STANDARD OIE COMPLAINT CONTINUES PROFESSIONAL DEVELOPMENT (CPD) MODULE ON LABORATORY DIAGNOSTIC TECHNIQUES IN ETHIOPIA

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CPD Module on veterinary laboratory diagnostic techniques in Ethiopia

l. Course Introduction

1.1**.** Development of standard OIE complaint Continues Professional Development (CPD) Module on veterinary laboratory diagnostic techniques in Ethiopia.

1.2. Course code:

1.3. Credit point:

1.4. Targeted trainees: Veterinary Professionals, and Para-professionals.

Unit ll. Veterinary Laboratory Diagnostic Techniques (35 hrs)

2.1. Brief description of the course

This course provides theoretical and practical aspects of training in key diagnostic skills for veterinary professionals and para-professionals, focusing on diagnosing infectious diseases in animals. At the end of this course, the trainees will acquire skills and knowledge on:

- Laboratory safety guidelines and how to collect, prepare, manage, and submit the specimens for laboratory analysis.
- How to diagnose various animal diseases by using different laboratory diagnostic approaches. Besides, the trainee will be confident in effectively managing, analyzing, interpreting, and reporting the results.
- Diagnostic laboratory-associated risk factors and how to control them. Furthermore, the participants will operate and use laboratory equipment effectively and safely.

2.2. The time spent on delivering the course

2.2.1. Modes of Delivery and time distribution

Lectures	10 hours
Oral presentation	10 hours
Assignments	5 hours
Self-reading	5 hours
Seminars	5 hours
Practical	10 hours

2.3. The course objectives

The objectives of the course are:

- ❖ To refresh the knowledge on Lab Biosafety & Biosecurity and effective operation of lab equipment's
- ❖ To deliver information about veterinary laboratory services to Veterinary Professionals and Paraprofessionals. Consequently, to improve the lifestyle of the community through expanding modern veterinary laboratory services in Ethiopia.
- ❖ To deliver and enrich veterinary and para-veterinary professionals with skills in different veterinary laboratory diagnostic techniques.

2.4. Learning outcomes

- Cognitive Skills
  - ✓ The participants will acquire knowledge of recent information on virological, bacteriological, mycological, parasitological, molecular, and serological diagnostic techniques including the theoretical aspects. Furthermore, the trainees will gain knowledge about veterinary vaccines and their proper use and guidance on laboratory safety principles.
- The new skills acquired
  - ✓ The participants will be able to read, understand and criticize scientific diagnostic methods regarding virological, bacteriological, mycological, parasitological, molecular, and serological diagnostic techniques. Besides, the participants will be able to justify the laboratory biosafety principles.
  - ✓ The participants will be able to assess articles dealing with procedures or practical applications to interpret the correct methods in veterinary laboratory diagnostic techniques.
  - ✓ The trainees will be able to gain personal confidence in searching data, information, and methodologies in order to set up and validate new laboratory diagnostic procedures.
  - ✓ The participants will be able to apply the knowledge acquired in the virological, bacteriological, mycological, parasitological, molecular, and serological diagnostic techniques to make decisions on laboratory test results.
  - ✓ The participants will be able to develop effective communication skills on the subject of general veterinary laboratory diagnostic techniques by practicing, reading, writing, and speaking clearly.
  - ✓ The participants will be able to receive, and safely manage the biological samples and perform different laboratory diagnostic tests, interpret the results and effectively report the final result to stockholders.

- ✓ The participants will be able to deeply understand the different laboratory diagnostic techniques and identify the disease causative agents accordingly.
- ✓ The participants will be able to design the experiments and perfectly manipulate them individually and in groups.
- ✓ The participants will be able to record and keep the recordings appropriately.
- ✓ The participants will be able to construct and perform the testing hypotheses.
- ✓ The participants will gain knowledge on diagnostic methods regarding virological, bacteriological, mycological, parasitological, molecular, and serological diagnostic techniques.

The trainees will be able to acquire additional laboratory diagnostic information:

- By learning from books.
- Newspapers.
- Magazines.
- Encyclopedias
- Internet, or school/college
- Training, workshops, visits.

2.5. The CPD module contents

Unit l. Introduction (5hrs)

Learning objective:

- To give a brief introductory concept about this CPD module
- To introduce laboratory techniques used in veterinary medicine

Learning outcome:

- Understand the basics and importance of the CPD module in laboratory diagnosis
- **U**nderstand, identify, and prioritize the diagnostic methods

Session question

- What is professional development?
- What is CPD?
- What are laboratory diagnoses and their importance?

The trainees are advised to read the following text to get the answers to the above session questions

Veterinary professionals across the globe are increasingly requiring disease pinpointing techniques and demonstrate their commitment to continuing professional development (CPD) to maintain proficiency in the ever-changing scope of practice and technological advances in veterinary services[1]. CPD can be defined as the practical updating of professional knowledge and the improvement of professional competence throughout a person's working life, a promise to be a professional, keeping up to date, and continuously seeking to improve. It also refers to the process of tracking and documenting the skills, knowledge, and experience gained both formally and informally based on the individual work and beyond any initial training. Likewise, it's a record of what someone experience, learn, and then applies.

CPD learning activities can be designated as categories I and II. Category I CPD learning activities comprise formal and structured learning opportunities offered by accredited CPD providers. The activities are usually scheduled and held at venues that would allow all concerned professionals to attend.

[1] Kasvosve et al.: Continuing professional development training needs of medical laboratory personnel in Botswana. Human Resources for Health. DOI: 10.1186/1478-4491, 2014 12:46.

While category II activities are essential of a self-learning nature or planned and conducted with a local or regional participant group in mind[2].

Diagnostic laboratories play a critical role in the delivery of quality health services which depends on the deployment of well-trained and competent laboratory professionals across the laboratory network of the veterinary facilities. The veterinary laboratory network assists member states to improve national laboratory capacities to early detect and control transboundary animal and zoonotic diseases threatening livestock and public health. Veterinary laboratory personnel must be up-to-date in knowledge, skills, and attitude to maintain good health service delivery through continuous professional development.

Laboratory diagnostic methods can identify disease-causing organisms directly using a microscope, by growing the organism in culture, using molecular techniques, and indirectly by identifying antibodies produced against the pathogen. Therefore, this is aimed to develop a standard OIE complaint CPD Module on the veterinary Laboratory diagnostic techniques and proper vaccine handling methods in Ethiopia to deliver adequate information to veterinary and para-veterinary professionals.

[2] Manley K, Martin A, Jackson C, Wright T. A realist synthesis of effective continuing professional development (CPD): A case study of healthcare practitioners' CPD. Nurse Educ Today. 2018; 69:134-41.

Unit ll. Laboratory diagnostic techniques (35 hrs)

2.5.1. Diagnostic techniques of viral diseases (5hrs).

Learning objective:

- To equip learners with the concept and principles of viral disease diagnostic methods.
- To introduce and equip the learners with sampling, sample transportation, and sample processing methods
- To introduce different viral isolation and identification methods used in veterinary medicine

Learning outcome:

- Understand the principle of viral disease diagnosis of veterinary importance.
- Understand and perform sampling and sample transportation efficiently.
- Understand, identify, prioritize, and perform viral diagnostic methods used in veterinary medicine.
- Understand and perform viral isolation and identification.
- Identify and characterize the viral effect on the cell after being infected by the virus.

Session questions

- What are the viruses, VTM, and viral diagnostic methods used?
- What is the method of sampling and sample transportation for viral diagnosis?
- What is sample processing?
- What are methods used for virus isolation and identification?
- What is the cytopathic effect?

The trainees are advised to read the following text to get answers to the above session questions.

Preparation of viral transport media

The viral transport medium (VTM) is a solution used to preserve virus specimens after collection so that they be transported and analyzed in the laboratory when needed[3]. Unless stored in an ultra-low temperature freezer or in liquid nitrogen, viral samples, and especially RNA virus samples, are prone

[3] Smith, K.P. 2020. Large-Scale, In-House Production of Viral Transport Media to Support SARS-CoV2 PCR Testing in a Multihospital Health Care Network during the COVID-19 Pandemic.

to degradation. Detailed information for the preparation of VTM is found at https:// www. cdc. gov/ coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf.

Isolation, Culture, and Identification of Viruses

Isolation of Viruses

Unlike bacteria, many of which can be grown on an artificial nutrient medium, viruses require a living host cell for replication. Infected host cells (eukaryotic or prokaryotic) can be cultured and grown, and then the growth medium can be harvested as a source of the virus. Virions in the liquid medium can be separated from the host cells by either centrifugation or filtration. Filters can physically remove anything present in the solution that is larger than the virions; the viruses can then be collected in the filtrate (https://bio.libretexts.org /Bookshelves/Microbiology/Microbiology_ (OpenStax)/06%3A_Acellular_Pathogens/6.03%3A_Isolation_Culture_and_Identification_of_ Viruses).

Cultivation of the viruses

Viruses can be grown in vivo (within a whole living organism, plant, or animal) or in vitro (outside a living organism in cells in an artificial environment, such as a test tube, cell culture flask, or agar plate). Bacteriophages can be grown in the presence of a dense layer of bacteria (also called a bacterial lawn) grown in a 0.7 % soft agar in a Petri dish (Figure 1 b,c,d) or flat (horizontal) flask (Figure 1a). The soft 0.7 % agar allows the bacteriophages to easily diffuse through the medium. For lytic bacteriophages, lysing of the bacterial hosts can then be readily observed when a clear zone called a plaque is detected. As the phage kills the bacteria, many plaques are observed among the cloudy bacterial lawn.

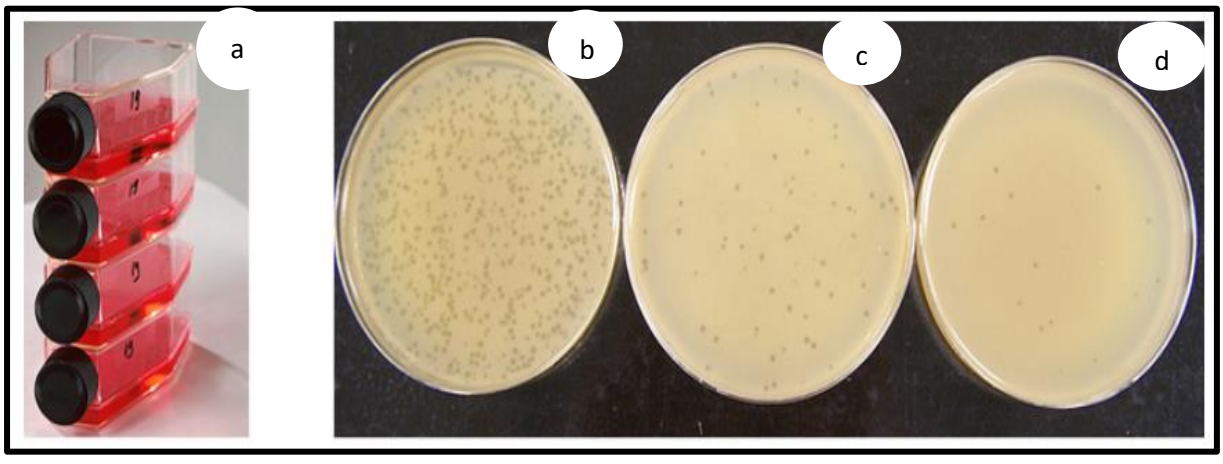

Figure 1. The growing of virus in different cell culture.

The figure shows the growth of the virus in the cell culture using a flask (Figure 1a) and Petri dish in Figures 1b, c, and d contains bacteriophage T4 grown on an Escherichia coli lawn. Clear plaques are visible where host bacterial cells have been lysed. Viral titers increase on the plates to the left. (Credit a: modification of work by National Institutes of Health; credit b, c, and d modification of work by American Society for Microbiology).

Animal viruses require cells within animal or tissue-culture cells derived from an animal. Animal virus cultivation is important for; the identification and diagnosis of pathogenic viruses in clinical specimens, the production of vaccines, and basic research studies[4]. In vivo host sources can be a developing embryo in an embryonated bird's egg (e.g., chicken, turkey) or a whole animal. For example, most of the influenza vaccine manufactured for annual flu vaccination programs is cultured in hens' eggs (https://www.healthline.com/health-news/why-we-grow-flu-vaccines-in-chicken-eggs).

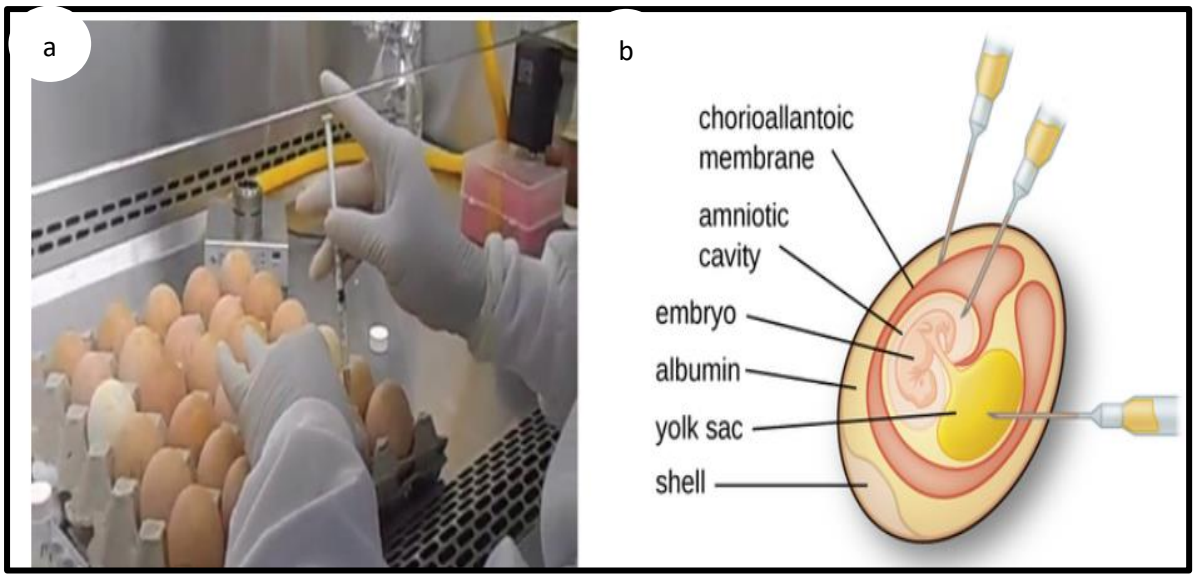

Figure 2**.** Inoculation of virus in chicken egg

The cells within chicken eggs are used to culture different types of viruses (Figure 2a). Figure 2b indicates where viruses can be replicated in various locations within the egg, including the chorioallantoic membrane, the amniotic cavity, and the yolk sac[5].

[4] Collection, submission, and storage of diagnostic specimens, OIE terrestrial manual, 2018. (https://www.healthline.com/health-news/why-we-grow-flu-vaccines-in-chicken-eggs).

[5] Lokhman, E., Rai, S., Matthews, W. (2020). The Preparation of Chicken Kidney Cell Cultures for Virus Propagation. In: Maier, H., Bickerton, E. (eds) Coronaviruses. Methods in Molecular Biology, vol 2203. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-0900-2_7., https:// www.ndvsu.org/images/StudyMaterials/Micro/Exercise-2-Micro.pdf

For in vitro studies, various types of cells can be used to support the growth of viruses. Primary cell culture is freshly prepared from animal organs or tissues. Cells are extracted from tissues by mechanical scraping or mincing to release cells or by an enzymatic method using trypsin or collagenase to break up tissue and release single cells into suspension. Because of anchorage-dependence requirements, primary cell cultures require a liquid culture medium in a Petri dish or tissue-culture flask so cells have a solid surface such as glass or plastic for attachment and growth. Primary cultures usually have a limited life span. For detail see https://bio.libretexts.org/Courses/ Mansfield_ University_ of_ Pennsylvania.

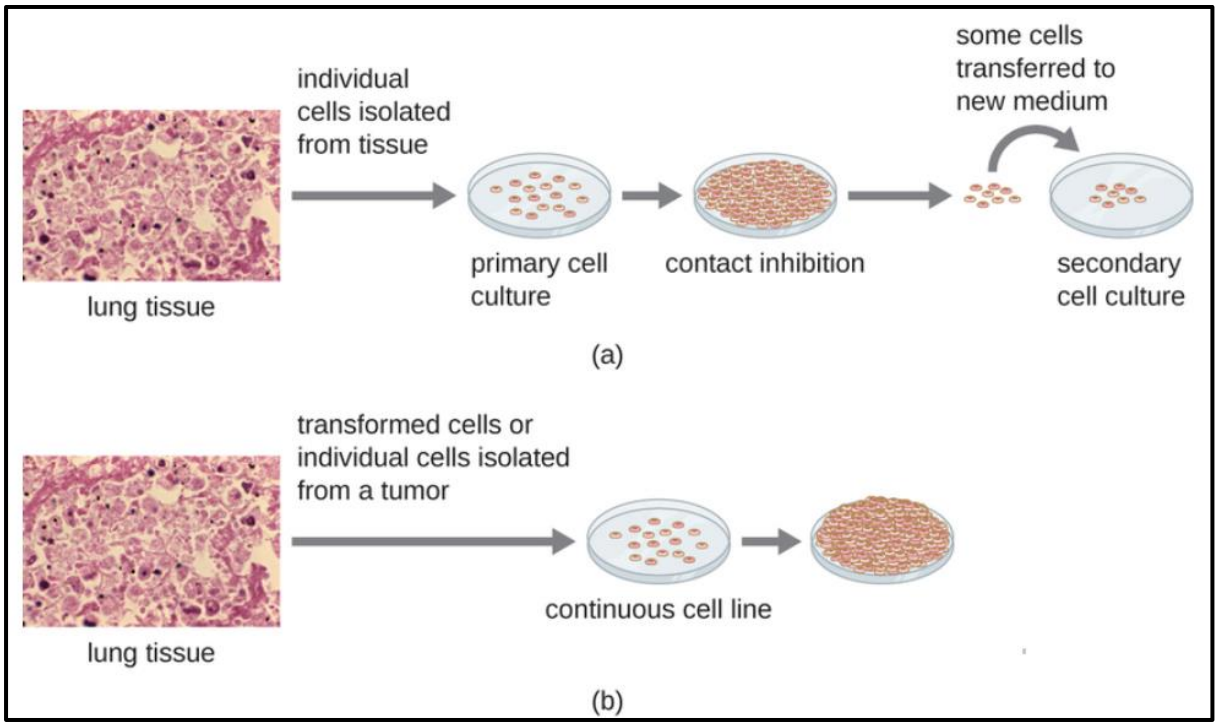

Figure 3. Some of the cells used for culturing viruses.

Cells used for culturing viruses are prepared by separating them from their tissue matrix (Figure 3a). Primary cell cultures grow attached to the surface of the culture container. Contact inhibition slows the growth of the cells once they become too dense and begin touching each other. At this point, growth can only be sustained by making a secondary culture. In Figure 3b continuous cell line cultures are not affected by contact inhibition. An immortalized or continuous cell line has acquired the ability to proliferate indefinitely, either through genetic mutations or artificial modification[6]. They continue to

[6] Bensch KG, Corrin B, Pariente R cf a/. Oat cell carcinoma of the lung: its origin and relationship to bronchial carcinoid. Cancer 1968; 2:1163-1172.

grow regardless of cell density. (Credit, micrographs modification of work by Centersa for Disease Control and Prevention).

Detection of the Virus

Nevertheless of the method of cultivation, once a virus has been introduced into a whole host organism, embryo, or tissue-culture cell, a sample can be prepared from the infected host, embryo, or cell line for further analysis under a bright field, electron, or fluorescent microscope. Cytopathic effects (CPEs) are distinct observable cell abnormalities due to viral infection (Figure 4). CPEs can include loss of adherence to the surface of the container, changes in cell shape from flat to round, shrinkage of the nucleus, vacuoles in the cytoplasm, fusion of cytoplasmic membranes, and the formation of multinucleated syncytia, inclusion bodies in the nucleus or cytoplasm, and complete cell lysis[7].

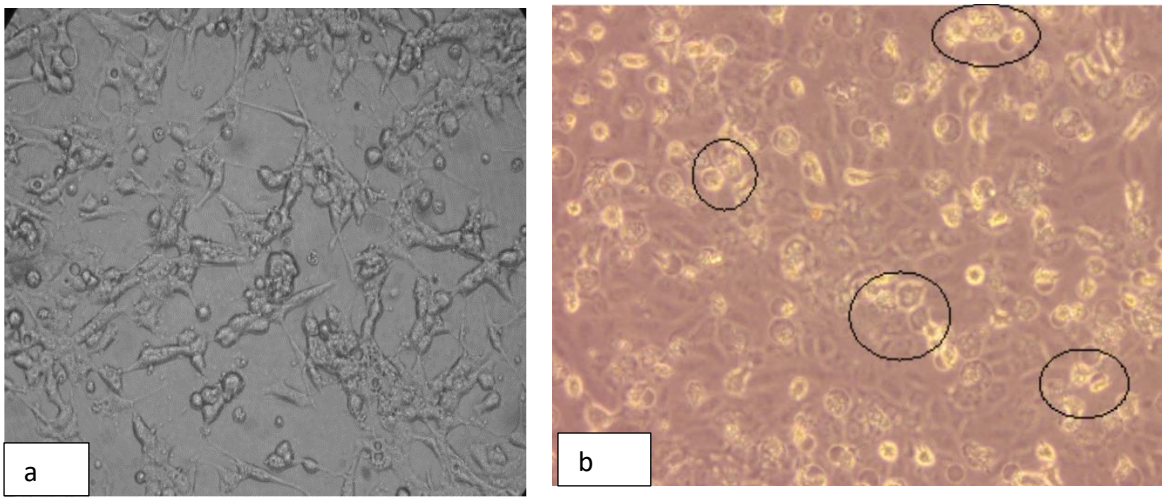

Figure 4: Cytopathic effect induced by Capripoxvirues.

Capripoxvirus-induced cytopathic effect detected in STF-R cells on day 4 post-infection (a). CPE (circled areas) on 36 hpi at 60th passage level, cells rounding and numerous small syncytia (2–3 cell) formation, × 82.5, phase contrast (b).

Identification of viruses

Viruses are extremely small in size and smaller than bacteria. They are ultra-microscopic, which are visible only with an electron microscope. They are considered to be the organisms that live at the edge of living and non-living things. They are dependent parasites that cannot survive on their own.

[7] Leland, D.S. 1992. Concepts of clinical diagnostic virology, p. 3-43. In E.H. Lennette (ed.), Laboratory Diagnosis of Viral Infections, second edition. Marcel Dekker, Inc., New York.

Virus identification is performed either by indirect immunofluorescence of virus-infected cells using group- and type-specific monoclonal antibodies, or RT-PCR on extracts of cell supernatants using specific primers or probes[8].

Chemical/Physical Methods of Virus Quantitation

- Direct visualization of virions by electron microscopy (EM).
- Hemagglutination (HA) assay.
- Serological assays (based on antigen-antibody interactions.
- Genome quantification by PCR.

2.5.2. Diagnostic techniques of bacterial diseases (6 hrs).

Learning objective:

- To equip the learners with the concept and principles of bacterial disease diagnostic methods.
- To introduce and equip learners with the method of sampling and sample transportation for the diagnosis of bacteria.
- To introduce different bacterial isolation and identification methods used in veterinary medicine
- To introduce primary and secondary biochemical tests used in bacterial genius and species identification.

Learning outcome:

- Understand the principle of bacterial disease diagnosis of veterinary importance.
- Understand and perform sampling, sample transport/shipment/.
- Understand, identify, prioritize, and perform bacterial disease diagnostic methods used in veterinary medicine.
- Understand, identify, and perform different bacterial isolation methods.
- To understand and perform primary and secondary biochemical tests used in bacterial genius and species identification.

Session questions

- What are bacterial diseases and diagnostic methods used?

[8] Kemeny, D.M.; Challacombe, S.J. (1988). ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects. *John Wiley and Sons*. ISBN 0-471-90982-3.

- What is sampling and how is it performed?
- What is the method used for sample transportation before being delivered to the laboratory?
- What are methods used for bacterial isolation and identification?
- What is the primary biochemical test?
- What are secondary biochemical tests?

The trainees are advised to read the following text to get answers to the above session questions

Collection and transportation of specimens for isolation of bacteria

Bacteria is a living organism, it can proliferate or die during the transportation of the specimens to the laboratory[9]. Survival of the pathogen is required for culture; however, the growth of organisms during transport is unwanted, if the quantity of bacteria is important or if overgrowth by flora makes the detection of a pathogen less likely[10]. The time for transport to the laboratory should be minimized to reduce the death or excessive growth of organisms. When transport time takes up to 24 hours a specialized transport media is essential for most specimens. Biological specimens like body fluids, tissues, and purulent materials are generally preferred to be collected on a swab. If the quantity of material, which can be collected on a swab, is small and bacteria may remain trapped on the swab, where they cannot be detected. Throat and genital specimens for bacterial culture are an exception to this rule and adequate specimens can be collected from these sites using swabs. If swabs must be submitted, submit one swab for each stain or culture ordered (https://microbeonline.Com/transport-medium-bacterial-viral-sample-transport-used-microbiology-laboratory/).

Obligate anaerobic bacteria are killed by the presence of oxygen, so special transport systems are used for specimens from infections that are likely to include these organisms. These include deep abscesses, fasciitis, and infections that have spread from sites heavily colonized by anaerobic bacteria, such as the oropharynx and intestine. Specimens from the oropharynx, intestine, and vagina, which are normally colonized by obligate anaerobic bacteria, generally should not be submitted for anaerobic culture, because the growth of these organisms is expected and susceptibility testing is not typically performed on anaerobic bacteria (https://anaerobesystems.com/products/transport-media/anaerobic-tissue-transport-medium-attm-and-attmsp/).

[9] Collection, submission, and storage of diagnostic specimens, OIE terrestrial manual, 2018.

[10] Sutton S: Quality control of microbiological culture media." *Pharmaceutical Microbiology Forum* 2006; 12 (1):2-5.Available at http://www.microbiologyforum.Org/pmf_newslet ers.asp. Accessed March 17, 2020.

In addition, superficial skin and wound infections are unlikely to include obligate anaerobic bacteria so, anaerobic culture is generally not useful for these infections. Unless the specimen will reach the laboratory very quickly (minutes for small specimens and up to 2 hours for larger specimens), a commercial anaerobic transport tube, jar, or bag should be used to protect the viability of the bacteria [11]. Fluids, transported without an anaerobic transport system, should be sent in a capped syringe from which all the air has been eliminated[12].

Laboratory detection and identification of bacteria

Detection and identification of bacteria can be performed by several methods, including microscopic examination of stained specimens, culture, antigen detection, and nucleic acid amplification tests (NAAT), which include polymerase chain reaction (PCR) and transcription-mediated amplification.

Methods of growing bacteria

Broth cultures

One method of bacterial culture is liquid culture, in which the desired bacteria are suspended in a liquid nutrient medium, such as Luria Broth, in an upright flask. This allows a scientist to grow up large amounts of bacteria for a variety of downstream applications. Liquid cultures are ideal for the preparation of an antimicrobial assay in which the experimenter inoculates liquid broth with bacteria and lets it grow overnight (they may use a shaker for uniform growth). Then they would take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein (antimicrobial peptides).

Agar based

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[11] J E Rosenblatt and P R Stewart: Anaerobic bag culture method. J Clin Microbiol. 1975 Jun; 1(6): 527–530. doi: 10.1128/jcm.1.6.527-530.1975c/articles /PMC275177/.

[12] D M Citron: Specimen collection and transport, anaerobic culture techniques, and identification of anaerobes. *Rev Infect Dis*, 1984 Mar-Apr;6 Suppl 1:S51-8. doi: 10.1093/ clinids /6.supp lement_1.s51.
https://anaerobesystems.com/products/transport-media/anaerobic-tissue-transport-medium-attm-and-attmsp/).

Miniaturized versions of agar plates implemented to measure formats, eg. Dip Slide, and Digital Dipstick show potential to be used at the point-of-care for diagnosis purposes. They have advantages over agar plates since they are cost-effective and their operation does not require expertise or a laboratory environment, which enables them to be used at the point of care.

Stab cultures

Motile and non-motile bacteria can be differentiated along the stab lines. Motile bacteria will grow out from the stab line while non-motile bacteria are present only along the stab line. Stab cultures are similar to agar plates, but are formed by solid agar in a test tube. Bacteria are introduced via an inoculation needle or a pipette tip being stabbed into the center of the agar. Bacteria grow in the punctured area. Stab cultures are most commonly used for short-term storage or shipment of cultures.

Staining of the isolated bacteria

Gram staining

Gram stain can differentiate bacteria into Gram-positive and Gram-negative bacteria. Gram-positive bacteria can be either cocci or bacilli or vibrios. Gram-positive pathogenic bacteria are staphylococci, streptococci, pneumococci, etc. Gram-negative bacteria can be either cocci or bacilli. Some examples of Gram-negative pathogenic bacteria include *E.coli, Klebsiella, Salmonella spp,* and *Shigella.* The detail is found in the module on microbiology (https://nios.ac.in › Microbiology › Lesson-11).

The Gram stain remains a valuable tool for the rapid detection and preliminary identification of bacteria. Gram-positive bacteria appear dark blue or purple because they have a thick cell wall composed of peptidoglycan, which retains crystal violet and iodine during distaining with alcohol (https://www.ncbi.nlm.nih.gov/books/NBK470553/).

In contrast, Gram-negative bacteria have a thin layer of peptidoglycan surrounded by an outer membrane, and the alcohol rinses and removes the crystal violet and iodine (https://en.wikipedia.org/wiki/Gram-negative_bacteria). After a counter stain with safranin, gram-negative bacteria appear pink. The Gram stain also reveals the shape and arrangement of bacteria.

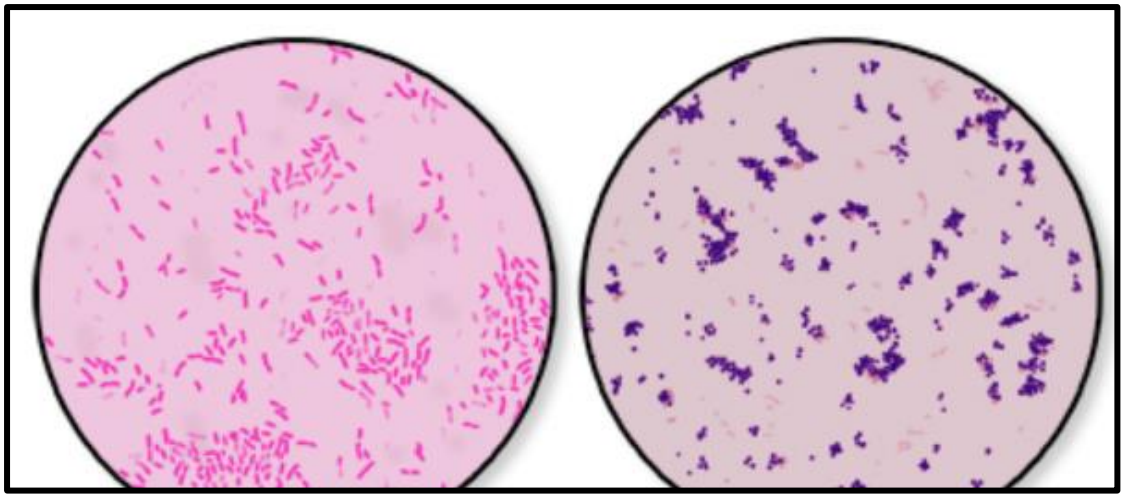


Figure 5.The morphology of gram positive and negative bacteria after staining.

Gram-positive bacteria shows blue or purple after gram-staining in a laboratory test (b). They have thick cell walls. Gram-negative bacteria show pink or red on staining and have thin walls (a).

Table 1.Some bacteria's and their morphological description after gram strain

Organism	**Morphology**
Streptococcus pneumoniae, Enterococcus species	Gram positive cocci in pairs and short chains
Streptococcus species other than S. pneumoniae	Gram-positive cocci in chains
Staphylococcus species	Gram-positive cocci in clusters
Listeriamonocytogenes (small, regular rods)	Gram positive bacilli
Neisseria species	Gram negative cocci in pairs
Many enteric bacteria, including Escherichia coli, Yersinia enterocolitica, Salmonella species, Shigella species	Gram negative bacilli

Albert staining

Albert staining can be performed when Corynebacterium species are suspected. Albert stain distinctly identifies metachromatic granules that are found in Corynebacterium diphtheriae. Corynebacterium is gram-positive, non-spore-forming that contains metachromatic (Volutin) granules which are intracellular inclusion bodies, found in the cytoplasmic membrane of some bacterial cells for storage of complexed inorganic polyphosphate (poly-P) and enzymes. When these granules are subjected to stain with methylene blue dye, they appear reddish-purple color and not the blue dye (https://mi_crobenotes.com/albert-staining/).

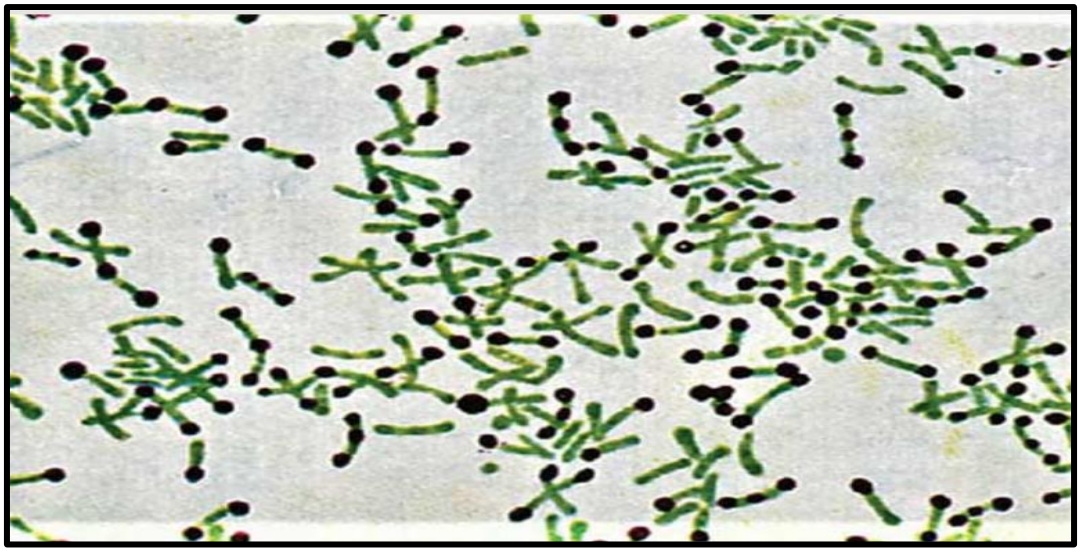


Figure 6.The morphology of Corynebacterium after stained by Albert staining.

Albert staining shows metachromatic (Volutin) granules which are intracellular inclusion bodies in Corynebacterium.

Acid-fast stain

Acid-fast stain is used to identify mycobacterium and Nocardia spp. Mycobacteria stained with carbol fuchsin and auramine O. These stains can be routinely performed on respiratory specimens and tissues and might be performed on other specimens at the choice of the laboratory. The detail is described below: https://www.ucsfhealth.org/medical-tests/acid-fast-stain,https://www.youtube.com/watch?v=yMxUHAsjo8k.

Motility testing

Motility testing is performed by preparing a wet mount and is then observed under the microscope. The motility of bacteria can also be tested by inoculating the bacteria in the semisolid motility medium. To test for motility, use a sterile needle to pick a well-isolated colony and stab the medium within 1 cm of the bottom of the tube. Be sure to keep the needle in the same line as it entered as it is removed from the medium. Incubate at 35 °C for 18 hours or until growth is evident.

Identification of bacteria using biochemical tests

The staining is followed by the use of various biochemical reagents and tests to get closer to the identification of bacteria. There are many biochemical tests available for bacterial identification. The test could be carried out depending on the bacteria (Figure 7). The commonly used biochemical tests are found at https://microbenotes.com/category/biochemical-test/.

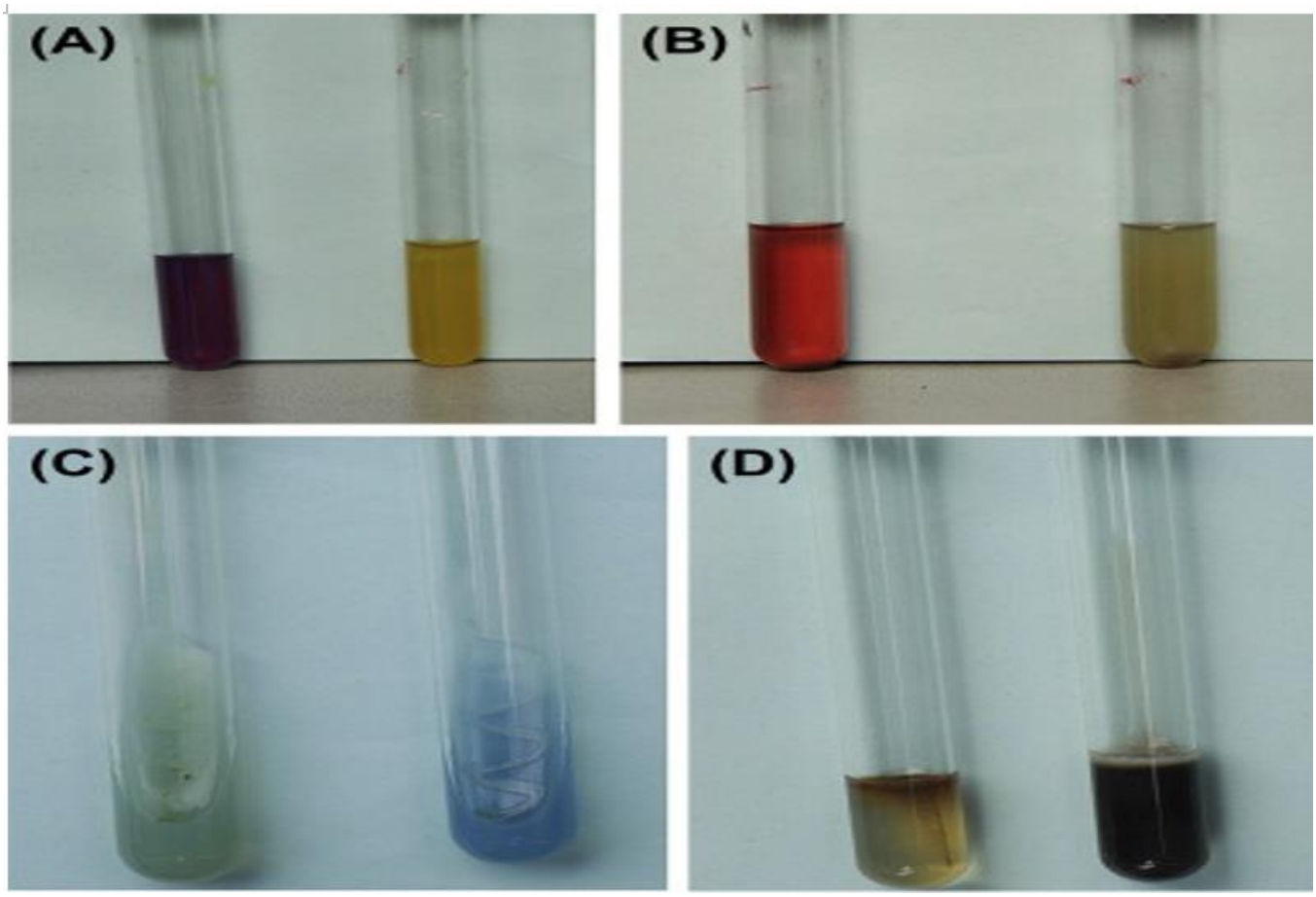


Figure 7. The result of different biochemical testes.

Carbohydrate fermentation test (A). Methyl red test (B). Citric acid utilization test(C). Hydrogen sulfide production test (D).

The isolated bacteria can be further processed by serological tests, phage typing, semi-automated and automated identification systems, and molecular techniques so as to identify the bacteria [13].

Antibiotic Susceptibility Testing

Antibiotic sensitivity testing or antibiotic susceptibility testing is the measurement of the susceptibility of bacteria to antibiotics. It is used because bacteria may have resistance to some antibiotics. Sensitivity testing results can allow a clinician to change the choice of antibiotics

[13] Abubakar I, Irvine L, Aldus CF, Wyatt GM, Fordham R, Schelenz S, et al. A systematic review of the clinical, public health, and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in feces and food. Health Technol Assess 2007; 11(36). https://microbenotes.com/category/biochemical-test/.

from empiric therapy, which is when an antibiotic is selected based on clinical suspicion about the site of an infection and common causative bacteria, to directed therapy, in which the choice of antibiotic is based on knowledge of the organism and its sensitivities.

Several methods are used for the determination of antibiotic susceptibility. The minimum inhibitory concentration (MIC) is the concentration of antibiotic that inhibits the visible growth of bacteria. The MIC can be determined by several methods, including culturing bacteria in a titration of antibiotics in agar or broth, or by using automated devices. The minimum bactericidal concentration (MBC) is the concentration of an antibiotic, which kills 99.9 % of bacteria. In practice, the MIC is easily determined and predicts the susceptibility of bacteria to antibiotics, while the determination of the MBC is technically heavy and rarely adds information beyond that from the MIC or disk diffusion testing, so MBC testing is seldom done. One circumstance in which determining the MBC may be useful in the selection of an aminoglycoside for the treatment of endocarditis with Enterobacteriaceae; is enteric gram-negative rods[14].

Disk diffusion susceptibility testing is performed by coating an agar plate with a known concentration of bacteria, and then placing paper disks impregnated with antibiotics onto the plate. The antibiotic diffuses out of the paper disk and a gradient of antibiotics forms around the disk. After 16–24 hours of incubation, the diameter of the zone of growth inhibition around the disk is measured. The zone of growth inhibition around the disk is inversely proportional to the MIC, and the relationship between these values is the basis for the interpretation of disk diffusion testing results. The interpretation of MIC values or disk diffusion zones as "susceptible," "intermediate," or "resistant" is performed according to guidelines that were published by the Clinical and Laboratory Standards Institute (CLSI) in the United States.

An organism is considered susceptible if it is inhibited by concentrations of antibiotic which will be achieved at the relevant body site with the recommended dosage, and it is considered resistant if it is not. An interpretation of intermediate means that failure of antibiotic therapy is more likely than if the organism is susceptible, but that drugs which are normally concentrated at the site of infection (e.g., penicillin in urine) or drugs given at higher doses than usual (e.g., penicillin for intermediate S. pneumoniae in meningitis) may be effective. The intermediate range also includes a buffer zone for

[14] Shubra Poonia, T. Shantikumar Singh, and Dechen C. Tsering: Antibiotic Susceptibility Profile of Bacteria Isolated from Natural Sources of Water from Rural Areas of East Sikkim *Indian J Community Med.* 2014 Jul-Sep; 39(3): 156–160. doi: 10.4103/0970-0218.137152.

technical variation in the test. The absorption, metabolism, and excretion of an antibiotic determine whether it will reach an effective concentration at the site of infection and so laboratories selectively report susceptibility testing based on the body site from which bacteria are isolated.

2.5.3. Laboratory diagnosis of fungal diseases (5hrs)

Learning objective:

- To equip the learners with concepts and principles of fungal disease diagnostic methods.
- To introduce and equip the learners with the method of sampling and sample transport methods used in fungal disease diagnosis.
- To introduce and equip the learners with methods of sample processing after delivered to the laboratory.
- To introduce different fungal isolation and identification methods used in veterinary medicine.

Learning outcome:

- Understand the principle of fungal disease diagnosis of veterinary importance
- Understand and perform sampling and sample transportation.
- Understand, identify, prioritize and perform fungal disease diagnostic methods used in veterinary medicine.
- Understand, identify, and perform different fungal isolation and identification methods.

Session question

- What are fungal diseases and diagnostic methods used?
- What is sampling and how is it performed in fungal suspected samples?
- What are the sample transportation and processing methods for the diagnosis of fungal pathogens?
- What are the methods used for fungal isolation and identification?

The trainees are advised to read the following text to get answers to the above session questions

Fungal infections, or mycosis, are diseases caused by a fungus (yeast or mold). Fungal infections are most common on the skin or nails, but the fungus can also cause infections in the mouth, throat, lungs, urinary tract, and many other parts of the body[15].

[15] Richardson MD, Warnock DW (2012). "1.Introduction". Fungal Infection: Diagnosis and Management (4th ed.). John Wiley & Sons. pp. 1–7. ISBN 978-1-4051-7056-7.

Whether opportunistic or primarily pathogenic, fungi occur as yeasts (e.g., *Candida* spp.) or molds (e.g., *Aspergillus* spp.). *Yeasts* generally exist as single-celled organisms that multiply through budding. *Molds,* or *filamentous fungi*, form hyphae or elongated structures that may or may not be septate.

Reproduction of filamentous fungi can occur through several mechanisms. Dermatophyte hyphae within the host may fragment to become *arthroconidia*, which are easily spread to other animals and the environment. Hyphomycetes, such as *Aspergillus* spp., can form complex asexual reproductive forms often called fruiting structures. Zygomycete asexual reproduction occurs through sporangium that contains sporangiospores.

Fruiting structures and sporangiospores are rarely found within tissues unless exposed to air, such as in the sinus or rough pocket[16]. These reproductive structures can occasionally be observed histologically and are essential to fungal identification in culture. Other fungi, such as *Coccidioides immitis* or *Sporothrix schenkii*, are dimorphic, having different structures in vivo (e.g., spherules or yeasts) and in vitro (hyphae).

Specimen collection and transport

For laboratory diagnosis of fungal infections, various specimens can be received in the laboratory. Specimens for fungal microscopy and culture include:

- Scrapings of scale, best taken from the leading edge of the rash after the skin has been cleaned with alcohol.
- Skin is stripped off with adhesive tape, which is then stuck on a glass slide.
- Hair that has been pulled out from the roots.
- Brushings from an area of scaling in the scalp.
- Nail clippings or skin scraped from under a nail.
- A skin biopsy.
- A moist swab from a mucosal surface (inside the mouth or vagina) in a special transport medium.
- A swab should also be taken from pustules in case of secondary bacterial infection

[16] Indranil Samanta (2015): Cutaneous, Subcutaneous, and Systemic Mycology. *Veterinary Mycology*: 11–153. doi: 10.1007/978-81-322-2280-4_4

Veterinary professionals, physicians, other personnel, and laboratory technologists need to work together in developing protocols that ensure the proper collection and prompt collection of specimens.The selection of appropriate collection devices and transport containers, labelling of the specimen, and complete requisition forms are important considerations in ensuring the correct diagnosis of fungal infections. In all cases, the specimen should be transported as early as possible to the laboratory. In general, the specimen that is not processed immediately are held at room temperature (for urine if delayed more than 2 hrs refrigerate at 4 °C). Cryptococcus neoformans, cytoplasm capsulatum, and Blastomyces dermatitidis do not survive well in frozen or iced specimens (https: /nios.ac.in /media /documents /dmlt/ Micro biology/Lesson-52.pdf).

Criteria for fungal specimen rejection

- Absence of identification number on the container or discrepancy between the information.
- Squamous epithelial cells as per low power field.
- A dried-out swab or if the material collected is insufficient.
- The sample was submitted in an improper container.

Specimen processing

Upon receiving the specimen, it should be promptly processed. The direct wet mounts or smears are prepared and for culture, the specimen is inoculated on culture media[17],[18].

Direct examination

The direct wet mounts or smears are prepared and for culture, the specimen is inoculated on culture media. Almost all the specimens could be processed for direct microscopic examination. This provides the presumptive diagnosis for the laboratory technologist and also aids in the selection of appropriate culture media. Various methods for direct examinations include;

[17] Chaya A K, Pande S. Methods of specimen collection for diagnosis of superficial and subcutaneous fungal infections. Indian J Dermatol Venereol Leprol 2007; 73:202-205.

[18] Hurst CJ, Crawford RL, Garland JL, Lipson DA, Mills AL, Stetzenbach LD (2007) Manual of environmental microbiology, 3rd edn. ASM Press, Washington, DC. 20036–2904, USA ISBN 978-1-55581-379-6.
https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_aphanomyces_invadans.pdf

- Direct wet mount of the specimen.
- India Ink.
- KOH/calcoflurol mounts.
- Lactophenol cotton blue (LPCB) mounts.
- Frozen section of tissue biopsies.
- Modified Kinyoun Acid Fast Stain for Nocardia.

Table 2. Direct Microscopic observations and presumptive identification of fungus

Direct Microscopic Observations	**Presumptive Identification**
Hyphae are relatively small (3-6 µm) and regular in size, dichotomously branching at 45° angles with distinct cross-septa	Aspergillus spp
Hyphae are irregular in size (6-50 µm), ribbon-like, and devoid of septa.	Zygomycetes (Phycoycetes) rhizopus-Mucor
Hyphae small (2-3 µm) and regular, some branching with rectangular arthrospores sometimes seen, found only in the skin, nail scrapings, and hair	Dermatophyte group Microsporum spp Trichophyton spp, Epidermophyton spp
Hyphae regular in diameter (3-6 µm), parallel walls, irregular branching, septate, dark yellow, brown or hyaline.	Phaeohyphomyces spp Hyalohyphomyces spp
Hyphae, distinct points of constriction, simulating link sausages (pseudohyphae), with budding yeast forms (blastospores) often seen.	Candida spp
Yeast forms, cell spherical and irregular, in size (5-20 µm) classically with a thick polysaccharide capsule (not all cells are encapsulated), with one or more buds attached by a narrow constriction.	Cryptococcus neoformans Cryptococcus spp, non-encapsulated
Small budding yeast, relatively uniform, in size (3-5 µm) with a single bud attached by a narrow base, extracellular or within macrophages.	Histoplasma capsulatum
Yeast forms, large (8-20 µm) with cells, appearing to have a thick, double-contoured wall, with a single bud attached by a broad base.	Blastomyces dermatitidis
Large, irregularly sized (10-50 µm) thick, walled spherules, many of which contain small (2-4 µm) round endospores.	Coccidiosis immitis

Preparation of mounts

The Lactophenol cotton blue (LPCB) wet mount, transparency tape method, and micro slide techniques are commonly used methods for microscopic examination. The mold colony is mounted in a drop of Lactophenol cotton blue stain on a glass slide and examined microscopically. The specimen is directly mounted in 40 % and 10 % KOH for skin and nail specimens respectively. The skin and nail

samples are mounted on the glass slide to which two drops of KOH preparation are added and kept for some time. KOH helps in dissolving the epithelial cells and thus aids in fungal visibility. For the detail see:
https://nios.ac.in/media/documents/dmlt/Microbiology/Lesson-52.pdf.

2.5.4. Diagnostic techniques of parasitic diseases (5 hrs)

Learning objective:

- To equip learners with the concept and principles of parasitic disease diagnostic methods.
- To introduce and equip the learners with the method of sampling and sample transportation used in parasitic disease diagnosis.
- To introduce and equip the learners with methods of sample processing after being delivered to the laboratory.
- To introduce different parasitic isolation and identification methods used in veterinary medicine.
- To introduce egg, larvae, and adult parasites for genius and species identification.

Learning outcome:

- Understand the principle of parasitic disease diagnosis of veterinary importance.
- Understand and perform sampling, sample transportation and sample processing in the laboratory.
- Understand, identify, prioritize and perform parasitic disease diagnostic methods used in veterinary medicine
- Understand, identify, and perform different parasitic isolation and identification methods.
- To understand and perform egg, larvae, and adult parasites identification for subsequent genius and species identification

Session question

- What are parasitic diseases and diagnostic methods used?
- What is sampling and how is it performed in the parasitic suspected sample?
- What is sample processing and methods of processing?
- What are methods used for parasite isolation and identification?
- What is the unique characteristic of individual eggs, larvae, and adult parasites after the test method is applied?

The trainees are advised to read the following text to get answers for the above session questions

A parasite is an organism that lives in or on another and takes its nourishment from that other organism or host. Parasites of animals and humans are available in many forms, including helminths (worms), arthropods, and protozoa. There are over 1,000 species of parasites affecting domesticated animals throughout the world. They can be broadly classified as external or internal depending on where they live on their host[19].

Collection, transportation, submission, and storage of specimens for parasitological diagnosis

Fecal Samples

A fresh fecal sample should be collected and subjected to examination. In large animals, faces can be collected directly from the rectum by using a disposable plastic glove. Although in small animals the feces should be collected immediately after defecation. Following collection feces should be placed in a sealed glass or plastic container and clearly labeled with the time and date of collection, species of animal, sex, and age, history of clinical disease, owner's name, and any other information relevant to the case. If the collected feces cannot be examined within a few hours, the sample should be stored at +4 ^{0}C until examined. Feces should not be frozen since freezing can distort parasite eggs and trophozoites. If the feces are inspected for the presence of protozoan, an examination should be undertaken immediately after collection. When the material is to be sent to another laboratory it should be packaged in cold packs. Helminth eggs may also be preserved in equal volumes of 10 % formalin or 70 % isopropyl alcohol[20].

Examination of the fecal samples

Gross examination

Consistency: The condition of the feces that is soft, watery (diarrheic), or very hard solid, this description will vary with the animal species, for example, cattle feces are normally softer than those of horses or sheep.

[19] Andrews, John S. 1987. "Animal Parasitology in the United States Department of Agriculture, 1886-1984." In *100 Years of Animal Health 1884-1984*, edited by Vivian D. Wiser, Larry Mark, H. Graham Purchase, and Associates of the National Agricultural Library, 113–65. Beltsville, MD: Associates of the National Agricultural Library, Inc.

[20] Collection, submission, and storage of diagnostic specimens, OIE terrestrial manual, 2018. https://pressbooks.umn.edu/cvdl/chapter/module-2-2-fecal-lab-procedure-1-direct-fecal-smear-wet-mount/

Color: Unusual fecal colours should always be reported.

Mucous: Mucous on the surface of fresh feces may be associated with intestinal parasitism or some other metabolic diseases.

Blood: blood may indicate severe parasitemia.

Age of feces: If the feces appear old and dry, this should be noted in the aged sample, parasite eggs have embryonated or larvated, oocyst may be sporulated or pseudo-parasites may be present. Gross parasites like tapeworm segments, roundworms, and larval arthropods (bots) may be present.

Microscopic examination of feces

1. Direct smear and its procedure

- Small amount of feces is placed directly on the microscope slide, by a Stick and diluted with water or normal saline.
- Mixed by using an applicator stick.
- A cover slip is placed and the smear is examined under the microscope.

Advantages: less costive equipment and a short time are required.

Disadvantages: Less sensitive and a positive result may be considered as negative. This method also leaves a lot of fecal debris on the slide (https://www.cdc.gov/dpdx/diagnosticprocedures/stool/microexam.html).

2. Concentration method

Qualitative: this method is used for the determination of the types of parasitic infection

Fecal flotation: This procedure is based on differences in the specific gravity of parasite eggs and larvae and that of fecal debris.

Specific gravity refers to the weight of an object (for example parasite eggs) compared with the weight of an equal volume of pure water.

Most parasite eggs have a specific gravity between 1.1 and 1.2, whereas tap water is only slightly higher than 1, therefore, parasite eggs are too heavy to float in tap water, to make the eggs float, a liquid with a higher specific gravity than the eggs must be used, such liquid is called flotation solution.

Sedimentation: This method is suitable for trematodes' eggs, and some cestodes and nematodes whose eggs do not float readily in common flotation solutions.

Procedure:

- Place 3-6 gm of the fecal sample in a 100 ml glass beaker.
- Add 30-40 ml of tap water or normal saline.
- Mix the water with the feces.
- Strain the solution through a fine sieve.

- Pour the strained mixture into the centrifuge tube and centrifuge for 1-2 minutes at 1500 rpm, if a centrifuge is unavailable, allow the mixture to sit undisturbed for 20-30 minutes.
- Pour off the liquid at the top of the tube without disturbing the sediment at the bottom.
- Using the Pasteur pipette, transfer a small amount of the top layer of the sediment to a microscope slide.
- Apply a cover slip to the drop and examine the slide microscopically.
- Pour off the liquid at the top of the tube without disturbing the sediment at the bottom.
- Transfer a small amount of the top layer of the sediment to a microscope slide using the pastor pipette.
- Apply a coverslip to the drop and examine the slide microscopically.

The detail is described here https: //www. rvc.ac.uk /review /parasitology /Faecal Sedimentation /Principle.htm, https://universe84a.com/collection/formal-ether, https://todaysveterinarynurse.com/clinical-pathology/the-veterinary-nurses-guide-to-fecal-flotation-techniques/

3. Baermann method

This method is used for the detection of lungworm larvae and the cultural method for specific identification of the third larval stage of the Strongyles and Trichostrongyles.

Procedure:

- Apply 5-20 gm of fresh feces or any suspected soil to gauze and placed it in the funnel.
- The sample is covered by warm water.
- Let the apparatus be at room temperature for 8-24 hours.
- Release the clip and collect the first 3-4 drops of water on a microscope slide and examine the slide, or collect 10 ml into a centrifuge tube, spin in the centrifuge for several minutes, and examine the sediment.

For detail information refer to: https://www.rvc.ac.uk/review/parasitology/Baermann/Principle.htm.

4. Faecal culture

Culturing faecal material is a useful technique when eggs or cysts cannot be properly distinguished or identified. Incubation of the culture at room temperature encourages larval hatching and allows for easy identification [21].

[21] Zajac A, Conboy G. Fecal examination for the diagnosis of parasitism. *Veterinary Clinical Parasitology*, 8th ed. Ames, IA: Wiley-Blackwell, 2012, pp 4-9, 12-14.

5. Stained faecal smear

As a confirmatory method of identifying protozoan trophozoites, the fecal smear may be stained. The stain allows the identification of oocysts and trophozoites by highlighting protozoan internal structures. Since the iodine stain will kill trophozoites, it is sensible to examine the fecal smear for motile organisms prior to staining. Non-moving organisms may be difficult to identify within the stained smear[22],[23].

6. Buffy coat method

The buffy coat method (BCM) is very important in order to optimize and facilitate the diagnostics of blood parasites. The concentration technique was developed for application in parasitology research. The method is based on blood centrifugation and the resulting separation of blood cells and parasites in different layers[24].

Procedure

- Prepare a haematocrit tube using whole blood. Be sure to seal one end of the tube with sealant clay.
- Process the tube in a micro haematocrit centrifuge at 1500 rpm for 5 minutes.
- Place the centrifuged tube on the microscope stage and, using 4x objective with low light, examine the area between the buffy coat and plasma for microfilariae movement.
- If desired, break the tube at the buffy coat layer and tap plasma onto a slide.
- Add a drop of methylene blue stain, a drop of physiological saline, and a cover slip and observe under 10x objective, with low to medium light, and scan for microfilariae
- Dirofilaria immitus microfilariae identified by concentrated filtering technique with staining at 10× and 40×.

[22] Hendrix C, Robinson E. Common laboratory procedures for diagnosing parasitism. *Diagnostic Parasitology for Veterinary Technicians*, 4th ed. St. Louis: Elsevier, 2012, pp 316-334, 336. https://todaysveterinarynurse.com/clinical-pathology/the-veterinary-nurses-guide-to-fecal-flotation-techniques/

[23] Zajac A, Johnson J, King S. evaluation of the importance of centrifugation as a component of zinc sulfate fecal flotation examinations. JAAHA 2002; 38:221-224.

[24] Chagas, C.R.F., Binkienė, R., Ilgūnas, M. *et al.* The buffy coat method: a tool for detection of blood parasites without staining procedures. *Parasites Vectors* **13**, 104 (2020). https://doi.org /10.1186/s13071-020-3984-8.

Identification protozoa in blood

Diagnosis of unicellular parasites is easily accomplished in a clinical setting by examination of a stained blood smear. The best samples to evaluate are those from animals with acute infection. A more definitive diagnosis or confirmatory testing (such as in the case of *Babesia*) may be achieved through diagnostic serology as well as the utilization of diagnostic laboratory services. Giemsa staining is one of the diagnostic methods for the identification of blood parasites. See the detail https:/ /today sveterina rypractice. com/ para sitology/todays-technician-diagnosis-of-internal-parasites/.

Giemsa staining

Giemsa stain is recommended and the most reliable procedure for staining thick and thin blood films. Giemsa solution is composed of eosin and methylene blue (azure). The eosin component stains the parasite nucleus red, while the methylene blue component stains the cytoplasm blue.

Steps for Giemsa staining from blood film

- Prepare air-dried blood film.
- Fix in methanol for 5 minutes and air dry again.
- Cover with Giemsa stain (1:20 Giemsa: distilled water ratio).
- Allow standing for 30 minutes undisturbed.
- Gently rinse the slide with tap water.
- Air dry and view.

Note: Parasite cytoplasm will stain blue while the nucleus will stain pink/magenta.

2.5.5. Molecular diagnostic technique (9 hrs)

Learning objective:

- To equip the learners with concept and principles of Molecular diagnostic methods.
- To introduce and equip the learners with the method of sampling and sample transport methods used in molecular diagnosis.
- To introduce and equip the learners with methods of sample processing for molecular diagnosis.
- To introduce different molecular diagnostic methods used in veterinary medicine.

Learning outcome:

- Understand the principle of molecular diagnosis.

- Understand and perform sampling and sample transportation for molecular diagnostic technique.
- Understand, identify, prioritize and perform molecular diagnostic methods used in identification of organism.
- To understand and perform nucleic acid extraction, PCR of different methods.

Session question

- What are molecular diagnostic method used in molecular laboratory?
- What is sampling and how is it performed for molecular diagnosis?
- What is sample processing and methods of processing?
- What are methods used for detection of microorganism using molecular methods?
- What is nucleic acid extractions and why is it important?
- What is the difference between DNA and RNA viruses?

The trainees are advised to read the following text to get answer for the above session questions

Molecular diagnostic is a collection of techniques used to analyse biological markers in the genome and how their cells express their genes as proteins. In Veterinary medicine, the technique is used to diagnose and monitor disease, detect risk, and decide which therapies will work best for the individual diseased animal[25].

Molecular tests developed for diagnostic use can either detect targeted genetic material like nucleic acid or identify specific protein signatures. A nucleic acid amplification test (NAAT) is a type of molecular test that may use polymerase chain reaction (PCR) to amplify a specific sequence of nucleic acid catalysed by rapid temperature cycling. PCR is a chemical reaction that exponentially amplifies the targeted nucleic acid to detectable levels. One cycle of PCR doubles the amount of genetic material in the starting sample. Running multiple cycles of PCR produces millions to billions of copies. A molecular PCR test can detect targeted nucleic acid even if it is only present in small amounts in the subject sample (https://www.bio fire dx.com /blog/how-molecular-diagnostic-testing-work/).

DNA Extraction and Purification Prototype

DNA Extraction from whole blood

[25] Burtis CA, Ashwood ER, Bruns DE (2012). Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. Elsevier. ISBN 978-1-4557-5942-2.

DNA extraction is a method to purify DNA by using physical or chemical methods from a sample separating DNA from cell membranes, proteins, and other cellular components.

Materials

Laminar hood, Eppendorf tube, marker, rack, centrifuge, water bath, micro pipete (200-1000 and 20-100), tips, mini column Reagents Buffers (ALT, Proteinase K, AL, AW1, AW2, elusion buffer), ethanol.

Procedure

In this laboratory session we used the QIAGEN DNA extraction kit. The procedure is undertaken based on the manufacturer's manual.

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml micro centrifuge tube.
2. Add 200 µl sample to the micro centrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 106 lymphocytes in 200 µl PBS. Buffer AL.
3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
4. Incubate at 56°C for 10 min.
5. Briefly centrifuge the 1.5 ml micro centrifuge tube to remove drops from the inside of the lid.
6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml micro centrifuge tube to remove drops from the inside of the lid
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. Place the QIAamp Mini spin column in a clean 1.5 ml micro centrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

RNA extraction

RNA extraction is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. For basic RNA isolation see: https://www.Thermofisher.com/et/en/home/references/ambion-tech-support/rna-isolation/general-articles/the-basics-rna-isolation.html.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a powerful core molecular biology technique that is an efficient and rapid *in vitro* method for enzymatic amplification of specific DNA or RNA sequences from various sources [26],[27]. A standard PCR consists of target DNA, a set of synthetic oligonucleotide primers that flank the target DNA sequence, a thermostable DNA polymerase (usually Taq polymerase), and nucleotides. Using thermal cyclers, there are three stages during each amplification cycle, including denaturing double-stranded DNA (dsDNA) into separate single-stranded DNA, annealing primers to the target DNA sequence, and extension, where DNA polymerase extends the DNA from the primers, creating new dsDNA with one old strand and one new strand. The strands synthesized in one cycle serve as a template in the next, resulting in a million-fold increase in the amount of DNA in just 20 cycles (https://www.Sigma aldrich.com/ET/en/applications/genomics/pcr).

Steps of Polymerase Chain Reactions (PCR)

The molecular technique called PCR is in vitro amplification of a specific segment of DNA using a thermostable enzyme. The polymerase chain reaction is able to produce many copies of the genes of interest as the above cycle can be repeated numerous times leading to an exponential increase in the number of new copies.

[26] Bermingham N, Luettich K. Polymerase chain reaction and its applications. Current Diagnostic Pathology. 2003; **9(3)**:159-164.

[27] Patel SV, Bosamia TC, Bhalani HN, Singh P, Kumar A. Polymerase chain reaction (PCR). Agrbios. 2015; **13(9)**:10-12. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4846334/

There are three basic steps in PCR

Denaturation: First, the template DNA is denatured; the strands of its helix are unwound and separated- by heating to 94-96°C. The high temperature disrupts the hydrogen bonds between the 2 complementary DNA strands, causing their separation.

Annealing: The second step is primer annealing. Short complimentary single stranded oligos are added that bind the denatured DNA and act as origins of replications for later DNA synthesis with Taq. Two primers are used, one for each strand of DNA. The reaction mixture is rapidly cooled to a temperature 45-65º C which allows the primers to bind to their complementary bases on the now single stranded DNAs.

Extension: In the third step, the temperature of the reaction is raised to the optimal temperature for the polymerase (72ºC) and the Taq DNA polymerase adds nucleotides to the hybridized primers, synthesizing new DNA, starting from the primer. The result is two new strands in place of the first, each composed of one of the original strands plus it's newly assembled complementary strand.

Once the first round is completed, the process is repeated by cycling back to the first reaction temperature and the next round of denaturation, annealing, and extension is started *(*an automatic process in thermocycler*)*. This 3 steps temperature cycle is repeated approximately 30 times which results in exponential amplification of the target gene sequence.

Agarose gel electrophoresis

Agarose gel is a technique used to separate and analyses the fragment of deoxyribonucleic acid according to their size. During gel electrophoresis, DNA migrates through agarose from negative charge (cathode) to positive electrode (anode) when current is applied. Agarose is a polysaccharide composed of agar and during gelation it forms a porous matrix that allows diffusion of nucleic acid (https://www.science.smith.edu/cmbs/wp-content/uploads/sites/36/2015/09/Gel-Electrophoresis-Protocol.pdf).

Materials

Microwave oven, Beaker or flask, Balance, Gel casting tray, power supply, Comb, Micro pipet with tip, Electrophoresis tank Reagents and chemicals 1x or 0.5x TAE/TBE buffer, Sample, Molecular Distilled water, agarose powder, Loading dye (Pico green), Para film.

Procedure

1. Weigh the agarose in to flask and added 1% agarose gel,1g agarose

2. Add 100 mL Tris acetate EDTA (TAE) or Tris-Borate-EDTA (TBE) Buffer (1x)

3. Mixed gently

4. Put the flask containing the agarose mix in the microwave and kept our eye on it and took it out when it starts to foam.

5. Remove the flask from the microwave and swirl very gently.

6. Combs are inserted into the gel casting tray.

7. Pour the gel in casting tray and then cooled for gel solidification.

8. After solidification the combs are removed and obtained wells.

9. DNA samples are pipetted into the wells and also molecular ladder added.

10. Then add to electrophoresis tank and power turned on and after migration is done power supply was turned off.

11. The gel is procured out and viewed under UV light.

Applications of PCR

- Identification and characterization of infectious agents
  - ✓ Direct detection of microorganisms in infected animal specimens.
  - ✓ Identification of microorganisms grown in culture.
  - ✓ Detection of antimicrobial resistance.
  - ✓ Investigation of strain-relatedness of a pathogen of interest.
- Genetic fingerprinting (forensic application/paternity testing).
- Detection of mutation (investigation of genetic diseases).
- Cloning genes.
- PCR sequencing.

Types of polymerase chain reaction (PCR)

- Real-Time PCR (quantitative PCR or qPCR). In this technique, DNA molecules are tagged using a fluorescent dye, which is used to monitor and quantify PCR products in real time.
- Reverse-Transcriptase (RT–PCR). This PCR technique creates complementary DNA (cDNA) by reverse transcribing RNA to DNA using reverse transcriptase.
- Multiplex PCR. This method uses a number of primers to multiply multiple fragments in a single DNA sample.
- Nested PCR. In this PCR technique, after the initial 25-35 PCR cycles, an additional PCR is conducted using new primers “nested” within the original primers, which reduces the risk of unwanted products.

- Hot Start PCR. In this method, heat is used to denature antibodies that are used to inactivate Taq polymerase.
- Long-range PCR. Longer ranges of DNA are formed by using a mixture of polymerases.
- Assembly PCR. longer DNA fragments are amplified by using overlapping primers
- Asymmetric PCR. Only one strand of the target DNA is amplified.
- *In situ* PCR. PCR takes place in cells, or in fixed tissue on a slide.

Essential PCR Components and steps

A standard polymerase chain reaction (PCR) setup consists of four steps:

- Add required reagents or master mix and template to PCR tubes.
- Mix and centrifuge. Add mineral oil to prevent evaporation in a thermal cycler without a heated lid.
- Amplify per thermo cycler and primer parameters.
- Evaluate amplified DNA by agarose gel electrophoresis followed by gel red staining.

This video shows the detail procidures and components of PCR:
https://www.youtube.com/watch?v=E6HfI76SO34.

Table 3. Reagents and recommended products needed for PCR

Reagents	Recommended products
Taq DNA polymerase	Select *Taq* DNA polymerase based upon user preference
PCR grade water	PCR Reagent Water
Primers diluted to working concentration	10 µM working stocks are sufficient for most assays
Oligos	Custom oligos
DNA to be amplified	Provided by researcher
Thermal cycler	With various well block sizes or in multiformat
Sterile 1.5 mL screw-top micro centrifuge tubes	Corning® micro centrifuge tubes with screw cap
PCR tubes or plates	Individual thin-walled 200 µL PCR tubes
	200 µL strip tubes
	Multiwell plates and plate seal
dNTP mix	Deoxynucleotide mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP *readymixes already include dNTPs

Additional information for Technical Guide to PCR Technologies is found on https://www.Sigma aldrich.com/applications/genomics/pcr.

Technical Considerations for PCR technique

Appropriate laboratory practice is important for all PCR based techniques. Accurate and careful sample handling and preparation helps to reduce carry-over contamination from one experiment to the next, as well as cross-contamination between samples. The guideline for preventing contamination described on https://www.sigmaaldrich.com/ET/en/technical-documents/technical-article/genomics/pcr/pcr-technologies-protocols-introduction.

PCR Assay Optimization and Validation

- Optimizing PCR Conditions.
- Validating Primer Design.
- Optimizing Probe Concentration.
- Optimizing Reaction Components and Multiplex Assays.
- Optimizing Mg2+ Concentration.
- Probe Fluorophore and Quencher Selection.
- Guidelines for Optimization of Quantitative Reverse Transcription PCR (RT-qPCR).
- Assay Evaluation

For detail information refer: https://www.sigmaaldrich.com/ET/en/technical-documents /tech nic al-article/genomics/pcr/assay-optimization-and-validation?.

Quality control of PCR

In a PCR laboratory, quality control is mainly concerned with the control of errors in the performance of tests and the verification of test results. It includes internal and external quality control. Internal quality control includes monitoring of laboratory performance by using control material and/or repeat measurements. External quality control can be undertaken by comparing the tests with other laboratories[28], [29].

[28] Rys PN, Persing DH (1993): Preventing false positives: Quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. *J Clin Microbiol* 31: 2356-2360.

[29] Kwok S, Higuchi R (1989): Avoiding false positives with PCR. Nature 339: 237-238.

2.5.6. Immunological diagnostic techniques (9 hrs)

Learning objective:

- To equip learners with concept and principles of serological diagnostic methods.
- To introduce and equip learners the method of sampling and sample transport methods used in serology diagnosis.
- To introduce and equip the learners with methods of sample processing for serology diagnosis.
- To introduce different serology based diagnostic methods used in veterinary medicine.
- To introduce and equip the learners with the different ELISA types
- To introduce and equip the learners with ELISA, CFT, HA, HI and agglutination method.

Learning outcome:

- Understand the principle of serological diagnostic methods used in veterinary medicine
- Understand and perform sampling, sample transportation and processing for serological diagnosis.
- Understand, identify, prioritize and perform serological diagnostic methods used in veterinary medicine
- To understand and perform serum purification and antigen quantification
- To understand and perform different ELISA types
- To understand and perform ELISA, CFT, HA, HI, SN and agglutination tests accurately.

Session question

- What are serological diagnostic method used in veterinary medicine?
- What is sampling and sample preparation in serological diagnosis?
- What is sample processing and methods of processing in the laboratory?
- What are methods used for antigen detection using serological diagnostic methods?
- What are methods use for antibody detection using serological methods?
- What are methods used for cellular immunity measurement using serology?

The trainees are advised to read the following text to get answer for the above session questions

Immunological methods are a set of screening techniques that are based on the reaction between antigen and antibody. One component of the reaction is always known and aimed to investigate the other. Immunology is used mainly in microbiology to diagnose pathogens. It is usually faster than cultivation and allows the capture of even poorly culturable agents (eg viruses). According to the

specific diagnostic procedure, serology can be classified as a direct and indirect method of detecting a pathogenic agent (https://www.Wikilectures.eu/w/Serological_methods), (https://www.woah.org/app/uploads/2021/05/401-04-cullinane.pdf).

Specimen used for serological tests

Blood serum is most often used for immunological analysis, but it is also possible to use another biological materials (sputum, urine, saliva, cerebrospinal fluid). There are three main principles in serological diagnostic approaches.

- Antigen detection.
- Antibody detection.
- Monitoring of cellular immunity.

Antigen detection

Antigens can be detected by using a set of known antibodies. Both polyclonal antibodies, which bind to several epitopes of the assumed antigen in the sample, and monoclonal antibodies which allow a more accurate determination of the pathogen type are used. The presence of antigenic molecules indicates the presence of an infectious agent[30].

Antibody detection

Antibodies are detected with a known antigen. The response to the presence of antigenic molecules is usually a response of the immune system. Due to the different dynamics of antibody production, their detection does not necessarily mean an acute disease. In the early stages of infection, IgM antibodies (sometimes IgA) appear, and later a class of IgG that can persist after the disease. For testing, it is necessary to make more samples and evaluate them over time[31].

[30] Bharath Wootla Aleksandar Denic, Moses Rodriguez: Polyclonal and monoclonal antibodies in the clinic. Methods Mol Biol. 2014; 1060:79-110. doi: 10.1007/978-1-62703-586-6_5.

[31] P.R. Hsueh, L.M, Huang, P.J. Chen, C.L. Kao and P.C. Yang (2004). Chronological evolution of IgM, IgA, IgG, and neutralization antibodies after infection with SARS-associated coronavirus. *Clin Microbiol Infect*. 10(12): 1062–1066.
https://www.woah.org/app/uploads/2021/05/401-04-cullinane.pdf

Monitoring of cellular immunity

Cellular immunity is a protective immune process that involves the activation of phagocytes, antigen-sensitized cytotoxic T cells, and the release of cytokines and chemokines in response to antigens (https://www.nature.com/subjects/cellular-immunity).

Types of serological test methods

Haemagglutinattion (HA)

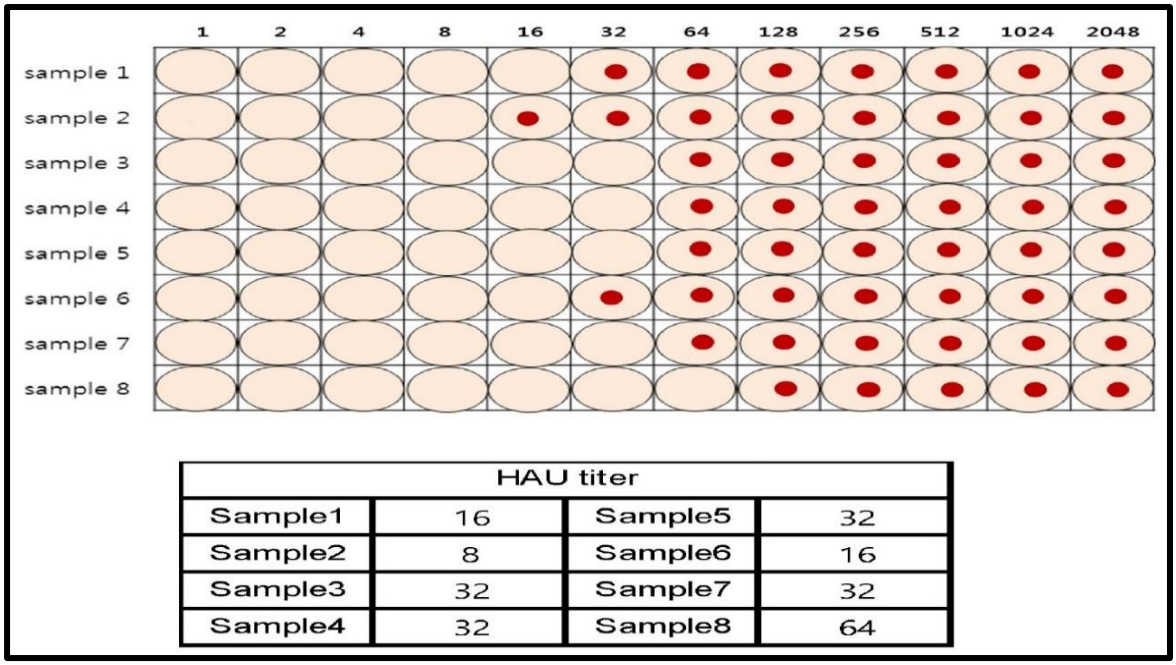
Hemagglutination, or haemagglutination, is a specific form of agglutination that involves red blood cells (RBCs). It has two common uses in the laboratory; blood typing in human and the quantification of virus dilutions in a haemagglutination assay. All strains of the Newcastle disease virus (NDV) are able to agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemaglutinattion and the result is visible macroscopically[32].

A general procedure for HA is as follows:

- Add 50 µl dilution buffer (PBS) to each wells.
- Add 50 µl of antigen (virus) and make serial dilution across the rows in a U or V- bottom shaped 96-well microtiter plate.
- The most concentrated sample in the first well is often diluted to be 1/5x of the stock, and subsequent wells are typically two-fold dilutions (1/10, 1/20, 1/40, etc.).
- The final wells serves as a negative control with no virus.
- Each row of the plate typically has a different virus and the same pattern of dilutions.
- After serial dilutions, a standardized concentration of 1% RBCs is added to each well and mixed gently.
- The plate is incubated for 30 minutes at room temperature.
- Following the incubation period, the assay can be analyzed to distinguish between agglutinated and non-agglutinated wells (Figure 7).

[32] Hirst, GK, 1942. "The quantitative determination of Influenza virus and antibodies by means of red cell agglutination". *J Exp Med*. **75** (1):464 doi: 10.1084/ jem.75.1.49.PMC2135212.PMID19871167.

- The images across a row will typically progress from agglutinated wells with high virus concentrations and a diffuse reddish appearance to a series of wells with low virus concentrations containing a dark red pellet, or button, in the center of the wells.
- The low-concentrated wells appear nearly identical to the no-virus negative control wells.
- The control appearance occurs because the RBCs are not held in the agglutinated lattice structure and settle into the low point of the U or V-bottom well.
- The transition from agglutinated to non-agglutinated wells occurs distinctively, within 1 to 2 wells.



HAU titer			
Sample1	16	Sample5	32
Sample2	8	Sample6	16
Sample3	32	Sample7	32
Sample4	32	Sample8	64

Figure 8. Haemaglutinattion assay.

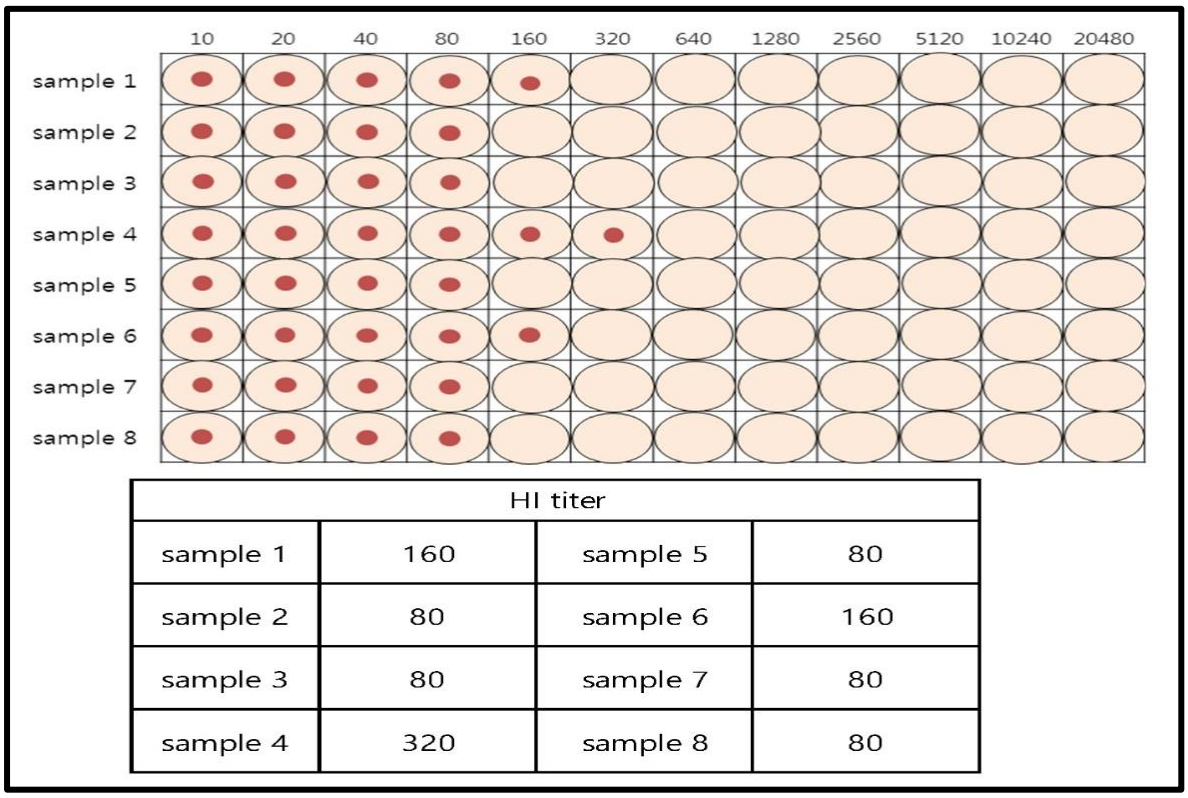
Hemagglutinin on the surface of the influenza virus binds to the sialic acid receptors of red blood cells and creates a lattice structure. The agglutinated lattice maintains the red blood cells in a suspended state and shows as a reddish solution. HAU titre is the dilution factor of the last well showing a reddish solution. HAU titre of sample 1 to sample 8 is 16, 8, 32, 32, 32, 16, 32, and 64.

Haemagglutination Inhibition (HI)

HI is closely related to the HA assay but includes anti-viral antibodies as inhibitors to interfere with the virus-RBC interaction. The goal is to characterize the concentration of antibodies in the antiserum or other samples containing antibodies[33]. The HI assay is generally performed by creating a dilution

[33] Noah, DL; Hill, H; Hines, D; While, EL; Wolff, MC (2009). "Qualification of the Hemagglutination Inhibition Assay in Support of Pandemic Influenza Vaccine Licensure". Clin Vaccine Immunol. **16** (4): 558–566. doi: 10.1128/cvi.00368-08. PMC 2668270. PMID 19225073.

series of antiserum across the rows of a 96-well microtiter plate. Each row would usually be a different sample. A standardized amount of virus or bacteria is added to each well, and the mixture is allowed to incubate at room temperature for 30 minutes. The last well in each row would be a negative control with no virus added. During the incubation, antibodies bind to the viral particles, and if the concentration and binding affinity of the antibodies is high enough, the viral particles are effectively blocked from causing hemagglutination[34] Webster R and Cox N, 2002. Next, a standardized amount of RBCs is added to each well and allowed to incubate at room temperature for an additional 30 minutes. The resulting HI plate images usually progress from non-agglutinated, "button" wells with high antibody concentration to agglutinated, red diffuse wells with low antibody concentration (Figure 8). The HI titre value is the inverse of the last dilution of serum that completely inhibited hemagglutination.



HI titer			
sample 1	160	sample 5	80
sample 2	80	sample 6	160
sample 3	80	sample 7	80
sample 4	320	sample 8	80

Figure 9. Haemaglutinattion inhibition test.

If antibodies bind to the viral particles, the influenza virus is effectively blocked from causing hemagglutination. The HI titre value is the last dilution factor of the antibody showing completely

[34] Webster, R; Cox, N; Stohr, K (2002). WHO Manual on Animal Influenza Diagnosis and Surveillance.

inhibited hemagglutination. HI, the titre of sample 1 to sample 8 is 160, 80, 80, 320, 80, 160, 80, and 80

Advantages of HA and HI

Both assays are simple, use relatively inexpensive and available instruments and supplies, and provide results within a few hours. They are also well-established in many laboratories around the world, allowing some measure of credibility, comparison, and standardization.

Serum neutralization test (SNT)

It is typically considered the gold standard serologic test for viruses. The pathogen is grown in cell culture, and serum from the infected animal is added to the culture. If antibodies are present in the serum, they bind to the pathogen and prevent invasion of the cells. As a result, the cytopathic effects (changes in the cells from infection) are only seen if the cells are infected. This requires cell culture, which is labour intensive and slow (days to weeks) and requires skills in virus cultivation. Due to these facts, limited laboratories are equipped to perform this gold-standard test method[35].

Materials Required

- Neutralizing antibodies: This may be purified antibody, ascites, antiserum, or tissue culture supernatant. Generally 5 mg/mL specific antibody is typically used for the test.
- Virus or cytokine to be neutralized: The virus is frequently measured in units between 1-100 of 50 % tissue culture infectious dose (TCID50).
- Target cells can be grown in 96 well tissue culture plates or another suitable assay format.
- Appropriate reagents to indicate the target effect with an appropriate platform for data collection. For detail procedure see https://www.scielo.br/j/pvb/a/JYbP5p46sX4zKMjLwKVMpm/?lang=en&format=pdf.

Slide agglutination test (SAT)

Slide agglutination tests are frequently used for initial confirmation of specific pathogens. Since antibodies to the target organism may cross-react with other organisms and auto agglutination may occur, these must be considered as screening tests and further confirmation will usually be necessary. In principle, this test is performed in a similar manner to other well-known slide tests. A certain amount

[35] Gauger, P.C., Vincent, A.L. (2014). Serum Virus Neutralization Assay for Detection and Quantitation of Serum-Neutralizing Antibodies to Influenza A Virus in Swine. In: Spackman, E. (eds) Animal Influenza Virus. Methods in Molecular Biology, vol 1161. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-0758-8_26.

of concentrated killed antigen and infected animal serum is mixed on a plate, slide, or card and allowed to react for a specified period, after which the presence of agglutination is determined by the naked eye. For detail, the procedure refers to http://tesla.rcub. bg.ac.rs/~lepto/lab/mat.html.

There are two forms of agglutination

- **Active agglutination**: With active agglutination, the antigen occurs naturally on the particle.
- **Passive agglutination**: With passive agglutination, the antigen must first be bound to an inert particle to detect an antibody.

Rose Bengal Plate Test (RBT).

Rose Bengal test is a rapid test that is used for the diagnosis of brucellosis in animals and humans. This test method is a simple and rapid slide-type agglutination assay performed with a stained *B. abortus* suspension at pH 3.6–3.7 and plain serum. Positive results are generally confirmed by the standard agglutination test (SAT). Although the overall sensitivity reported for RBT varies widely, with the use of good quality antigens made by experienced or reference laboratories, the sensitivity of RBT can be increased.

The procedure of the Rose Bengal Plate Test:

- Test Serum (0.03 ml) is mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
- The mixture is agitated gently for four minutes at ambient temperature, and then observed for agglutination.
- Any visible reaction is considered to be positive (Figure 9).

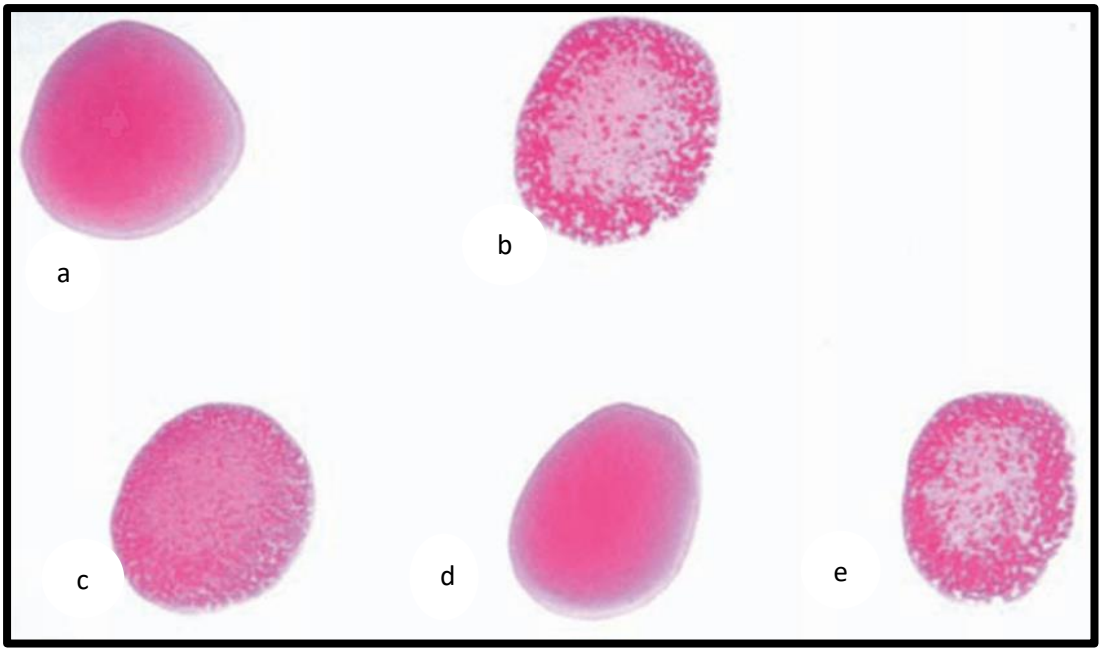


Figure 10. Rose Bengal test.

As indicated on the pictures b, c and e reveals positive agglutination, a and d shows negative result.

Latex agglutination test (LAT)

In the latex agglutination test, a group of passive agglutination tests carried out by coating either antigen or antibody on an artificial carrier particle called a latex bead.

Coating of Latex (For detection of antibodies).

- To 20 µl of latex beads taken in a 1.5 ml vial add 40 µl of the glycine-saline buffer.
- Add 60 µl of antigen to the latex and incubate at 37 ºC for 2 hours.
- Spin down at 5000 rpm for 10 minutes and carefully aspirate the supernatant.
- Resuspend the pellet in 1 ml of blocking buffer and spin down at 5000 rpm for 10 minutes.
- Repeat the washing once more.
- Add 90 µl of blocking buffer to the pellet, and mix well.
- Incubate at 4 ºC, overnight.

Interpretation

Positive: Agglutination of the beads evident by clumps in any of the dilutions is considered a positive result (figure 10 [1, 2, 3, 4, 5]).

Negative: No agglutination or formation of clumps (Figure 10 [6]).

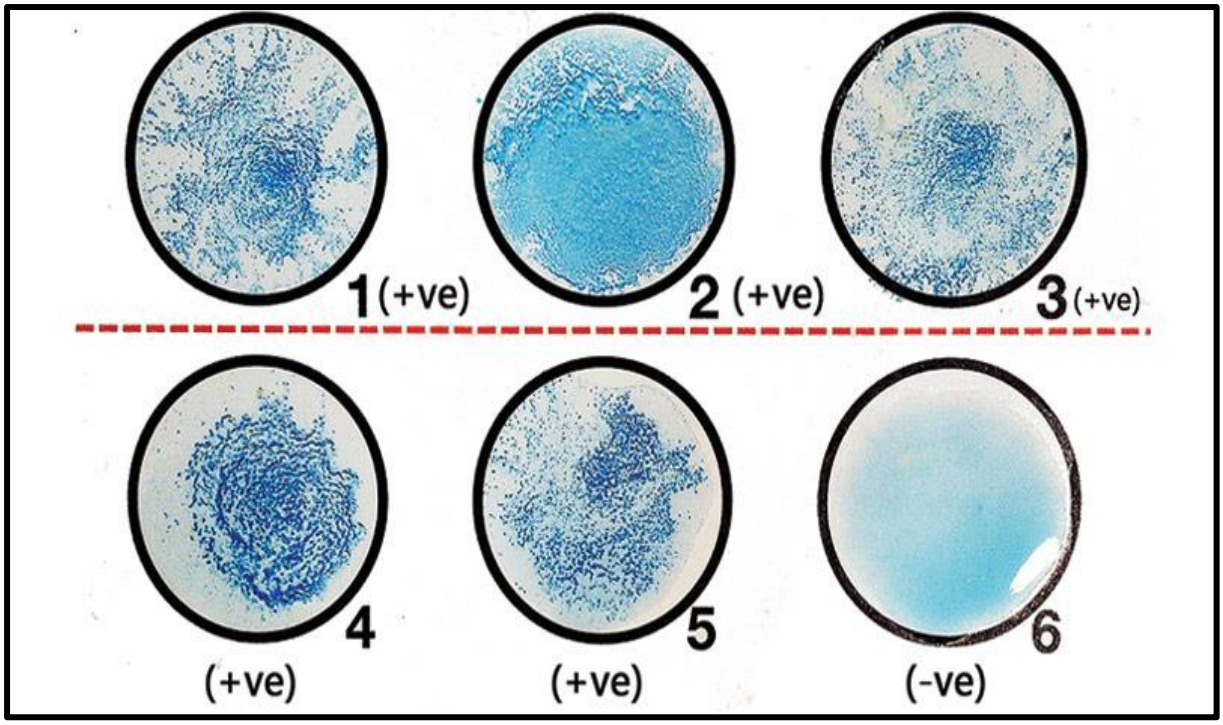


Figure 11. Figure showing latex agglutination test.

As indicated on the figure only figure 6 revealed negative and the others shows positive test result.

Complement fixation test (CFT)

A complement fixation test is a diagnostic technique for the detection of the presence of a particular antibody in the serum of infected animals[36]. In a complement fixation test (CFT), the infected animal serum is heat-treated to inactivate the complement at 56 °C and mixed with the test antigen. Any specific antibody in the serum will be complex with the antigen. Complement is then added to the reaction. If antigen antibody complexes are present, the foreign complement will be fixed. The detection of the presence or absence of complement fixation by the addition of a suitable indicator system. The CFT is often used for the screening of antibodies against a variety of possible pathogenic microbes, especially viruses (https://www.science direct.com /topics/nursing-and-health-professions/complement-fixation-test.

The principle of the complement fixation test

If there is the presence of antibodies in the serum, the complement is fixed due to the formation of the Ag-Ab complex. If no antibody is present then the complement remains free (Figure 11). To

[36] Public Health England. (2013). Complement Fixation Tests. UK Standards for Microbiology Investigations. V 18 Issue 3.2. http://www.hpa.org.uk/SMI/pdf.

determine whether the complement has been fixed, sheep RBCs and antibodies against sheep RBCs are added.

In the positive test: The available complement is fixed by the Ag-Ab complex and no haemolysis of sheep RBCs occurs. So the test is positive for the presence of antibodies.

In the negative test: No Ag-Ab reaction occurs and the complement is free. This free complement binds to the complex of sheep RBC and it's an antibody to cause homolysis, causing the development of pink colour. For detailed information refer https://laborator yinfo.com/complement-fixation-test/.

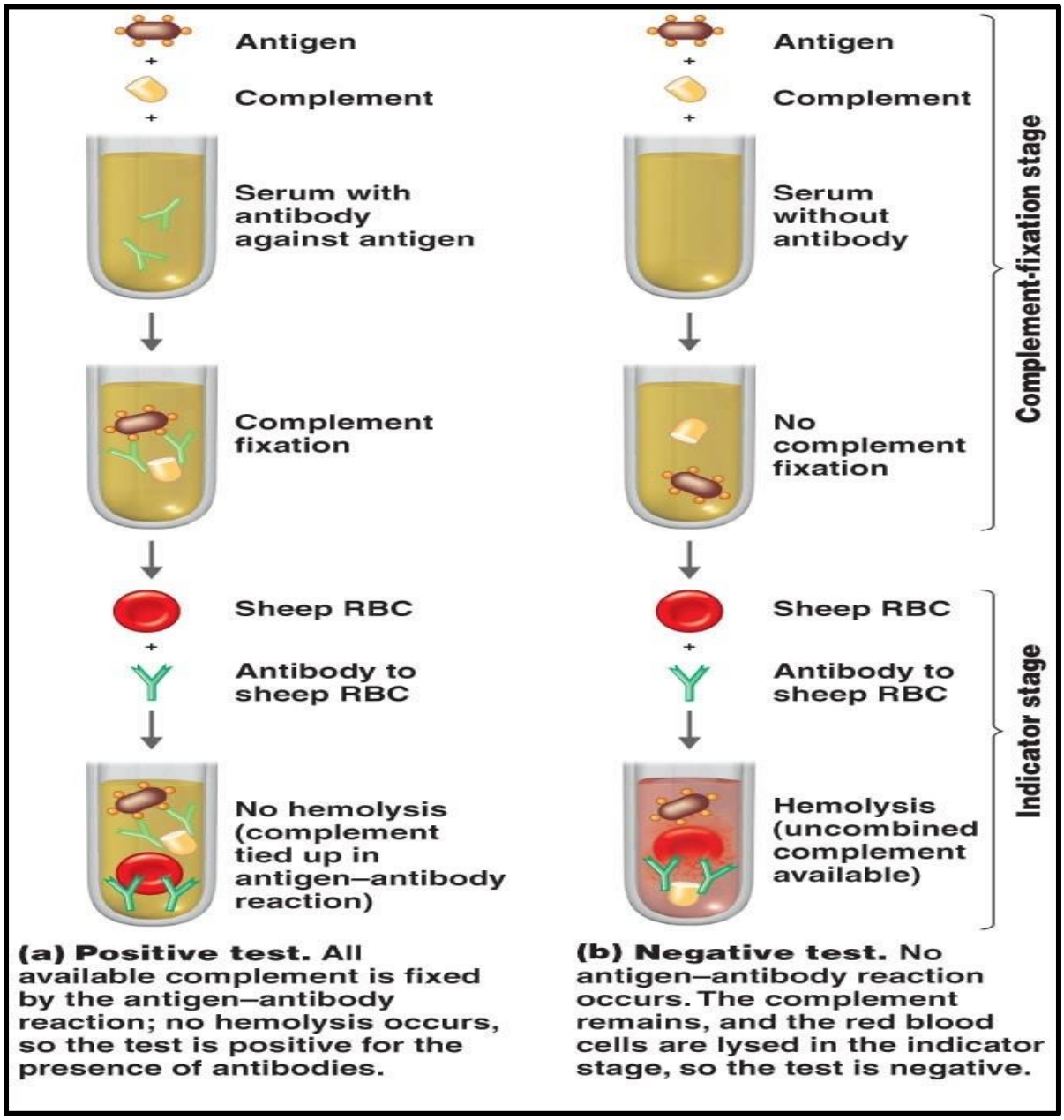


Figure 12. Complement fixation test technique and its result.

Whenever there is antibody in the sample, antibody-antigen binding occurs and the complement fixes to the reaction and no RBC haemolysis happens (Figure a). Whereas if there is no antibody in the sample, the complement stays free without binding and cause haemolysis of RBC (figure b).

ELISA (enzyme-linked immunosorbent assay)

ELISA is a plate-based assay technique designed for detecting and quantifying soluble substances such as peptides, proteins, antibodies, and hormones. ELISA is a commonly used analytical biochemistry assay, first described by Eva Engvall and Peter Perlmann in 1971[37].

Types of ELISA

Direct ELISA: In a direct ELISA, the antigen is bound to the bottom of the microplate well, and then it is bound by an antibody that is specific to the antigen and also conjugated to an enzyme or other molecule that enables detection (Figure 12).

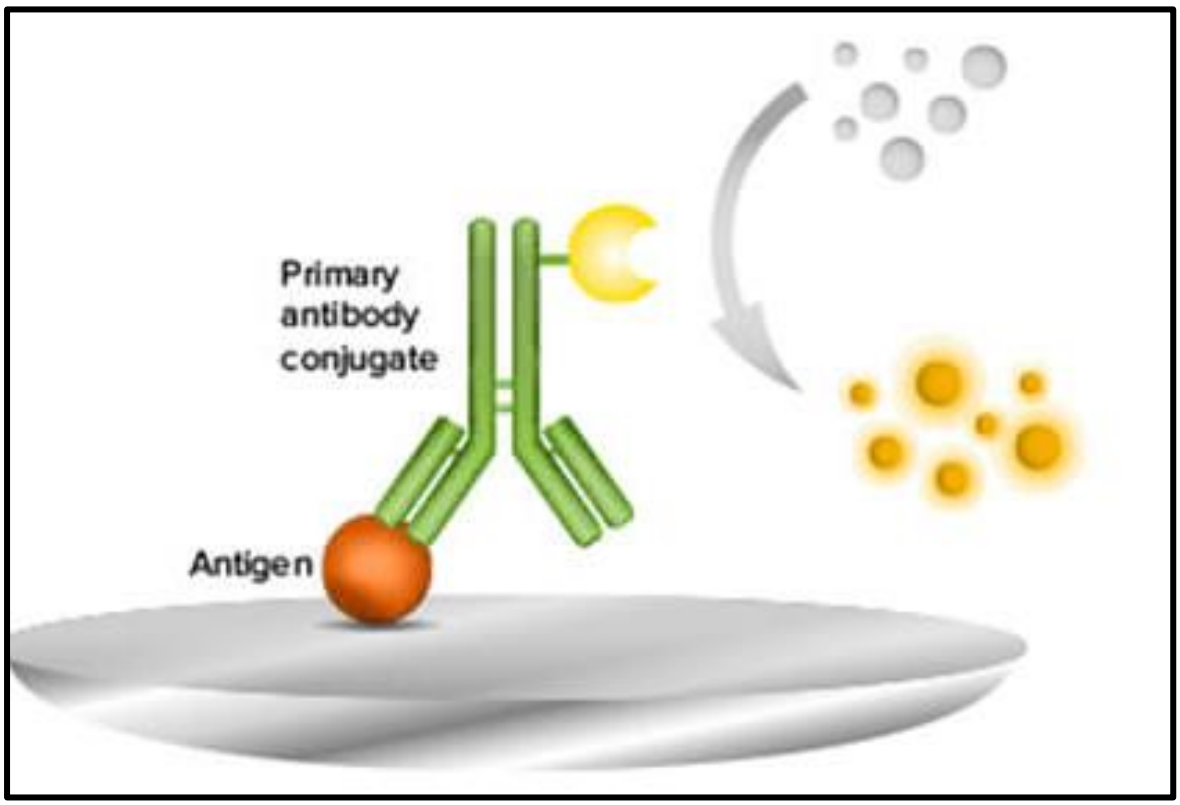


Figure 13. Direct ELISA method.

In this ELISA method the antigen is binded to the microplate the conjugated primary antibody added and the higher signal OD value indicate the better antigen present in the sample.

[37] Engvall, Eva, ELISA: enzyme-linked immunosorbent assay, univ., Diss. Stockholm: Univ. Stockholm, 1975; LIBRIS record.

Indirect ELISA: In an indirect ELISA, the antigen is bound to the bottom of the microplate well, and then an antibody specific to the antigen is added. A secondary antibody, conjugated to an enzyme or other detection molecule, is then bound to the first antibody (Figure 13).

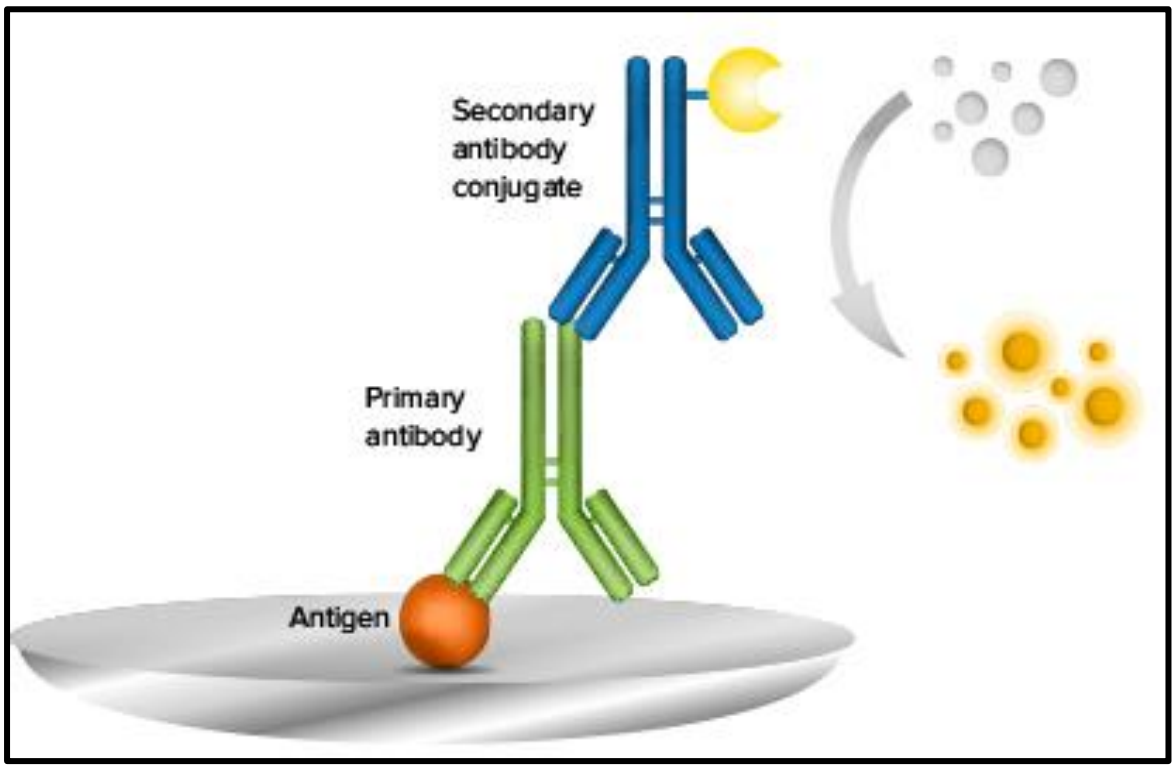


Figure 14. Indirect ELISA method.

This figure shows indirect ELISA technique in which the antigen is coated to the microplate then antibody in the sample added to the plate. Finally the secondary antibody which is conjugated with enzyme added. High signal indicates the better antibody present in the sample.

Competitive ELISA: The antibody in the sample competes with a specific amount of labeled antibody for the coated reference antigen to the micro well. The reference antigen is pre-coated on a multi-well plate and the sample is incubated with labeled antibodies and added to the wells.
Unbound material is washed away. The more antibody in the sample, the less antibody ends up bound to the bottom of the wells by the reference antigen, and the lower the signal (Figure 14).

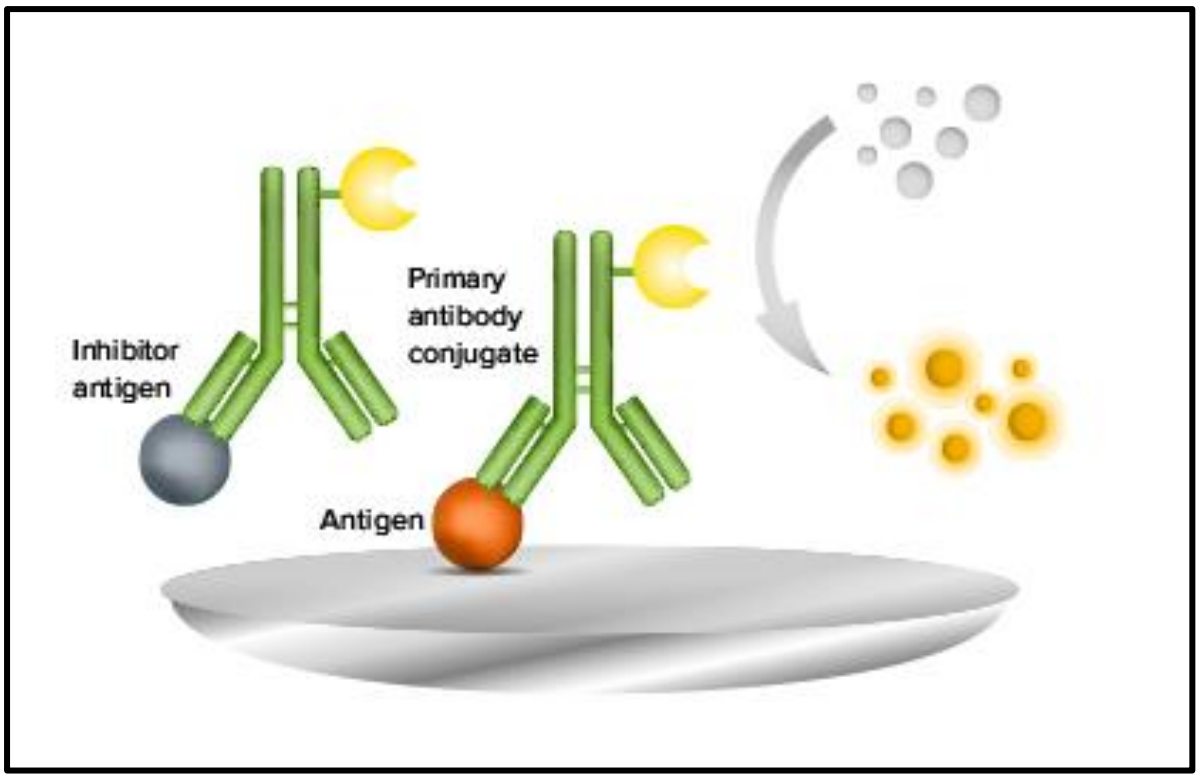


Figure 15. Competitive ELISA method.

Regarding competitive ELISA the antigen bounded to microplate and two antibodies compute for the coated antigen and the signal of OD value is inversely proportional to the amount of anybody present in the sample.

Sandwich ELISA: For the sandwich ELISA, two antibodies specific to two different epitopes on the target antigen are used. The capture antibody is bound to the bottom of the microplate well and binds one epitope of the antigen. The detection antibody binds to the antigen at a different epitope and is conjugated to an enzyme that enables detection (Figure 15). (If the detection antibody is unconjugated, then a secondary enzyme-conjugated detection antibody is required).

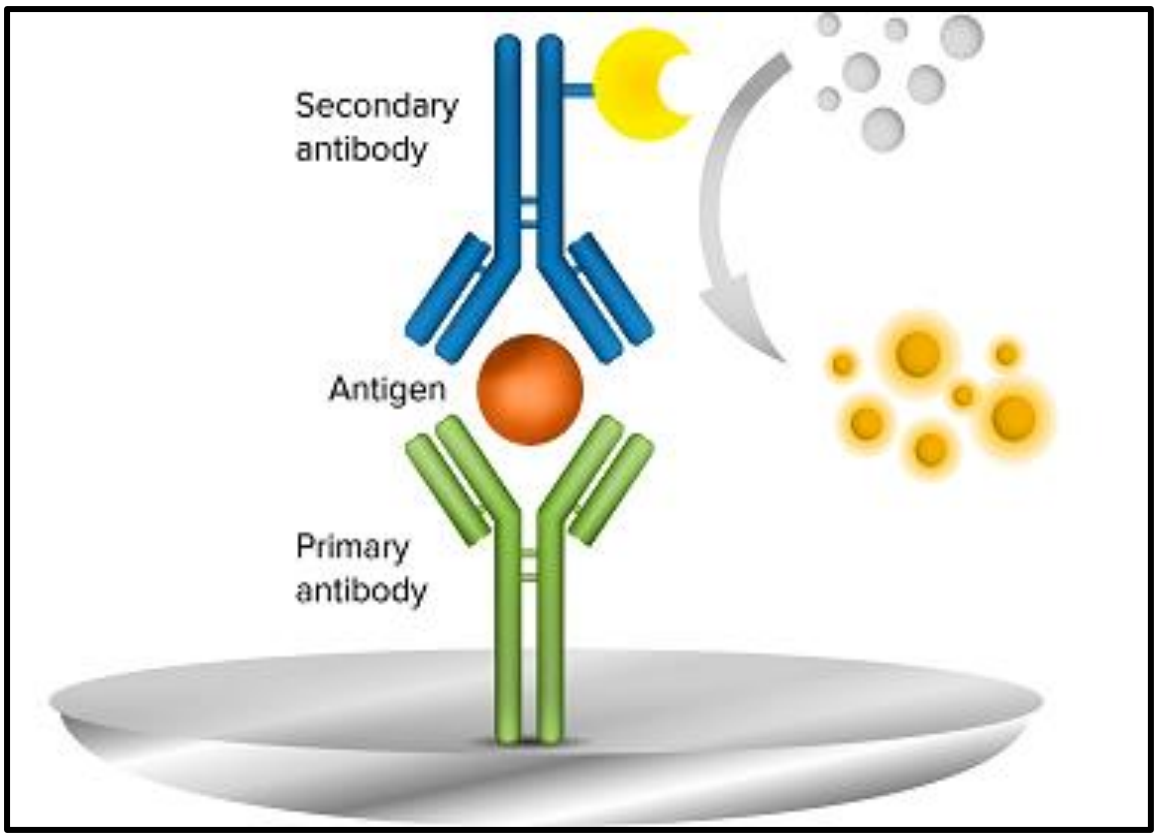


Figure 16. Sandwich ELISA method.

In this ELISA technique the primary antibody binded to the microplate and the target antigen or antibody captured between two antibodies.

ELISA Procedure

1. An antigen is attached to a polystyrene plate which is a solid surface.
2. A microtiter coated with antigen is filled with this antigen-antibody mixture after which free antibodies are removed by washing.
3. A second antibody specific to the primary antibody is added which is usually conjugated with an enzyme.
4. Free enzyme-linked secondary antibodies are removed by washing the plate.
5. Finally, the substrate is added. The substrate is converted by the enzyme to form a colored product, which can be measured by spectrophotometry.

Note: the procedure depends on the ELISA techniques described https://byjus.Com biology/elisa-technique/.

2.5.7. Fundamental Safety Practices in Diagnostic Laboratories (3hrs)

Learning objective:

- To introduce the learners with the principle of safety and its practice in diagnostic laboratory.
- To familiarise the learners with best set up for sample reception to avoid risk of personnel and environmental contamination.
- To acquaint the learners with concepts of sample transportation, packaging and receipt.
- To introduce the learners with the principle and use of safety cabinets to avoid dissemination of harmful microorganisms.
- To introduce the learners about the occupational health safety (OHS) in the laboratory such as use of UV- fitted apparatus, Bunsen burner, Autoclave and dry oven.
- To introduce learners with good laboratory practise (GLP).

Learning outcome:

- Understand the principle of safety and its practice in a laboratory.
- Understand and perform a good sample reception set up that avoids environmental and personnel contamination.
- Understand different methods of sample transportation, packaging and receipt.
- Understand and use of different safety cabinet levels.
- Understand and follow occupational health safety practices (OHS).
- Understand and perform/follow Good laboratory practices (GLP).

Session question

- What is Laboratory safety?
- What are risks at sample reception and in the laboratory during sample processing and performing the test?
- How is sample packed, and transported?
- What is safety cabinet and what is the principle behind it?
- What is OHS?
- What is GLP?

The trainees are advised to read the following text to get answer for the above session questions

Many safety procedures, guidelines, and principles should be applied to all sections of diagnostic laboratories. The recommendations presented in this section represent a broad view of safety throughout

the laboratory. Refere the following link. https://rr-asia.woah.org/wp-content/ uploads/ 2020/02/ seacfmd-manual-8.pdf

Diagnostic laboratories, state and local health departments, CDC, and the American Society for Microbiology have established and/or published guidelines to follow when suspected agents of bioterrorism have been or could be released into the community. However, routine laboratory testing may provide the first evidence of an unanticipated microorganism.

Predictable clinical specimens also may harbour unusual or exotic infectious agents that are dangerous to amplify in culture. These agents are often difficult to identify, and the routine bench technologist might continue work on the culture by passage, repeated staining, nucleic acid testing, neutralization, and other methods. This continued workup places are dangerous for the technologist and others in the laboratory (https://www.cdc.gov/labs/pdf/CDC-Biosafety microbiologicalBiomedicalLaboratories-2009-P.pdf).

Perfectly, these specimens are not to be processed or tested in the routine laboratory, and they can be removed from the testing stream if the suspected agent is known. Relationships with the state public health laboratory, and subsequently with the Laboratory Response Network, are critical in this effort. Once the testing process has begun, the bench technologist must have clear and concise instructions about when to seek assistance from the laboratory supervisor and/or director.

Specimen receiving setup station

- Microbiology specimens are to be received in uncontaminated containers that are intact and consistent with the laboratory specimen collection policy.
- Use of pneumatic tubes for the transport of specimens is acceptable for most specimens but might be contraindicated for specimens without sealed caps.
- Preferably, all specimens in a biosafety level (BSL) 2 or higher facility are to be processed in a biological safety cabinet (BSC) adhering to safe BSC practices.
- If a BSC is unavailable in the laboratory, the professionals processing intake specimens must wear a laboratory coat and gloves, employ an effective splash shield, and continue to follow universal precautions.
- Additional precautions may be necessary if warranted by site-specific risk assessments.
- Limit the use of a 4-foot-wide BSC for inoculating plates and preparing smears to one employee at a time, wearing appropriate personal protective equipment (PPE).
- Six-foot-wide BSCs may accommodate additional testing equipment or materials. Check the manufacturer's recommendations before allowing two employees to work simultaneously in the larger cabinet, and then allow only after a risk assessment.

- Minimal PPE for the general setup area is a gown and gloves. In microbiology, a surgical-type mask is recommended, but optional if the BSC is used.
- For mycobacteriology and virology laboratories where organism manipulation is conducted, workers should wear a fit-tested N95 respirator or select other appropriate respiratory protection, as indicated by the risk assessment.
- An N95 respirator is usually not required for biocontainment levels up through BSL-2, although it provides a higher degree of protection than a surgical mask. Safe BSC practices are to be adhered to at all times. Mycobacterial, fungal, viral, and molecular specimens may require specific additional safeguards.

Containers required for sample transportation

- Submit specimens to the laboratory in a separate container that is labelled and limit the container to one sample to prevent misidentification and cross-contamination.
- Request a new specimen if a container is broken or has spilled its contents. These containers are unacceptable for culture because the contents may have become contaminated.
- Document the incident, and notify the supervisor if an exposure occurred.
- Visually examine containers for leaks upon arrival and before placing them on rockers, in centrifuges, in racks, in closed-tube sampling (cap-piercing probe) systems, in automated aliquot stations or automated slide preparation systems.
- Track and document all incidents of cracked tubes, loose caps, and leaking containers. Increases in documented events may indicate the need to clarify or strengthen specimen acceptance policies or improve specimen collection or transportation practices, or they might identify defective container lot numbers.
- If the sample is suspected of tuberculosis or pneumonia, handle it with care. External contamination caused by inappropriate lid closure can contaminate the gloves of the laboratorian and all contents of the BSC. If the specimen is leaking or contaminated, consider rejecting it and requesting another specimen if feasible. Change and discard gloves after disinfection and clean up. (A 1:10 bleach solution or appropriate disinfectant is recommended.) Document the external contamination for reporting purposes.
- Viral specimens with damaged or leaking containers may need to be discarded before opening. Contact the supervisor for instructions on whether or not to continue processing, and be prepared to notify the submitter and request another specimen.

Visible contamination of the outside of containers

- Consider all specimen containers as potentially contaminated and do not rely on visible external contamination to confirm the potential source of contamination.

- Wipe off visible contamination by using a towel or gauze pad moistened with acceptable decontaminant, such as a 1:10 dilution of household bleach, or use the established laboratory disinfectant. Ensure the label and bar code are not hidden before advancing the specimen for analysis.

Unfastened caps

- Always hold the tube or outside of the specimen container, not the stopper or cap, when picking up tubes or specimen containers to prevent spills and breakage.
- Ensure tops are tightly secured on all specimen containers, blood-collection tubes, and sample tubes before advancing for analysis or storage.

Operational procedures

- Confirm that specimen placement, specimen flow, and bench operational workflow are unidirectional (i.e., from clean areas to dirty areas) and uniform for all operators to maximize the effective use of engineering controls.
- Determine appropriate PPE on the basis of documented risk and hazard assessments of all the operations performed at each bench. Try to incorporate engineering controls and PPE information in the same location in all procedure manuals, and clearly post the information for each operation carried out at the bench.
- Ensure that workstation procedure manuals include instructions for the organization of all instruments, materials, and supplies in each area as well as instructions for any cleaning and disinfection and the frequency of cleaning and disinfection for all surfaces and instruments.
- The cleanliness of the bench should be routinely inspected by the laboratory supervisor.
- Have written procedures for non-laboratory operations, e.g., technical instrument maintenance, in-house or contracted maintenance, emergency response, housekeeping, and construction and utility operations, to mitigate exposures associated with assigned operational tasks and write non-laboratory operation procedures for non-laboratory service providers with their input and consultation.
- Document the training and assess the competency of service providers and bench operators for all written non-laboratory operational bench procedures.
- General bacteriology stains may constitute both a chemical or biological hazard. Chemicals and reagents used for Gram stain; crystal violet, methylene blue, potassium iodide, and ethanol are all irritants. Crystal violet is also carcinogenic and somewhat toxic whereas ethanol is hepatotoxic. Other risks associated with Gram stain procedures include handling live organisms, the potential for creating aerosols, and the potential for skin and environmental contamination.

- Eye protection (safety glasses or chemical splash goggles) and disposable gloves are recommended during staining or preparing stains. Gloves provide protection from live organisms as smears are prepared and provide protection from accidental exposure to stains.
- Place contaminated waste in a biohazard bag for disposal. Use biohazard bags only once and then discard them. Never wash or reuse them.
- For all other stains, including fluorescent conjugates, refer to the Material Safety Data Sheets associated with each stain or chemical.
- Equipment decontamination. Examine equipment contaminated with blood or other potentially infectious materials before servicing or shipping, and decontaminate as necessary. Contact the manufacturer for the decontamination process. If decontamination of equipment or portions of such equipment is not feasible, do the following;
    - ✓ Label the equipment with a biohazard symbol and a second label specifically identifying which portions remain contaminated.
    - ✓ Deliver this information to all affected employees and examine representatives before handling, servicing, or shipping so that appropriate precautions will be taken.

Manual removal of sealed caps; specimen aliquoting and pipetting.

- Always remove caps behind a bench-fixed splash shield, or wear additional PPE appropriate to protect from splashes and aerosols.
- Place a gauze pad over the cap, and then slowly interfere or push the cap off with an away-from-body motion. Never reuse a gauze pad; doing so might contribute to cross-contamination.
- Use automated or semi-automated pipettes and safety transfer devices.

Pneumatic tube systems

- Establish SOPs for the use and decontamination of the pneumatic tube system (PTS).
- Place all specimens sent through a PTS in a sealed zip-lock bag.
- Test bags, and ensure they are leakproof under the conditions in the PTS.
- Protect requisition forms in separate pouches, or enclose them in a separate secondary bag to prevent contamination.
- Handle contaminated pneumatic tube carriers in accordance with standard precautions.
- Disinfect contaminated carriers with a bleach solution or another disinfectant following the protocol recommended by the manufacturer[38].

[38] Laboratory Chemical Waste Management Guidelines, Revision: November 2020, EHRS, Environmental Health & Radiation Safety, 3160 Chestnut Street, Suite 400, Philadelphia, PA 19104-6287.

Personal Precautions

If engineering controls are in place to prevent splashes or sprays, the requirement for PPE can be modified on the basis of a risk assessment and evidence of the effectiveness of the engineering control to prevent exposure from splashes or sprays. Examples of engineering controls include the use of a BSC, having sealed safety cups or heads in centrifuges, and negative airflow into the laboratory[39].

Biological Safety Cabinet

- The Class II-A1 or II-A2 BSC is best suited and recommended for the diagnostic laboratory.
- Every diagnostic microbiology laboratory needs one or more BSCs as a primary means of containment for working safely with infectious organisms. The lack of a BSC is a Phase II deficiency for microbiology departments that handle specimens or organisms considered contagious by airborne routes. The three basic types of BSCs are designated Class I, Class II, and Class III.
- The Class I cabinet is similar to a chemical fume hood and is usually hard-ducted to the building exhaust system. It protects personnel and the room environment but is not designed to protect the product inside the cabinet. The Class I BSC could be used in the general laboratory setup area as a second choice of cabinet.
- For most diagnostic laboratories where volatile chemicals and toxins will not be manipulated within the cabinet, the Class II-A2 BSC would be appropriate and easiest to install without a hard duct to the outside. This cabinet can be used at the specimen-processing station; in the mycobacteriology, mycology, and virology laboratories; and in chemistry and hematology if needed. Air can be recirculated back into the room through high-efficiency particulate air (HEPA) filters with little risk if the cabinet is maintained properly and certified annually. The A-1 or A-2 BSC in the mycobacteriology laboratory is also an option with a cover connection to a building exhaust duct and annual certification. Never hard-duct the Class A BSC to the building exhaust system because building airflow patterns cannot be matched to the cabinet. HEPA filters remove at least 99.97 % of 0.3µm particles, which include all bacteria, viruses, spores, and particles or droplets containing these organisms.
- The Class III cabinet is designed for highly infectious agents, such as the Ebola virus and monkeypox virus.
- Some rules and proper usage of BSC by the laboratorian are described below

[39] Bio-risk management/ Laboratory biosecurity guidance, WHO/CDC/ EPR/, 2006.6.

- ✓ Do not twist your arms into or out of the cabinet. Move arms in and out slowly, perpendicular to the face opening.
- ✓ Install the BSC in the laboratory away from walking traffic, room fans, and room doors.
- ✓ Do not block the front grill where the down flow of air is conducted or the rear grill where the air is removed from the cabinet.
- ✓ Let the blowers operate at least 4 minutes before beginning work to allow the cabinet to remove unwanted.
- ✓ At the beginning and end of the day, with the blower running, disinfect all surfaces with a 1:10 dilution of household bleach, and remove residual bleach with 70 % alcohol, or use another disinfectant appropriate for the organisms encountered.
- ✓ Do not use open flames inside the cabinet.
- ✓ To decontaminate the BSC before maintenance, engage a BSC certification technician to use either formaldehyde gas, hydrogen peroxide vapour, or chlorine dioxide gas when the BSC is not in use.
- ✓ Open sealed rotors or safety cups on high-speed and ultracentrifuges in a BSC, particularly when respiratory pathogens are manipulated.
- ✓ Where safety cups or sealed rotors cannot be used, place centrifuges in a containment device or BSC designed for this purpose.
- ✓ Collect waste generated inside the BSC in bags or sharps containers. Seal these before removal and place them in waste containers outside the BSC.

Ultraviolet radiation is a form of non-ionizing radiation, and biological effects from it vary with wavelength, photon energy, and duration of exposure. The 100-280 nm wavelength band is designated as UV-C, which is used for germicidal purposes. The sterilization /decontamination activity of UV lights is limited by a number of factors, including:

- Penetration: In the dynamic air streams of BSCs, microorganisms beneath dust particles, plastics, and work surfaces are not affected by UV light because they cannot penetrate particles so far from the UV source.
- Relative humidity: The germicidal effects of UV light drop off quickly when relative humidity is above 70 %.
- Temperature and air movement: The optimum temperature for the UV lamp to be effective is 77-80 degrees F. Temperatures below this range result in reduced efficacy, and air movement can aggravate this.
- Cleanliness: Dust and dirt block the germicidal effectiveness of the UV lamp, so weekly cleanings are necessary.
- Age: Check UV lamps every six months to assure proper function, as the amount of germicidal wavelength emitted decreases with bulb age and hours of use.

- Overuse: UV lights are routinely left on overnight or longer in an effort to decontaminate workspaces, but this practice can result in the germicidal wavelength no longer being produced by the bulb.

For these reasons and other concerns, the National Sanitation Foundation (NSF) does not recommend the use of UV lights in BSCs. Retrofitting any equipment (e.g., UV lights) into a cabinet may alter the airflow characteristics, invalidate the manufacturer warranty, and is not recommended. Laboratories are solely responsible for maintaining any UV lights in their cabinets and will need to use outside contractors for services. Be aware that environmental health and safety do not support the use of UV lights in BSCs.

Laboratories are solely responsible for maintaining any UV lights in their cabinets and will need to use outside contractors for services. If you are using a UV light, please be advised:

- No one should be present in a room where a UV light is on, due to the short time for UV overexposure to occur.
- Less than one minute of direct exposure can result in severe eye damage and additional health effects including skin cancer. BSCs with UV lamps should be labeled with a UV Light Source Caution sticker.

Good work practices

- Regardless of the method, the purpose of decontamination is to protect the laboratory worker, the environment, and any person who enters the laboratory or who handles laboratory materials that have been carried out of the laboratory.
- Instructions for disinfecting a laboratory workbench are to be a part of each SOP and must include what PPE to wear, how to clean surfaces, what disinfectant to use, and how to dispose of cleaning materials.
- Contact time is a critical and necessary part of the instructions. Post the instructions in the bench area for easy reference.
- Routinely clean environmental surfaces before setting up work areas and again before leaving work areas.
- Clean any item (e.g., timer, pen, telephone, and thermometer) touched with used gloves.
- Do not use alcohol or alcohol-based solutions alone to disinfect surface areas. These evaporate readily, which substantially decreases efficacy. Use disinfectants recommended for environmental surfaces, such as Environmental Protection Agency (EPA) registered disinfectants use a 1:10 dilution of household bleach. For the detail refer to https://www.ehs.washington.edu/about/latest-news/trouble-uv-light-your-biosafety-cabinet.

2.6. Learning approach/Methods

Teaching approaches/methods in laboratory diagnosis encompass

- Using laboratory animals as a model

Laboratory animals are used for researching the efficacy and safety of new medicinal products, testing biological and chemical substances, and developing knowledge about human and animal biological processes.

- By conducting different testes
- Demonstrations; Demonstrations are mostly designed to illustrate and consolidate theoretical principles outlined in lectures.
- There are three basic types of learning systems:
  - ✓ Behaviorist

    Behaviourist: is a systematic approach to understanding the behaviour of humans and animals. It assumes that behaviour is either a reflex evoked by the pairing of certain antecedent stimuli in the environment
  - ✓ Intellectual constructivist

    It is based on the idea that people actively construct or make their own knowledge, and that reality is determined by your experiences as a learner. Basically, learners use their previous knowledge as a foundation and build on it with new things that they learn
  - ✓ Social constructivist.

    Social constructivism focuses on the collaborative nature of learning. Knowledge develops from how people interact with each other, their culture, and society at large. Students rely on others to help create their building blocks, and learning from others helps them construct their own knowledge and reality.
- Practical exercises.
- Using projects.
- Clarifying the objectives.
- Precise Instructions.

2.6.1. Description of laboratory ethics

Several ethical issues exist within the diagnostic laboratory. The major ethical challenges include: permission, confidentiality, codes of conduct, conflict of interest, lab utilization, proficiency, and direct access testing are sometimes more prevalent in resource-limited settings.

2.6.2. Laboratory precautions

The safety issues in the laboratory during lab practices include:

- No food or drink in the laboratory.
- Wearing Proper Laboratory clothing is mandatory.
- Good Hygiene is mandatory.
- Use Proper Storage Containers.
- Label your work Space and apparatus.
- Don't work alone in the laboratory.
- Stay focused and aware of your surroundings.
- Participate in safety exercises.

2.6.3. Demonstration of laboratory equipment

Some of the laboratory equipment includes Microscope, Centrifuge, Autoclave, incubator, Hot air oven, Analytical balance, Water, bath, Spectrophotometer, Distiller, and others.

2.6.4. Practicing how to use laboratory equipment

Laboratory equipment can have dangerous side effects if not handled properly. Something as simple as a Bunsen burner can light surrounding objects on fire, cause property damage and potentially harm fellow lab inhabitants if it is handled incorrectly or carelessly.

2.6.5. Sample collection and transportation

Specimens may be transported through safely secured tubes. The tubes include blood culture bottles, Vacutainer tubes, and swabs. Specimens should be in tightly sealed, leak-proofed containers and transported in sealable, leak-proof plastic bags.

2.6.6. Sample reception and processing

- The nature of the sample should be checked.
- Samples should be labelled correctly.
- Sample containers should be placed sequentially.
- Proper container should be used for sample collection.
- Finally identical number (ID) should be given to the sample at the reception site and delivered to the laboratory workers.

2. 7. Measurement of the courses

2.7.1. Trainee's assessment

- Technical and manipulative skills in using laboratory equipment, tools, reagents, and computer software.
- Un understanding of laboratory procedures, including health and safety, and scientific methods.
- A deeper understanding of abstract concepts and theories gained by experiencing and visualizing them as reliable phenomena.
- The skills of scientific inquiry and problem-solving, including:
  - ✓ Recognizing and defining a problem.
  - ✓ Formulating hypotheses.
  - ✓ Designing experiments.
  - ✓ Collecting data through observation and/or experimentation.
  - ✓ Interpreting result.
  - ✓ Testing hypotheses.
  - ✓ Drawing conclusions.
  - ✓ Communicating processes, outcomes, and their implications.
- The complementary skills of collaborative learning and teamwork in laboratory settings.
- Understanding, and being prepared for, future possible roles in laboratory-based work.

2.7.2. Conducting interviews and monitoring behaviour

Stages of an Interview

- Introductions. One of the most important steps in the interview process just so happens to be the first.
- Small talk. After introductions are finished, it is a good idea to conduct a bit of small talk with the candidate.
- Information gathering.
- Question/Answer.
- Wrapping up.

2. 8. Required reading materials

- ❖ Nottinghamam.ac.U/vet/cpd.
- ❖ National veterinary Institute Manuals and Standard operating procedures (SOP).
- ❖ www.rcvs.org.uk/lifelong-learning/continuing-professional-development-cpd/.

- Guidance on Continuing Professional Development (CPD) for veterinary surgeons.
- Veterinary continuing professional development in Ethiopia framework and curriculum development: Revised consultancy report to ILRI/EVA, Addis Ababa, Ethiopia
- Guidelines for Animal Disease Control, World Organization for Animal Health (OIE), 2014.
- Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018, Volume 1, eighth edition, URL: https://www.oie.int/standard-setting/terrestrial-manual/.
- Livestock diseases outbreak investigation guidelines, MoA, 2010.

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Acronyms

LPCB=Lacto phenol cotton blue
VTM= Viral transport medium
CPD=Continuous Professional Development
CPE=Cythopatc Effect
hpi= hours post-infection
NAAT= Nucleic acid amplification tests
PCR=Polymerase Chain Reaction
MIC= Minimum inhibitory concentration
MBC= Minimum bactericidal concentration
BCM= Buffy coat method
DNA=Deoxy Ribo Nucleic acid
RT–PCR= Reverse-Transcriptase Polymerase Chain Reaction
cDNA= Complementary Deoxy Ribo Nucleic Acid
IgM=Immunoglobulin M
IgA= Immunoglobulin A
IgG= Immunoglobulin G
HA= Haemaglutinattion
RBCs= Red blood cells
NDV= Newcastle disease virus
PBS=Phosphate Buffered Saline
HI= Haemaglutinattion Inhibition
RBPT= Rose Bengal Plate Test
SAT= Standard Agglutination Test
SNT= Serum Neutralization Test
LAT= Latex Agglutination Test
CFT= Complement Fixation Test
ELISA=Enzyme-linked immunosorbent assay
CDC= Centers for Disease Control and Prevention
BSL= Biosafety Level
BSC= Biological Safety Cabinet
PPE= Personal protective equipment
IAEA= International Atomic Energy Agency
FAO= Food and Agriculture Organization