10× microscope objective or processed the sample in 10% KOH. Several slides may need to be examined before mites are found, especially in cases of Sarcoptes infestation. If mites need to be transported to veterinary laboratory for identification, they should be stored in 70% alcohol. Skin scrapings and mites that are stored dry cannot usually be successfully identified.

Immunodiagnostic Methods
Immunodiagnostic methods for a range of parasitic infections are available. There are two basic approaches in designing an immunologic test. Antigen detection tests identify specific parasite-associated compounds in blood, serum, or fecal suspensions that indicate the presence of the organism in the host. Alternatively, antibody detection tests show the host immune response to a parasite through the production of specific antibodies. To have a positive test result, it is assumed that the host animal is immunologically competent to react to the pathogen and that a sufficient time of exposure has occurred for the animal to produce detectable antibodies. There are a variety of test formats for immunodiagnostic tests. The enzyme-linked immunosorbent assay (ELISA) Dot ELISA, Immunochromatographic assay. These test formats can be designed as antigen or as antibody detection assays, and test formats have been developed for use with blood, serum, or faeces. The other commonly used format is the indirect fluorescent antibody (IFA) test, which is an antibody detection test designed for use with serum or plasma. These tests are routinely performed in the diagnostic lab setting because a compound microscope equipped with
appropriate barrier filters and a UV light source is needed to evaluate the test. Other less common immunodiagnostic test formats include direct or indirect hemagglutination (HA or IHA), complement-fixation (CF) tests, and Western blot tests. World wide many immunodiagnostic tests are available for pet and domesticated animals for the detection of pathogenic organism (Table 1).

Table 1. Immunodiagnostic test available for parasitic diseases in human and animals.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Host affected</th>
<th>Commercial test</th>
<th>Basis of test</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Giardia intestinalis</em></td>
<td>human</td>
<td>1. MeriFluor Crypto &amp; Giardia test (Meridian Bioscience, Cincinnati, OH, USA), 2. Giardia II test (TechLab, Inc., Blacksburg, VA, USA)</td>
<td>ELISA</td>
</tr>
<tr>
<td><em>Strongyloides stercoralis</em></td>
<td>human</td>
<td>1. IVD-ELISA [IVD Research, Inc 2. Bordier-ELISA [Bordier Affinity Products SA)]</td>
<td>ELISA</td>
</tr>
<tr>
<td><em>Schistosoma haematobium</em></td>
<td>human</td>
<td>1. urine-circulating cathodic antigen (CCA) strip 2. soluble egg antigen enzyme-linked immunosorbent assay (SEA-ELISA)</td>
<td>ELISA</td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td>human</td>
<td>1. microwell enzyme linked immunoabsorbant assay (ELISA) detection kit</td>
<td>ELISA</td>
</tr>
<tr>
<td><em>Dirofilaria imitis</em></td>
<td>Dog</td>
<td>1. Difil test (EVSCO Pharmaceuticals)</td>
<td>ELISA</td>
</tr>
<tr>
<td><em>Wuchereria bancrofti</em></td>
<td>human</td>
<td>1. Tro-Bio ELISA and ICT card test</td>
<td>ELISA</td>
</tr>
<tr>
<td><em>Leishmaniasis</em></td>
<td>human</td>
<td>1. dipstick test based on a highly specific recombinant antigen 2. latex agglutination test</td>
<td>CFT</td>
</tr>
<tr>
<td><em>Babesia equi / Theileria equi</em></td>
<td>horse</td>
<td>1. COFEB-kit</td>
<td>CFT</td>
</tr>
<tr>
<td><em>Toxocara canis</em></td>
<td>human</td>
<td>1. IgG-ELISA commercial kit (Cypress Diagnostic, Belgium)</td>
<td>ELISA</td>
</tr>
</tbody>
</table>

Molecular Diagnosis

Several molecular tests to detect parasites have been developed in the last decade. Their specificity and sensitivity have gradually increased, and parasites that were previously difficult to diagnose using conventional techniques began to be identified by molecular techniques. As a
result, currently, these parasites can be easily treated before causing major harm to the infected population. Current molecular diagnostic methods for the identification of parasites include: polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), microsatellite marker method, loop-mediated isothermal amplification (LAMP), and the real-time PCR.

**Polymerase Chain Reaction (PCR)**

The PCR makes it possible to perform selective amplification from complex genomes. PCR-based techniques have revolutionized many areas of study because the enzymatic amplification of DNA can be performed in vitro from small amounts of material. This is particularly relevant to parasitology because it is frequently impossible to obtain or isolate a sufficient amount of material from parasites at their different life-cycle stages for conventional analysis. These techniques provide alternative methods for detecting specific pathogens in stool. The detection sensitivity of PCR is higher than that of light microscopy; therefore, this technique is useful for detecting a low number of parasites in stool samples. The PCR technique has also been used to investigate non-intestinal parasites. The effectiveness of PCR in detecting parasite species such as *Leishmania* and *Plasmodium* has already been studied. Despite the benefits of PCR-based technologies, such as high specificity and sensitivity to detect some parasites, the main disadvantage of these methods is that they are very time-consuming and do not provide quantitative data.

**Real-Time Polymerase Chain Reaction (RT-PCR)**

This technique was developed in the early 1990s and enables the monitoring of PCR amplification in real time. RT-PCR is a simple, fast, closed, and automatized amplification system responsible for decreasing the risk of cross-contamination typical of conventional PCR. Using RT-PCR it is possible to quantify parasitic nucleic acids from environmental samples or tissues, as well as to estimate the intensity of infection and/or viability of parasites. In spite of the disadvantage presented by the high costs of RT-PCR, which may prevent this technique from being used in resource-poor settings, its advantageous performance characteristics and rapid results warrant its use as a diagnostic adjunct for parasitic infection.

**Loop-Mediated Isothermal Amplification (LAMP)**

LAMP is a technique that enables DNA amplification with high specificity, sensitivity, and rapidity under isothermal conditions. DNA amplification can be achieved using simple incubators (water bath or block heater) because of these isothermal conditions. Such simple requirements make LAMP easily available for small laboratories, especially in rural endemic areas; thus, it seems to be a promising tool. Recently, parasitologists have adapted the LAMP technique to detect several parasitic diseases, including the human parasites *Cryptosporidium*, *Entamoeba histolytica*, *Plasmodium*, *Trypanosoma*, *Taenia*, *Schistosoma*, *Fasciola hepatica* and *Fasciola gigantica*, and *Toxoplasma gondii*, and animal parasites such as *Theileria* and *Babesia*.

**Random Amplified Polymorphic DNA (RAPD)**

Known as AP-PCR (arbitrarily primed PCR), RAPD has been extensively used for description of strains in epidemiological studies. The surveying of genomes of parasites is enhanced by the advantage that RAPD is a very simple, fast, and inexpensive technique that does not require either prior knowledge of the DNA sequence or DNA hybridization. Studies on parasitic nematodes of plants and humans have demonstrated its high efficiency in the differentiation of amplification profiles, as well as its ability to distinguish polymorphisms.
between microorganisms. RAPD markers have been used to map genes for the characterization of species, to stimulate the genetic variability and determine the genetic structure of populations of different microorganisms. Studies using this technique show that it is able to differentiate species of *Leishmania*, in addition to being used to study polymorphisms of parasites of medical importance such as *Plasmodium* and *Trypanosoma*. This technique enabled the differentiation of endemic strains of *Wuchereria bancroftii*, *Leishmania donovani*.

**Amplified Fragment Length Polymorphism (AFLP)**

The AFLP technology is also a technique that allows detection of a DNA polymorphism without prior information on the sequence. This technique is highly efficient because of the possibility of analyzing a large number of bands simultaneously, with extensive coverage of the genome. This method of molecular diagnosis employs PCR to selectively amplify the groups of restriction fragments of totally digested genomic. It consists of four steps: DNA digestion, ligation, amplification, and gel analysis. The advantages of this technique are the ability to search an entire genome for polymorphisms, the reproducibility of the method, and the possibility of being used against parasites about which there is no prior genetic information. Analysis based on AFLP polymorphic markers have revealed high genetic variability among the genome species of *Leishmania major*, *L. tropica* and *L. donovani*, which was sufficient to distinguish between CL and VL.

**Restriction Fragment Length Polymorphism (RFLP)**

The RFLP technique is currently one of the most commonly used molecular methods for diagnosis of species and genotypes of parasites such as *Toxoplasma gondii* and other parasites. This reaction is based on the digestion of the PCR products by restriction enzymes or endonucleases. These enzymes cleave DNA into fragments of certain sizes, whose analysis on agarose or polyacrylamide gel results in different patterns of fragment sizes, enabling the identification. The RFLP technique is suitable for environmental samples because it permits the detection of multiple genotypes in the same sample. Importantly, the RFLP technique can also be used in the differentiation of animal parasites, such as species of *Theileria*.

**Microsatellites**

Microsatellites are short DNA sequences (about 300 base pairs) composed of tandem repeats of one to six nucleotides, with approximately one hundred repeats. Microsatellites have been described in parasitology and used in some parasites of both humans and animals. Microsatellites are abundant in eukaryotic genomes and can mutate rapidly by loss or gain of repeat units. The wide variety of applications of microsatellites is mainly because they show frequent polymorphism, codominant inheritance, high reproducibility and high resolution, require simple typing methods, and can be detected by PCR. Despite their potential usefulness, microsatellite markers were developed only for some parasitic nematodes such as species of *Trichostrongyloid nematodes*. The low popularity of these genetic markers may be explained by the high number of microsatellites, which cause technical difficulties in isolating parasites by PCR.

**Reference**


Demonstration of post mortem examination and histopathology techniques

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Necropsy examination
- Post-mortem Examination (PM) of carcass is conducted by Veterinarian or a Veterinary Pathologist to ascertain the cause and nature of disease in fatal cases of diseases. The term autopsy is preferred in human medicine for PM examination and necropsy in Veterinary Medicine.
- **Autopsy** means seeing with one’s own eyes.
- **Necropsy** means seeing a carcass.
- **Autopsist** is one who conducts the PM examination

Types of necropsy
- Where no necropsy is conducted
  - If the blood smear from ear vein (cattle, sheep and goats) or smear from oedematous fluid from throat or abdominal region (pings, horse) reveals *anthrax bacilli* no necropsy should be conducted on the carcass since the organisms are aerobic spore formers. The spores survive as long as 18 years.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Particulars</th>
<th>Anthrax bacilli</th>
<th>Anthracoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Organism</td>
<td><em>Bacillus anthracis</em></td>
<td>Other than <em>B. anthracis</em></td>
</tr>
<tr>
<td>2.</td>
<td>Capsule</td>
<td>Predominantly pink stained</td>
<td>Less predominant</td>
</tr>
<tr>
<td>3.</td>
<td>Spores</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>4.</td>
<td>Length of chain</td>
<td>Short-usually 2 to 3 organisms</td>
<td>Long chains</td>
</tr>
<tr>
<td>5.</td>
<td>End of bacilli</td>
<td>Truncated</td>
<td>Rounded</td>
</tr>
</tbody>
</table>

- Partial necropsy
  - In case of rabies only the brain of the ‘carcass’ is examined for diagnosis. Here only a part of the body (head) is opened for the purpose. Other parts of the body are not opened.

- Complete necropsy
  - All parts of the body are thoroughly examined to arrive at an aetiological diagnosis.

- Cosmetic necropsy
  - Examination of the carcass is done with very less mutilation. Cuttings and incisions are sewed together and the body is washed to appear as nearly intact as possible. It is done in case of pet and wild animals.
Necropsy as a factor in diagnosis

- Necropsy actually accomplishes to bring into open previously unseen or merely surmised lesions and even certain etiological agents not observable from the animal’s exterior. Quite frequently, the necropsy may be compared with opening and reading a book, the title of which conveys a certain meaning; but it is the text that really portrays the plot, the sequence of events and the conclusions. The necropsy like the text book may reveal items of a surprising or unexpected nature thus explaining previously unknown or baffling events.
- Clinical diagnosis would be more accurate if the clinician follows the animal which failed to respond to therapy to the necropsy.
- The veterinarian holds a district advantage over the physician in the matter of Post-mortem diagnosis since he/she may employ euthanasia in order to hasten the process of diagnosis.

Time of necropsy

- The post-mortem examination (PME) should be conducted as soon as possible after the death of the animal. If delayed, various PM changes including autolysis and putrefaction may set in which sometimes may confuse with morbid lesions and distort the diagnosis. However, even if PM changes have advanced considerably, still from a stand point of gross pathology the deterioration is not as serious as many believed. If the disease was one that could have been diagnosed originally by the gross pathological changes it probably can still be diagnosed by distinguishing PM changes from morbid lesions.

Place of necropsy and site for disposal

- The veterinary practitioner needs but little space for the conduct of necropsies. For small animals, a well ventilated room of the hospital may be set aside for euthanasia and for necropsies. In large animals, the veterinarian should choose an outdoor large area least likely to allow contamination to spread.
- Sanitary conditions and intended disposition of the carcass are factors which outweigh convenience in deciding where to perform the necropsy. If there is any possibility that the animal may have died of a contagious disease, it is imperative to avoid contamination of ground accessible to susceptible livestock or their food.
- If the necropsy has to be performed near farm building or on ground from which livestock is not excluded, it may be feasible to have an extremely deep bed of straw prepared on which to place the carcass. The straw absorbs the fluids and can be burned or buried afterwards.
- More frequently, it will be decided to transport the carcass to some distant field not used for livestock atleast during the current year. If the animal is to be buried, a deep grave layered with lime can be dug where the carcass can be easily rolled into, to be followed by the contaminated layer of the earth.

**DISINFECTANTS AND PRECAUTIONS**
Disinfectants

- Although not effective as steam / heat sterilisation, chemical disinfectants are usually employed for necropsy instruments, boots and gloves as well as the tables and premises connected with necropsy. To be effective any such disinfection must be preceded by thorough mechanical cleaning. The commonly used chemicals are Lysol, cresol, chlorine, quaternary ammonium compounds, mercury in the form of bin-iodide combined with potassium iodide, iodophores, phenol etc. The choice of disinfectant to be used for the disposal of carcass where the cause of death suspected to be infectious and contagious (Anthrax) is quick lime.

Precautions

- Obtain written permission from the owner before post mortem examination.
- Request from local police is a must in vetero-legal cases
- Conduct postmortem as early as possible to avoid putrefaction.
- Examine the smear from peripheral blood to rule out anthrax. Besides anthrax bacilli, examination of blood smear may reveal blood parasites, other bacterial and/or postmortem invaders.
- Postmortem examination should be done in day time to appreciate the accurate changes in the colour of tissues. This is not possible with artificial light.
- Conduct postmortem far away from animal houses and farm premises and preferably in a government land to avoid litigation.
- Obtain history, symptoms and treatment done etc.
- Wear gloves, mask, aprons and gum boots to avoid contact with zoonotic agents.
- Record the postmortem findings immediately.
- Bury the carcass in deep ditches layered with lime. Carcass can be burnt to ashes if incinerator is available.

Note before necropsy

- Ruminants: Place the carcass on the left side to avoid the interference of rumen during examination.
- Horses: Place the carcass on the right side to avoid the interference of colons during examination.

Necropsy Techniques and Observations

Steps in Necropsy

1. Record the kind of animal and to whom it belongs.
2. Write the precis of the case.
3. Carry out the external examination of the carcass. Then proceed to internal examination.
4. Secure the carcass on its back.
5. Make incision in the mid ventral line from chin to anus going round about the external genitalia in male and incision is also made on the medial aspect of all legs and flay the skin.
6. Examine the subcutaneous tissue.
7. Open the cavities of the body. Look for exudates, transudate etc.
8. Examine the position of the organs.
9. Separate lungs from heart and palpate for any abnormalities. Incise and examine the lungs.
10. Examine the pericardial sac. Open the pericardium, examine the nature of contents.
11. Cut through heart and the vessels. Examine the wall and chambers for the nature of content, valves and the lumen of vessels.
12. Examine the diaphragm.
13. Examine the abdominal visceral organs-liver, spleen, kidneys, adrenals, pancreas before and after incising the organs.
14. Open the bile ducts and gall bladder and examine.
15. Divide the kidney symmetrically by longitudinal incision. Remove the capsule, examine the cortex, medulla and pelvis.
16. Open the mouth to examine the gum, tooth, tongue and buccal cavity. Then open and examine esophagus.
17. Open the nasal cavity and examine. Examine the pharynx, larynx, trachea and bronchi.
18. Open the stomachs/forestomachs and abomasum (ruminants) and examine the nature of contents and the wall.
19. Open the intestine. Examine the contents and the wall.
20. Open and examine the urinary bladder for the nature of content and the wall.
21. Examine the genital organs.
22. Open the skull and vertebral column to examine the brain and spinal cord.
23. Examine the skeleton and musculature.
24. Record the findings.
25. Summarize the appearances found.
26. Collect suitable materials for microbiological, histopathological, parasitological and chemical examination as required.
27. Arrive at an etiological diagnosis based on the PM findings and the results of the materials examined.

**PRECISE OF THE CASE**

- This includes date of admission, ward, case number if treated in Veterinary Hospital or admission as carcass with case number, date and time to death reported, date and time of making PM, history with symptoms, treatment details and clinical diagnosis

**EXTERNAL EXAMINATION OF THE CARCASS**

- Record the class of animal as bovine, equine, porcine, ovine, caprine, canine, feline etc., sex, age; if not known, assess based on teeth or ossification of bones; breed - specify the breed or record it as not-descript. Descriptive marks – natural colour and markings (whirls) and artificial marking-tattoo number, brand marks, tag number, wing band or leg
band number etc., In vetero-legal cases, measure the distance between horns at the level of base, midlevel and tips, and the length and direction of horns.

- Record the condition of body as fair (well-fed), poor, emaciated/cachectic (hide and bone condition).
- Record rigor mortis as present or absent
- Natural orifices-nature of discharge-in anthrax tarry coloured blood oozes from natural orifices and blood fails to clot. Abortion or metritis- discharge from genital orifice.
- Visible mucous membrane –Pink, pale or blanched (anaemia), icteric (jaundice), congested, haemorrhagic, cyanotic (local or systemic disturbances). Skin and coat - hair loss (patchy or complete); look for the presence of wounds, abrasions, lacerations, perforations, swelling, abscess and tumours. Check umbilicus for omphalitis, mammary gland and external genitalia for any change.
- Snake bite; Fang marks with swelling, haemorrhage or necrosis; Foot and Mouth Disease vesicles, maggots wounds in the interdigital spaces; Blue tongue: coronet region congestion and haemorrhage; Swine erysipelas: diamond like lesion; swine fever; erythema or purplish discoloration of skin; Canine distemper: pustules on the ventral side of the abdomen; Pock lesion on the udder and teats in cattle and face and also underneath the tail in sheep.
- Examine the skin-look for dermatomycosis (ring worm) and scabies or mange and look for ectoparasites-ticks, lice, fleas etc., Examine the superficial lymph nodes - prescapular, precrural etc., swollen (theileriosis) purulent inflammation (glanders, strangles).

INTERNAL EXAMINATION OF THE CARCASS

- Subcutaneous tissue: normally fair and moist but may be dry, congested, haemorrhagic (contusions), icteric, edematous, arboriform congestion and haemorrhages (electrocution).
- Abdominal and thoracic cavities-record the nature of exudates, if present, examine the organs in situ to appreciate any dislocation (abomasal displacement, intussusception, hernia etc.) adhesion of serous membranes etc.
- Pericardial sac-moist, record the nature of content –cattle –traumatic pericarditis with fibrinopurulent exudates and foreign body may be seen: cardiac tamponade-blood clot covering the heart-rupture of aorta (spirocercosis).
- Heart – epicardial and endocardial haemorrhage (septicaemia, toxemia, enterotoxaemia, impaction, pyometra); purulent pericarditis in salmonellosis, fibrinous pericarditis in colisepticaemia, tigroid appearances, i.e pale streaks in myocardium in Foot and Mouth Disease in calves and pigs; Chambers may contain, unclotted blood, partially clotted blood, clotted blood “current jelly” and “chicken fat” clot. Vegetative endocarditis affect valves (swine erysipelas, streptococcal and corynebacterial infections etc.)
- Larynx, trachea, bronchi-mucosa may be congested: trachea and bronchi contain frothy exudates in pulmonary edema, aspirated substances, caseous and haemorrhagic exudates with haemorrhagic mucosa (ILT), Lungs: pink and spongy normally, areas of emphysema, collapse, congestion, subpleural/parenchymatous haemorrhages, infarcts, red or grey hepatisation, granulomatous nodules may be caseous, calcified or uncalcified in contagious bovine pleuroneumonia), black spots (anthracosis), brown induration (chronic cardiac failure): pleura-shaggy appearance in serofibrinous inflammation.
- Spleen -enlargement, infarction, abscesses, tumour (lymphoid).
• Kidney-Capsule should peel off easily: but is adherent to cortical surface of kidney in inflammation: kidney –enlarged, shrunken and hard (chronic nephritis), cystic, hydropnephrosis, congested, petechial haemorrhages (“turkey egg appearances” in swine fever), pale areas (infarcts or lymphoid cell collection-theileriosis), tumours; Lesions may involve medulla and pelvis; Pelvis-surface smooth, eroded, congested, calculi.

• Ureter –distended, indurated; Gout –tortuous with urates and uric acid and distended.

• Urinary bladder –may be empty or distended with urine, examine nature of contents [urine straw coloured to colourless normally, deep yellow (icterus)], reddish to coffee coloured (haematuria and haemoglobinuria); Mucosa –congested, haemorrhagic, bladder – thickened (chronic cystitis, bovine hill haematuria). Look for calculi (may be few to many).

• Adrenals-enlargement, tumour, thinning or widening of cortex (stress)

• Mouth: Look for vesicles and ulcers on the gums, dental pad, tongue etc., in foot and mouth disease, Bran-like deposits on the gums and tongue in rinderpest, cyanotic tongue in blue tongue, wooden tongue in actinobacillosis, condition of teeth: worn out, sharp teeth etc., and tumours.

• Esophagus- Check patency (stenosis and dilatation or diverticulum) and for foreign bodies (choke), spirocerca nodules (distal end of oesophagus) in dogs, pustule-like lesion in vitamin A deficiency in chicken.

• Forestomachs (ruminants) –bloat, examine nature of content-solid semisolid, liquid/watery, impacted, exudates etc., nature of exudates, worms, trichobezoars, phytobezoars and foreign bodies. Examine mucosa and submucosa for erosions, ulceration, congestion, haemorrhage, perforation etc.

• Forestomach or stomach: Impaction and bloat –congested and haemorrhagic, vesicles in FMD; abomasums –ulcers in theileriosis; Habronema nodules in horses Ranikhet disease – proventricular haemorrhages around glandular papillae; IBD – haemorrhage in the proventriculus –gizzard junction.

• Intestine: Coccidiosis ballooning up of intestine with red and white spots seen through serosa with blood mixed porridge like content present in lumen. Rinderpest serofibrinous foul smelling contents; streaks of haemorrhages in the intestine and rectum (Zebra marking); Swine fever –button like ulcers in the caecum and colon. Nodules –E.coli infection, lymphoid leucosis, TB, worms; gangrenous inflammation (intussusception, volvulus, torsion), calculi. Mesentery: vessels for congestion, surface for deposits (Traumatic peritonitis, egg peritonitis) and tumours.

• Liver—may become pale, yellowish (icterus, fatty changes) normal sharp borders become rounded(swelling) soft and friable(fatty changes) cooked up appearance, hard in consistency in cirrhosis/chronic hepatitis, nutmeg appearance (chronic venous congestion), surface may show congestion, necrotic foci (grayish-white), telangiectasis, haemorrhages, cysts, abscesses, tumours which may extent into parenchyma, liver flukes in bile ducts with clay-pipe cirrhosis/biliary cirrhosis, colisepticaemia-fibrinous exudates covers the surface.

• Gall bladder –thickened wall, nature of content (bile) –think, thick, greenish, yellowish green etc., calculi.

• Generative organs: testicles – Cryptorchid, swelling, balanoposthitis; Accessary glands – prostate-enlargement; ovaries enlargement, cystic, and tumours, uterus –tear, torsion,
congestion, haemorrhage, nature of content, mummified or macerated fetus, pyometra (common in dogs), vagina and vulva (IBR/IPVV).

- **Skeleton:** Actinomycosis –granuloma of mandibular bones: fractures, osteoporosis, osteopetrosis, osteodystrophy, rickety rosary (vitamin deficiency), Bone marrow –for hyperplastic or hypoplastic activity.
- **Musculature:** degeneration, necrosis, gangrene (BQ) pale streaks (Vitamin E and Selenium deficiency, white muscle disease) and abscesses. Brain, spinal cord and meninges for congestion, haemorrhage (heat stroke), cyst-coenurus, encephalomalacia (Vitamin E deficiency) Nature of cerebrospinal fluid –clear, cloudy etc.
- **Examine all lymph nodes in general, particularly regional lymph nodes if any changes are detected in the organs or tissues. Lymph nodes - enlarged, oedematous, congested, haemorrhagic, granulomatous (TB, JD) purulent (strangles, glanders) and tumours (lymphosarcoma). Examine bursa in birds – enlarged, haemorrhagic, contain creamy, caseous or haemorrhagic exudates and shrunken (IBD).

**NECROPAY PROFORMA OTHER THAN POULTRY**

**No. /20**

**POSTMORTEM REPORT**

Notes on the postmortem examination on the body of a ___________________

belonging to ………………………………………………………………………………...

Result of examination of blood smear taken after death :

**A. Precis of the case**

1. Date of admission, ward and case No.
2. Date and time of death reported
3. Date and time of making P.M.
4. History
5. Clinical diagnosis

**B. External Examination**

1. Class of animal, sex, age, breed
2. Descriptive marks
3. Condition of the body
4. Rigor mortis
5. Natural orifices
6. Visible mucous membranes
7. Presence of wounds, if any
8. Superficial lymph nodes
9. Any other abnormalities
C. Internal Examination

1. Subcutaneous tissue
2. Abdominal cavity
   1. Peritoneal cavity and peritoneum
   2. Position of organs (organs inspected *in situ*)
3. Thoracic cavity
   1. Pleural cavity and pleura
   2. Position of organs
4. Pericardial sac
5. Heart
   1. Gross appearance, colour, size etc.
   2. Chambers
   3. Valves/Endocardium
   4. Myocardium
   5. Blood vessels
6. Larynx, Trachea and Bronchi
   1. Abnormalities
   2. Parasites
   3. Bronchial lymph nodes
7. Lungs
   1. Gross appearance, colour, size etc.
   2. Palpable abnormalities
   3. Section
   4. Parasites
   5. L. nodes
8. Diaphragm
9. Liver
   1. Gross appearance, colour, size etc.
   2. Surface
   3. Borders
   4. Parenchyma
   5. Portal lymph nodes
   6. Gall bladder (wall and contents)
   7. Parasites
10. Spleen
11. Kidneys
   1. Gross appearance, colour, size etc.
   2. Capsule
   3. Cortical surface
   4. Section
   5. Renal pelvis
   6. Parasites and calculi
12. Adrenal glands
13. Head
1. Mouth (lips, teeth, gums, tongue, palate, salivary gland etc. and associated lymph nodes)
2. Eyes
3. Ears

14. Nasal cavity
   1. Mucous membrane
   2. Sinuses
   3. Pharyngeal mucous membrane
   4. Tonsils
   5. Guttural pouches
   6. Retropharyngeal lymph nodes

15. Neck
   1. Oesophagus
   2. Thyroids
   3. Parathyroids
   4. Thymus

16. Stomach (s) – Foregut in ruminants
   1. Serous surface
   2. Mucosa and contents
   3. Parasites

17. Intestines
   1. Mesentery, mesenteric blood vessels, lymph nodes and parasites
   2. Surface (serous and mucous)
   3. Ileocaecal valve
   4. Contents (Parasites)

18. Pancreas

19. Pelvic cavity
   1. Urinary bladder
   2. Generative organs
   3. Accessory sexual glands
   4. L. nodes

20. Brain and spinal cord

21. Skeleton and musculature

22. Clinical Laboratory Examination

D. Appearances found

Diagnosis:

Materials collected:

Result of examination:
POULTRY POSTMORTEM REPORT

- Name of the institution:
- Poultry necropsy No.:
- Date & Time of making necropsy:
- Owner Name and Address:
- Reference:
- Date, time and place of death:
- Particulars of the bird:
- Species: Breed: Type of bird: Commercial/Breeder/Fancy/Wild Broiler / Layer
- Age:
- Sex: M / F
- Identification / Wing /Leg band No:
- Colour:
- History:
- Total stock:
- Mortality:
- Pattern for last 7 days:
- Percentage:
- Signs:
Demonstration of Cell Culture Techniques
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ATCC cell lines and hybridomas are shipped frozen on dry ice in cryopreservation vials or as growing cultures in flasks at ambient temperature. Upon receipt of frozen cells, it is important to immediately revive them by thawing and removing the DMSO and placing them into culture. If this is not possible, store the cells in liquid nitrogen vapor (below −130°C). Do not store frozen cells at temperatures above −130°C as their viability will decline rapidly. Prepare for reviving cell lines by assembling the appropriate medium, serum, and additional reagents required for growth. Many of these products are available from ATCC and can be ordered with the cell lines. These are the same reagents used by ATCC for cell growth and preservation.

Initiating Frozen Cultures

- Prepare a culture vessel so that it contains the recommended volume of the appropriate culture medium as listed on the Product Sheet, equilibrated for temperature and pH (CO₂).
- Thaw the vial by gentle agitation in a water bath at 37°C or the normal growth temperature for that cell line. Thawing should be rapid, approximately 2 minutes or until ice crystals have melted.
- Remove the vial from the water bath and decontaminate it by dipping in or spraying with 70% ethanol. Follow strict aseptic conditions in a laminar flow tissue culture hood for all further manipulations.
- Unscrew the top of the vial and transfer the contents to a sterile centrifuge tube containing 9 mL of the recommended medium.
- Remove the cryoprotectant agent (DMSO) by gentle centrifugation (10 minutes at 125 × g). Discard the supernatant, and resuspend the cells in 1 or 2 mL of complete growth medium. Transfer the cell suspension into the culture vessel containing the complete growth medium and mix thoroughly by gentle rocking.
- Examine the cell cultures after 24 hours and subculture as needed.

Adapting to a New Medium or Serum

To ensure that the characteristics of your cell line remain constant, maintain your cells in the same medium, serum, and supplements with the same subculturing regimen used to establish the culture. Any change to the culturing conditions has the potential to change the characteristics of the cell line. Be particularly cautious when working with a new cell line as media formulations vary among suppliers, even for media with similar or identical names. Read descriptions, formulations, and labels carefully to ensure that the appropriate medium is used or the cell line
may be inadvertently adapted to a new medium. All ATCC cell lines come with information on their growth medium. In most cases, the recommended medium and serum can be purchased from ATCC along with the cell line.

Use the following procedure to adapt a cell line to a new medium:

- Subculture the line at a 1:2 split ratio (split the culture in half) into two vessels. Maintain one with the original medium and continue to subculture these cells for the entire adaptation process. Use a 1:1 mix of the original and new medium in the second vessel. The culture grown in the original medium serves as a reference point as well as a safeguard in case the adapting cells do not survive the process. The low split ratio helps mitigate the stress associated with subculturing as well as with the new medium.
- Monitor cell growth in the two media and watch for any change in morphology or growth rate. If they are identical, subculture the adapting cells at the next passage with a 1:2 split ratio in a 1:3 medium mix (25% original, 75% new).
- Monitor the growth rate and morphology of the original and adapting cultures. At the next passage, split the adapting cultures 1:2 in a 1:7 medium mix (12.5% original, 87.5% new).
- Monitor the growth rate and morphology of the original and adapting cultures. If the cells are identical, then at the next passage split the adapting cells 1:2 in 100% new medium. At this point, the culture should be adapted to the new medium.

To confirm complete adaptation to the new medium, perform functional tests on cells derived from the original and new medium. If at any point in the process the adapting culture fails to perform as well as the reference culture, then allow the adapting culture more time and a few more passages in their current medium mix (e.g., 1:3, 1:7, etc.) until they match the reference cells. The same approach can be used to adapt cells to serum-free medium; simply decrease the serum level in the medium by half with each passage until a 0.06% (or lower) serum level is reached. At this point, the cells can be maintained in serum-free medium. If at any point the growth rate declines, then the serum level should be increased to the level where the cells grew normally. In this procedure, start with the “serum-free” medium supplemented with serum so that only the level of serum changes with each passage.

**Examination of cell cultures**

Observe the morphology and viability of cultures regularly and carefully. Examine the medium in the vessel for macroscopic evidence of microbial contamination. This includes unusual pH shifts (yellow or purple color from the phenol red), turbidity, or particles. Also, look for small fungal colonies that float at the medium-air interface. Specifically check around the edges of the vessel as these may not be readily visible through the microscope. With an inverted microscope at low power (40×), check the medium for evidence of microbial contamination and the morphology of the cells. Bacterial contamination will appear as small, shimmering black dots within the spaces between the cells. Yeast contamination will appear as rounded or budding particles, while
fungi will have thin filamentous mycelia. For nonadherent cells grown in flasks, such as hybridomas, this is a simple matter of viewing the flask directly on the microscope. For cells grown in spinner flasks or bioreactors, a sample of the cell suspension will need to be withdrawn and loaded into a microscope slide or hemocytometer for observation. Most adherent cells should be attached firmly to the surface. In some cases, healthy cells will round up and detach somewhat during mitosis and appear very refractile. Following mitosis, they will reattach. Some of these will float free if the culture vessel is physically disturbed. In contrast, dead cells often round up and detach from the monolayer and appear smaller and darker (not refractile) than healthy cells. Cells in suspension culture grow either as single cells or as clusters of cells. Viable cells appear round and refractile whereas dead cells appear smaller and darker. Occasionally, a portion of the cells will attach and grow on the side of the culture vessel and appear round or flattened. The percentage of attached cells varies with the culture conditions and the cell density. Cellular debris may also be observed in healthy cell populations. Some cell lines grow as mixed adherent and suspension cultures. As a reference, photomicrographs for some ATCC cell lines are available on the website. Cells are shown at two different densities: just after subculturing (low) and just before they need to be subcultured (high). In addition to daily examinations, periodically test a sample of the culture for the presence of fungi, bacteria, and mycoplasma. There are several methods that can be used to check for these contaminants. For additional information, refer to the section on microbial contamination.

**Subculturing Monolayer Cells**

Anchorage-dependent cell lines growing in monolayers need to be subcultured at regular intervals to maintain them in exponential growth. When the cells are near the end of exponential growth (roughly 70% to 90% confluent), they are ready to be subcultured. The subculturing procedure, including recommended split-ratios and medium replenishment (feeding) schedules, for each ATCC cell line is provided on the Product Information Sheet. Subcultivation of monolayers involves the breakage of both intercellular and intracellular cell-to-surface bonds. For some cells that are loosely attached, a sharp blow with the palm of your hand against the side of the flask can dislodge them. Many require the digestion of their protein attachment bonds with proteolytic enzymes such as trypsin/EDTA. For some cell lines mechanical forces such as scraping to dislodge the cells is preferred. After the cells have been dissociated and dispersed into a single-cell suspension, they are diluted to the appropriate concentration and transferred into fresh culture vessels with the appropriate growth medium where they will reattach, grow and divide. The procedure below is appropriate for most adherent cell lines. However, since every cell line is unique, incubation times and temperature, number of washes or the solution formulations may vary. In all cases, continually observe the cells with a microscope during the dissociation process to prevent damage by the dissociation solution. The amounts used in this procedure are for a 75-cm² flask. Adjust volumes as appropriate for different sized vessels.

- Bring the trypsin-EDTA solution (ATCC® No. 30-2101), balanced salt solution [Dulbecco’s Phosphate Buffered Saline without calcium or magnesium, ATCC® No. 30-
2200], and complete growth medium to the appropriate temperature for the cell line. In most cases, this is the temperature used to grow the cells (usually 37°C). For some sensitive cells, the trypsin-EDTA solution may need to be used at room temperature or 4°C.

- Remove and discard the cell culture medium from the flask.
- Rinse the cell monolayer with Dulbecco’s PBS without calcium or magnesium and remove.
- Add 2 mL to 3 mL of the trypsin-EDTA solution and incubate at the appropriate temperature. Check the progress of cell dissociation by microscopy. To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for them to detach.
- Once the cells appear to be detached (5 to 15 minutes for most cell lines; they will appear rounded and refractile under the microscope), add 6 to 8 mL of complete growth medium with a pipette to the cell suspension to inactivate the trypsin. Gently wash any remaining cells from the growth surface of the flask. Check the cells with the microscope to be sure that most (>95%) are single cells. If cell clusters are apparent, continue to disperse the cells with gentle pipetting.
- Add 12 mL to 15 mL of fresh culture medium to a new flask and equilibrate this medium to the appropriate pH and temperature.
- Count the cells in suspension and determine their viability or simply divide them according to a routine split ratio and dispense them into the medium of the newly prepared flask. Do not add a concentrated cell suspension to an empty culture vessel as this can result in uneven cell attachment and growth.
- Place the flask back into the incubator. Examine the culture the following day to ensure the cells have reattached and are actively growing. Change the medium as needed; for most actively growing cultures two to three times per week is typical.
The immune system accounts for a relatively minor portion of total nutritional requirements in the normal healthy animal but activation of the immune system in response to an immune challenge has a major impact on nutritional status and requirements for most nutrients.

Nutrient deficiencies increase susceptibility to most infectious diseases, including bacterial, viral and parasitic diseases. Once disease has developed, nutritional deficiencies increase the severity of the disease and increase the probability of secondary infections. Deficiencies of vitamins or trace minerals significantly depress immune function and resistance to stress even when animals are otherwise well fed with sufficient energy and protein.

Stress such as handling, transportation, transition, that during peri parturition period, physical trauma, surgery, general infection, haemorrhagic bowel syndrome, fatigue, fasting, and unfamiliar environment etc increases requirements of many nutrients essential for immune function and leads to multiple short term nutrient deficiencies. Consequently, nutrition has the largest impact on morbidity and mortality in stressed animals. For example the immune system is suppressed in stressed cattle, contributing to the high incidence of respiratory disease in the first 45 days on feed after transportation. Some nutrients such as vitamin E can be fed at levels above normal requirements to stimulate immunity of immuno suppressed animals.

All animals require energy, amino acids, fatty acids, fat soluble vitamins (A, D, E, K), water soluble vitamins (B-vitamins), trace elements and macro minerals for both health and growth. When availability of any of these nutrients is limited, deficiency symptoms occur most quickly in tissues with the highest rates of protein synthesis or metabolic activity. The immune system is particularly sensitive to a nutrient deficiency because any immune response requires rapid synthesis of proteins for immune cells and immune products. Most nutrient deficiencies including vitamins and trace minerals directly or indirectly affect synthesis of these proteins. Other nutrients regulate the immune response (vitamins A and D), or act as antioxidants to protect immune cells and other cells from the toxic effects of a wide variety of enzymes and chemicals used by the immune cells to destroy bacteria and infected cells. Compared to other systems in the body, the immune system has especially high requirements for antioxidant nutrients but these nutrients are quickly depleted when animals are stressed.

Nutrition has the greatest impact on immunity during stress. It can be difficult to get enough nutrients into cattle, particularly those that need it the most. Nonetheless, proper nutrition during immune function directly, and also indirectly by improving resistance to stress. Providing key nutrients can reduce stress-induced weight loss and immunosuppression, improve weight gain and reduce morbidity and mortality from stress induced immune challenge such as during that of bovine respiratory disease.

**Background: What is Immunity to Infection?**

Animals resist infection using non-specific mechanisms (‘innate’ immunity) and specific mechanisms (acquired immunity). Almost all immune mechanisms are less effective in stressed animals.
Innate immunity

Innate immunity includes:
1. Epithelial tissue which covers body surfaces – ex. skin or hide, hoof, cornea
2. Epithelial tissues lining body cavities – ex. respiratory, urogenital, gastrointestinal tract. These epithelial lining cause a physical barrier to keep infectious agents away from entering the body. Breaks in this covering layer caused by physical damage or by nutrient deficiencies such as vitamin A and zinc can result in infectious agents gaining access to the body;
3. Secretions that block infectious agents and numerous antimicrobial compounds in body fluids and secretions frequently affected by stress – ex. Mucus
4. Secretions that wash out infectious agents, and numerous antimicrobial compounds in body fluids and secretions; frequently affected by stress - ex. saliva, tears
5. Cells in blood and tissue that engulf, kill and digest infectious microorganisms. These are called phagocytes and include macrophages, monocytes, and neutrophils. They continually circulate through blood and migrate into tissues in search of invading pathogens. This important defence is seriously impaired by stress;
6. Receptors on the surface of these cells which recognize and bind to pathogens
7. Signalling molecules (e.g., chemokines, cytokines) which communicate sites of infection and regulate expression of immune genes
8. Normal microbial flora which can compete with disease causing organisms, thereby holding them in check; is less effective in stressed animals.

Specific or acquired immunity:

Exposure to a foreign substance (antigen) results in the development of immune cells and antibodies specific against that particular antigen only. Specific immunity takes time to develop, and therefore is effective in preventing infection only if the animal was previously exposed to that antigen. It is also important for recovery from many infections. The foreign substance (antigen) may be a live virus or bacteria but does not have to be infective or alive to induce immunity. Weakened or dead viruses or bacteria, and even specific fragments of pathogens or pieces of their DNA can be used in vaccines to induce immunity with reduced risk of disease. Exposure to the antigen triggers growth and development of two types of blood cells specific for eliminating the antigen. These are the T-cells (responsible for cell-mediated immunity) and the B-cells responsible for antibody production (humoral immunity).

T-cells secrete numerous hormones (such as interleukins and interferon) which up regulate or down regulate the entire immune system as needed, and increase effectiveness of other immune cells including phagocytes and B-cells. In addition, some T-cells directly destroy target cells. Antibodies are fairly large proteins that are secreted by B-cells and found in blood plasma, nasal secretions and other fluids. Because of their size, they cannot enter into bovine cells so are not effective against intracellular pathogens. All antibodies act by binding to the foreign substance. In many cases, this prevents the virus or bacteria from replicating or being infective, but also makes it much easier for other immune products or cells to destroy the pathogen. Vaccines may be designed to stimulate primarily an antibody response or an increase in cell-mediated immunity, depending on which type of immune response is most effective against a specific pathogen.
A nutrient deficiency does not affect all immune mechanisms equally, nor does it always affect the same mechanism equally for different antigens. A group of deficient animals may have adequate antibody responses to some antigens (or vaccines), and depressed antibody responses to other antigens. Similarly, under practical conditions, deficient animals may be more susceptible to some but not all disease causing organisms.

**Nutritional Requirements of Stressed Animals**

Acute or prolonged stress makes animals more susceptible to disease by increasing levels of hormones which suppress the immune system and by depleting nutrients critical to an effective immune response. Stressful stimuli in cattle include handling, transport, physical trauma, fatigue, fasting, and unfamiliar environment etc. These induce hormonal responses which control and alter animal metabolism. Cortisol, epinephrine, nor epinephrine, aldosterone, beta-endorphin, and enkephalins are released in large amounts in response to stress. A short-term stress may have relatively minor effects on metabolism and nutritional status. A long-term, chronic stress may cause substantial metabolic changes. Metabolic changes during stress are designed to control and reserve energy and other nutrients for use in the most vital processes needed to combat the stress. Metabolic pathways shift from anabolic processes (growth) to primarily catabolic (tissue breakdown of proteins and fat).

The first response of any category or any species of stressed animal is – drop in feed intake. Stressed animals have higher nutritional requirements, but do not consume as much feed as unstressed animals. Feed intake is the foremost nutritional problem for highly stressed loads of cattle. The first objective is to get enough feed into the animal so that it can stop breaking down its own tissues for fuel and begin to eliminate nutrient deficits. Cattle produce blood glucose essential for the central nervous system and red blood cells by breaking down body proteins in liver, kidney, intestine, and skeletal tissue to provide glucose precursors. Body fat is broken down to provide fatty acids, the primary energy source for most other tissues. These free fatty acids can be converted to ketone bodies, primarily β-hydroxybutyric acid and acetoacetic acid, which can lead to a metabolic acidosis. The nutrient concentration of the diet has to be increased to compensate for the low intake. Stressed cattle are often deficient in energy, amino acids, vitamin A, B-vitamins, calcium, phosphorus, potassium, magnesium, zinc, and copper (Nockel, 1990). Antioxidant requirements are markedly increased by both stress and disease, because both result in accelerated production of highly reactive oxygen by products, peroxides, and free radicals. Free radicals damage healthy tissues. Antioxidants such as vitamin E and vitamin C are quickly depleted in stressed animals, particularly in the white blood cells where they are critically important for immune function. Antioxidants scavenge free radicals thereby, checking tissue damage by them.

Stressed, previously fasted cattle differ from normal cattle in ability to utilize certain feeds and nutrients because of changes in rumen function. Stressed cattle such as calves have a lower tolerance for non protein nitrogen (e.g. urea) than normal calves, which should be limited to 30 grams or less per day during the first 2 weeks of feeding. Stressed calves also have a low tolerance for dietary fat, and for silage compared to non fermented feeds. Feed intake, gain, and immune function are improved when stress calves are fed sufficient undegradable intake protein.
There is some indication that stressed calves may differ from normal cattle in having some bypass of soluble nutrients that are normally completely degraded in the rumen by bacteria. If so, it may be possible to take advantage of reduced ruminal function in the first 1-2 days on feed to supplement nutrients such as glucose, choline, ascorbic acid, and various B-vitamins when these requirements are known.

**Effects of Disease on Nutritional Status**

Infection results in a complex array of metabolic responses which affect the nutritional status of the animal. Feed intake decreases more than 50% in cattle with respiratory disease and fever, and takes 10 to 14 days to return to normal. During this time, requirements of virtually all nutrients increase, even though nutrients are deflected from growth to immunity. Proteins must be synthesized for the immune response, development and resolution of fever, and repair of cell and tissue damage. Body proteins are broken down to provide energy and amino acids for the immune system. Losses of important minerals such as magnesium, potassium, phosphate, and zinc are also increased.

Immune activation increases requirements for antioxidant nutrients, trace minerals, and vitamins similar to the effect of stress. Stress aggravates or predisposes animal to immune challenge. Consequently, when cattle are stressed before an immune challenge, nutrients critical to the immune response are likely to be in short supply. Trace minerals and B-vitamins are required as co-factors for the chemical reactions involved in breaking down body tissues and synthesizing new proteins. The major extracellular electrolytes, sodium and chloride, are influenced by hormonal changes. Urinary excretion of salt may increase during the onset of infection, and may lead to dehydration. The kidneys may then begin to retain body salt and water. Diarrhoea can result in substantial direct faecal losses of sodium, chloride, bicarbonate and potassium.

**Effect of Nutritional Status on Resistance to Infection**

Good nutrition improves disease resistance of stressed cattle, by helping to counteract the suppression of the immune system caused by stress hormones and by providing nutrients essential for maintaining and activating the immune system as required.

**Energy** - Stressed calves seem to have an altered eating pattern—unlike normal calves, they won’t eat more of a lower energy diet, and given a choice, they select a diet with about 72%. Consequently, performance of lightweight stressed calves is increased with high-concentrate receiving diets (>60% grain) but morbidity rate may increase as well. Generally morbidity and severity of illness increase with increasing grain in the diet. The optimum concentration of grain in the receiving diet depends on the age and weight of animal, previous management, stress level, and other factors; cattle with lower intakes (calves) can safely consume diets with a higher proportion of grain than can cattle with higher intakes (yearlings).

**Protein** - Diets that contain relatively low or high levels of dietary proteins adversely affect immunity to infection compared to diets with moderate protein levels (*Galyean et al.*, 1999). Morbidity rate has been found to be lowest for diets containing 12 to 14% protein, and increased...
as protein increased to 22% of DM. However, the best performance is usually achieved at higher levels of dietary protein (16 to 20%). Morbidity was better when less soluble, higher bypass proteins were fed (distillers dried grains, blood meal). Nissen et al., 1989 reported that gain and feed efficiency improved as metabolizable protein concentration increased but serum cortisol increased linearly, and the proportion of calves responding to the IBR vaccine decreased linearly.

Vitamins and minerals: Vitamins and minerals, such as Vit A, vitamin E, selenium, copper, Chromium and zinc, when properly supplemented, can enhance a cow’s immunity against diseases, such as mastitis, by increasing resistance to infections and by decreasing severity of infections when they do occur. Cow requirements for vitamins and minerals are influenced by several factors, including age, stage of pregnancy, and stage of lactation. For some vitamins and minerals, the amount required for optimal immune response is greater than the amount required for growth and reproduction. Cattle can have sufficient vitamin and mineral intake for adequate growth and reproductive performance but not have optimal immune performance. By the time clinical signs of deficiency become apparent, immunity, growth and fertility already may have been compromised.

Vitamins: Vitamins A and D within the ranges that are normally fed, are important in regulating immunity. Vitamin A deficiency reduces resistance to all types of disease, including parasites. Vitamin A supplementation is essential for cattle fed grain-based diets. In ruminants, for example, supplementation with β-carotene at dry-off reduced mammary gland infections (Chew, 1987). β-carotene increased lymphocyte blastogenesis (Daniel et al., 1990) and increased neutrophil killing activity (Michal et al., 1994). Under practical conditions, vitamin D deficiency is unlikely to be a concern even when cattle are not supplemented, unless they also do not have access to sunlight.

Antioxidant nutrients are crucial to the immune response, becoming rapidly depleted during infection. These key nutrients include dietary antioxidants such as carotenes, vitamin E, and vitamin A, and trace minerals such as selenium, zinc, copper, and manganese used to synthesize antioxidant enzymes. Antioxidants protect immune cells and surrounding tissue from damage caused by the immune response, which otherwise would damage the animal as much or more than the disease organisms. Antioxidants are particularly important for the effectiveness of phagocytes, which are the front line of defense against invading pathogens. If phagocytes are deficient in antioxidants, microbial killing is ineffective. Vitamin E is currently the most important antioxidant in diets. Vitamin E and Se play overlapping and essential roles in support of the immune system in ruminant animals. Vitamin E supplementation increases lymphocyte proliferation (Reddy et al., 1986). Supplementation with vitamin E has improved growth, feed efficiency, morbidity, and antibody titres of stressed cattle, but not consistently. In studies with dairy calves, vitamin E supplementation decreased (P < 0.05) serum cortisol concentrations, an indicator of stress. Dietary vitamin E in excess of requirements for growth increases resistance to stress and disease. The vitamin E status of the cattle, exposure to stress and disease, and other factors may influence the response. Supplementation with vitamin E decreases the incidence of mastitis, and selenium decreases the duration of these infections. Combining the two supplements results in the greatest
increase in defense against mastitis. Deficiencies of vitamin E and selenium also have been found to increase the incidence of retained placenta.

B-vitamins and vitamin C affect immunity and resistance to stress. Rumen microbes can synthesize B vitamins. Production of B-vitamins by rumen microbes is directly related to energy availability in the rumen; synthesis of vitamin C in the animal is directly related to energy availability in animal tissue. The combination of stress and low intake can result in low availability of B-vitamins which may affect rumen microbes and/or the animal itself. B-vitamin supplementation of receiving diets has improved performance and reduced shipping fever in several studies. In a study at Kansas, B-vitamin supplementation in combination with vitamin E tended to improve gain and significantly improved feed efficiency, without significantly affecting morbidity. Vitamin B₁₂ deficient lambs had higher fecal egg counts than vitamin B₁₂ supplemented one after natural infection with gastrointestinal nematodes.

Magnesium and phosphorus are the most important macro minerals associated with the immune response; their supplementation levels have influenced mortality in other animal species but have not been studied in this regard in cattle.

**Trace mineral supplementation**: It is important for maintaining resistance to infectious disease. Trace minerals have numerous functions as components of vital proteins. Young growing animals are depositing protein at high rates and are therefore more vulnerable to trace element deficiencies than older animals. Copper, zinc, manganese, and selenium are required for the production of antioxidant enzymes. Feeding elevated levels of Se to ruminant animals reduces incidence of diseases including intra-mammary infections. For example, Hogan et al. (1990) reported that Se enhanced neutrophil killing activity. Maddox et al. (1999) have reported that Se deficiency increases neutrophil adherence and altered adherence could affect ability of neutrophils to attack and sequester pathogen. Cao et al. (1992) reported that Se and vitamin E increased antibody responses of dairy cattle. In a more recent study, Parnousis et al. (2001) reported that injection of Se either alone or in combination with vitamin E significantly improved the production of specific antibodies against *E. coli*, and that the production of specific antibodies was greater after the administration of Se alone. Selenium deficiency can increase the incidence of embryonic death and uterine infections and can decrease fertility. Deficiencies of copper and selenium impair the ability of bovine phagocytes to kill invading microorganisms. Cu supports immunity as it is associated with many proteins. Deficiencies of copper also have been associated with retained placenta, embryonic death, and decreased conception rates. Inadequate copper status may be related to an increased incidence of infections at calving. Cu increased neutrophil killing activity of a common mold: *Candida albicans*. Ward et al. (1997) reported that Cu enhanced cell mediated immunity (DTH-response). Zinc is also an integral part of the immune system. Zinc is important for production of keratin, which lines the inside of the teat duct and helps to keep out micro-organisms that can cause mastitis. Zinc deficiencies also will delay sexual maturity and may also cause fetal abnormalities. A zinc deficiency increases nonspecific infections, and inhibits normal healing of wounds. Supplementation with zinc or with high levels of trace minerals has improved recovery from respiratory disease in several studies. Zinc builds up a successful immune response against gastrointestinal nematodes.

Although chromium is depleted in stressed animals, and chromium supplementation has improved immune function in some studies, more information is needed to determine when

*ASCAD Training on “Advanced approaches for diagnosis of livestock diseases” (25-30 Nov., 2013)*
supplementation would be cost-effective. Several studies have indicated that supplementation of Cr to dairy cattle, in a biologically-available form (e.g., Cr-amino acid complex or Cr-yeast), benefits immunity. Supplementation of Organic Chromium to transit-stressed calves and early lactation dairy cows, improved the immune status and milk production. Chromium seems to reduce blood cortisol concentration during stress and promoted insulin or insulin like growth promoter sensitivity in target tissues such as muscle, mammary gland and immune system. Improved performance and lower disease occurrence can be found.

Iron supplementation - meet the loss through blood during gastrointestinal parasitic infections (Koski and Scott, 2003).

Mo in diet containing 4-8 mg/kg DM in sheep reduces worm burden (McClure et al., 1999).

Organic iodine is extensively used for foot rot control at levels exceeding those required for growth. Young, growing cattle may be most susceptible to footrot.

**Suggested Feeding Levels in Total Diet**

- Vitamin E: 1000 IU/day for dry cows, 500 IU/day for lactating cows
- Selenium: 0.3 ppm
- Copper: 20 ppm
- Zinc: 40-60 ppm

Additionally, Omega-3 and Omega-6 fatty acids, Feed additives, Bypass protein etc. have been shown to improve immunity against various diseases.

**Omega-3 and -6 fatty acids (ω-3 and ω-6).** Dietary fatty acids can affect immunity through the production of the cytokines (Lessard et al., 2003). A mechanism by which fatty acids affect immunity is through production of eicosenoids (e.g., prostaglandins) and leukotrienes. Diets rich in the ω-6 fatty acids, such as linoleic acid (C18:2), lead to the formation of arachidonic acid; whereas diets rich in the ω-3 fatty acids (such as linolenic acid, C18:3, flaxseed, and fish oils) lead to the formation of, for example, eicosapentaenoic acid (EPA). Eicosenoids synthesized from arachidonic acid tend to have strong inflammatory potential; whereas those synthesized from EPA have lesser potential. Hence, feeding fatty acid mixtures which are enriched in the ω-3 fatty acids reduces inflammatory reactions and reduces production of pro-inflammatory cytokines including IL-1, IL-6, and TNFα.

**Feed additives - fungus**

- **Fungi**: Feeding of fungi such as Duddingtonia spp., Harposporrium spp. and Arthrobotrys spp. as a feed additive control gastrointestinal parasite

- **Probiotics** - such as lactobacilli and bifidobacteria, yeast culture (saccharomyces spp.) helps to improve growth rate and feed conversion efficiency in calves, microbial protein flow and DM intake, particularly in poor managemental conditions.
Prebiotics – Such as Lactulose, lactitol, a variety of oligosaccharides and inulin Prebiotics have shown promise in the prevention and control of exogenous and endogenous intestinal infections and good health of the animals.

Feeding By-pass protein : Improves resistance and expression of immunity to gastrointestinal parasites and increase the resistance of sheep to *Haemonchus contortus*.

**Beneficial Effects of Nutrients in Excess of Growth Requirements**

Several nutrients such as vitamin E and selenium enhance immunity above normal levels when fed in excess of requirements for growth. These same nutrients stimulate immunity when immune function is below normal, as in animals that are stressed. Some nutrients such as vitamin E and chromium influence production of stress hormones such as cortisol. Antioxidant nutrients in general, including vitamin E and selenium, also reduce the susceptibility of cells throughout the body to the cortisol that is produced in response to stress. Vitamin E is depleted in stressed cattle whereas vitamin E supplementation appears to counteract stress-induced immunosuppression (see vitamin article). A small excess of selenium may also be beneficial.

**Optimal immunity**

The immune system appears to have priority for nutrients over growth. Immune activation is costly. Resources devoted to immune activation cannot be used for growth. As a result, any immune response will depress growth rate and feed efficiency. The optimum immune response is the one that is the shortest in duration and the lowest in intensity while successfully eliminating the pathogen. An extreme immune response not only wastes resources but may cause local cell damage and depress productivity. Reducing challenges to the immune system by providing a clean environment and reducing stress can improve growth rate. In normal healthy cattle, there is a trade-off between growth and immunity – both cannot be maximized at the same time. This is not usually a practical concern because the immune system functions very well at nutrient levels ideal for growth. Some very promising research has been done in this area but much more is needed to evaluate products and economics of use.

However, cattle feeders dealing with less than ideal facilities or management, limited labour, or lightweight, high risk cattle may prefer to sacrifice some growth but reduce disease risk by adapting cattle to full feed more slowly than usual. This can reduce the incidence of respiratory disease, death loss, and medical costs during the receiving period.

**Balancing diets for immunity**

Rations of stressed animals should be adequately supplemented with trace minerals, vitamin A and vitamin E. Feeding higher levels than normal of these nutrients in the receiving period can compensate for reduced intake and marginal deficiencies. In order of priorities, providing a palatable diet and encouraging feed and water consumption is most important. There is little benefit from adding expensive nutrients to an unpalatable diet. In situations where the cattle are most vulnerable to disease (high stress, younger animals, poor environmental conditions, etc.) and the disease risk is high, supplementing high levels of nutrients such as vitamin E during the receiving period may improve gain and reduce morbidity substantially. A large number of
nutrients have shown promise for improving feeder calf health, including chromium, zinc methionine, B-vitamins, vitamin E, HMB. After cattle have recovered from stress-induced immuno suppression and are adapted to the finishing diet, balancing diets to meet requirements for growth usually provides adequate nutrients for immune function.

References


Faculty and Participants of ASCAD Training Course

Sitting (from L to R): 1. Dr. B. S. Mathapati, Asstt. Prof., 2. Dr. D. B. Barad, Asstt. Prof., 3. Dr. P. H. Vataliya, Principal & Dean, 4. Dr. B. B. Javia, Head of Dept., 5. Dr. H. L. Kacha, Veterinary Officer

Standing (from L to R)- Veterinary Officers: 1. Dr. H. S. Kher, 2. Dr. G. M. Prajapati, 3. Dr. B. M. Solanki, 4. Dr. H. G. Vamja, 5. Dr. J. A. Chavda, 6. Dr. B. D. Patel 7. Dr. R. G. Mali