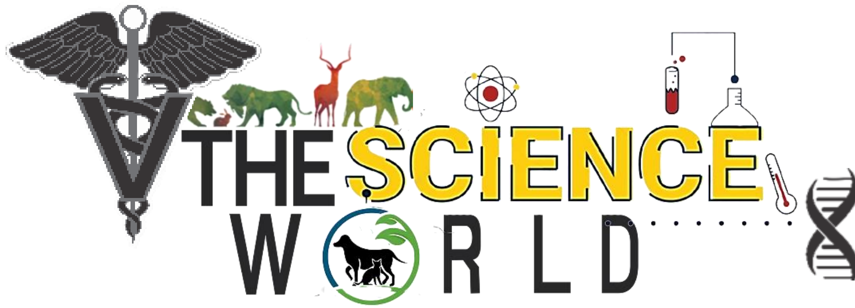

Popular Article

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Collection, Preservation, Identification and Diagnosis of Veterinary Importance Protozoa

Indu Yadav[1*], Dushyant Yadav[2]

[1]PhD Scholar, Division of Veterinary Parasitology, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P.

[2]Asstt. Prof., Department of Livestock Farm Complex, Bihar Veterinary College, BASU, Patna.

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Protozoa require different techniques for their identification than helminth parasites. Due to lack of detailed identifications, our understanding has hampered. For an exact, reliable determination of protozoans, a thorough record of the life stages must be made. In most cases, this includes the use of both fresh smears and stained material.

Stool Specimens - Specimen Collection

Collect the stool in a dry, clean, leakproof container. Make sure no urine, water, soil or other material gets in the container.

1. The image on the right demonstrates the distribution of protozoa in relation to stool consistency and should be taken into consideration when specimens are received.
2. Fresh stool should be examined, processed, or preserved immediately. An exception is specimens kept under refrigeration when preservatives are not available; these specimens are suitable for antigen testing only.
3. Preserve the specimen as soon as possible. If using a commercial collection kit, follow the kit's instructions. If kits are not available, the specimen should be divided and stored in two different preservatives, 10% formalin and PVA (polyvinyl-alcohol), using suitable containers. Add one volume of the stool specimen to three volumes of the preservative.
4. Ensure that the specimen is mixed well with the preservative. Formed stool needs to be well broken up

5. Ensure that the specimen containers are sealed well. Reinforce with parafilm or other suitable material. Insert the container in a plastic bag.
6. Certain drugs and compounds will render the stool specimens unsatisfactory for examination. The specimens should be collected before these substances are administered, or collection must be delayed until after the effects have passed. Such substances include: antacids, kaolin, mineral oil and other oily materials, non-absorbable antidiarrheal preparations, barium or bismuth (7-10 days needed for clearance of effects), antimicrobial agents (2-3 weeks), and gallbladder dyes (3 weeks).

Specimen collection may need to be repeated if the first examination is negative. If possible, three specimens passed at intervals of 2-3 days should be examined.

Blood sample collection for Haemoprotozoa

Blood was collected from jugular vein with anticoagulant EDTA. The blood samples were used to prepare thin blood smears for microscopic examination and the remaining was stored at -20° C until performing DNA extraction for PCR analysis.

Preservation of specimens

Necessary when stool specimens cannot be examined within the prescribed time interval. Various preservatives are available, with the two most commonly used being 10% aqueous formalin and PVA (polyvinyl-alcohol).

Stool Preservation

While visualization of motility in unpreserved specimens may facilitate diagnosis, this technique is impractical for most laboratories, as transport of fresh stool to the laboratory for testing is rarely within the requisite time frame for examination (i.e., 30 to 60 min). A variety of stool fixatives have been developed and modified in recent decades for use with traditional microscopic examination. Those that remain widely used and commercially available include formalin, sodium acetate-acetic acid-formalin (SAF), Schaudinn's fluid, polyvinyl alcohol-containing fixatives (mercury, copper, or zinc based), and mercury-free/formalin-free fixatives. A two-vial collection system, consisting of one vial containing 5 to 10% buffered formalin for use in concentrated wet mounts and a second vial containing a polyvinyl alcohol-based preservative for permanent stained smears, is considered the “gold standard.” However, concern over working with toxic formalin in the laboratory and the environmental impact and disposal costs associated with the use of mercury-based fixatives have led many to consider alternate preservatives and single-tube collection systems (*Pietrzack Johnston sm et al,2000)*. SAF may be used to achieve this goal, if coupled with iron hematoxylin for the permanent

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stained smear; however, for laboratories desiring to maintain the trichrome stain, SAF is not a valid option, as poor-quality results have been documented.

Alternative stool preservatives

Zinc- and copper-based polyvinyl alcohol (PVA) formulations have been developed and are commercially available to replace the mercury-based fixatives ,zinc sulfate-PVA versus mercuric chloride-PVA with trichrome stain(*Garcia LS et al,2006)*

Identification

In vivo observations are indispensable for identifying members of most groups, since fixation induces an uncontrollable degree of shrinkage, distorts the shape of most protozoa, and makes some important diagnostic characters unrecognizable. Measurement of myxosporean and microsporidian spores, as well as coccidian oocysts, should always be done using fresh material. Certain protozoa, such as flagellates and ciliates, have characteristic motility, which is readily observed and can be recorded on video tape, or in digital format. For flagellates, ciliates and opalinids, Protargol (silver protein) staining is essential to visualize the arrangements of flagella, cilia, and nuclei. In the mobile peritrich ciliates, (the trichodinids), a Klein's silver nitrate stain is used to demonstrate the components of the adhesive disc. Electron microscopy is a valuable tool for characterization of protozoa, with scanning EM being used to visualize surface features, and transmission EM being used to visualize internal ultrastructural features. In some taxa, such as the microsporidia, TEM is essential for determination of genus; in many taxa, such as the diplomonad flagellates, EM is essential to distinguish species within a genus.

Protozoa	**Stain**	**Features**
flagellates	Gomori's methenamine	nuclei
ciliates	silver	opalinids
Apicomplexa	Acid-fast	mature oocysts of cryptosporidium and myxosporeans
	Giemsa	polar capsules and spores
	Ziehl Nielsen acid fast	polar capsules and spores

GIARDIA- is suspected but not detected in stool, duodenal specimens, such as those collected by a string test, may be used for permanent stains and concentrated wet mounts. Tear drop-shaped trophozoites range from 10 to 20 µm in length, 9 to 12 µm in width, and contain two

nuclei, a sucking disk, 4 pairs of flagella, 2 axonemes, and 2 median bodies. Cysts contain 4 nuclei, 4 axonemes, and 4 median bodies and range from 11 to 14 µm in length and 7 to 10 µm in width (*Feng Y et al,2011).*

CRYPTOSPORIDIUM- Upon MAF staining, Cryptosporidium spp. oocysts appear as bright red spheres (4 to 6 µm) containing four crescent-shaped sporozoites (which may or may not be seen in all oocysts). Additionally, oocysts may also occlude stain, resulting in transparent “ghost” cells (*Garcia LS,1997).*

ENTAMOEBA HISTOLYTICA- in nonendemic areas are still primarily diagnosed via microscopy on the permanent stained smear. Organisms may be accompanied by clubbed RBCs in cases of dysentery. On the permanent stained stool smear, E. histolytica trophozoites are 12 to 60 µm in diameter and contain a single, well-defined nucleus. Spherical cysts measure 12 to 15 µm, contain 2 to 4 nuclei, and occasionally have cigar-shaped, cytoplasmic chromatoidal bars. Nuclei of both forms are surrounded by an obvious nuclear membrane, a compact, central karyosome, and evenly distributed peripheral chromatin (*Fotedar R et al,2007).*

BALANTIDIUM COLI- is the largest infectious intestinal protozoan, at 50 to 100 µm in length and 40 to 70 µm in width. Trophozoites have fine, visible cilia and a large, kidney-bean-shaped macronucleus. A single, polar cystosome, or oral groove, can also be detected on some cells. The cyst form also has a visible macronucleus, but is smaller (50 to 70 µm long, 40 to 60 µm wide) and rounder than the trophozoites. Cysts have a thick cyst wall and often do not have visible cilia. While molecular or serologic-based diagnostics might improve detection sensitivity compared to microscopic diagnosis (*Schuster FL,2008).*

BABESIA- On the basis of morphology, babesias are divided into two groups – small babesias (1.0–2.5 µm long) which included B. bovis, Babesia gibsoni, B. microti, Babesia rodhaini, etc., and large babesias (2.5–5.0 µm long) which included B. bigemina, Babesia caballi, Babesia Canis, etc., The orientation of the parasite in the red blood cells (RBCs) depends on its size because large pyriform parasites meet at their pointed ends at an acute angle to each other and small forms make an obtuse angle to each other(*Ruprah NS,1985).*

CATTLE AND BUFFALOES

- B. bigemina - large form (4.5 µm × 2.0 µm) of Babesia. The parasites are characteristically pear shaped. Round (2–3 µm in diameter) oval or irregularly shaped form may also be found.

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- B. bovis - small form (2.0 µm × 1.5 µm) of Babesia. Slightly larger than B. divergens, vacuolated signet ring forms are particularly common (*Soulsby EJ,1986)*.
- B. divergens - small form (1.5 µm × 0.4 µm) of Babesia. Generally remained as paired form, superficially lie on the RBC, stout and pyriform or circular forms may be found.
- B. major - large form (3.2 µm × 1.5 µm) of Babesia. Pyriform bodies, the angle between the organism is <90°. Round forms with a diameter of about 1.8 µm are also available.

CANINE

Canine babesiosis is caused by two species of Babesia viz; B. canis and B. gibsoni, which are morphologically differentiated on the basis of their size.

B. canis - large form (4–5 µm long) of Babesia. Pyriform in shape, pointed one end, and round other. In a single RBC, multiple infection that is, more than one organism up to 16 may be found.

Stool Specimens - Molecular Diagnosis

1. 1.Microscopic examination is still considered the “gold standard” for the diagnosis of parasitic diseases. If an unequivocal identification of the parasite can not be made, the stool specimen can be analyzed using molecular techniques such as polymerase chain reaction (PCR). PCR amplified fragments can be analyzed by using restriction fragment length polymorphisms (RFLP) or DNA sequencing if further characterization is needed.
2. 2.If PCR is being requested on a stool specimen, the specimen must be collected in a preservative that is compatible with molecular detection. Fixatives/preservatives with acceptable performance for PCR include TotalFix, Unifix, modified PVA (Zn- or Cu-based), and Ecofix. Stool specimens in these preservatives can be stored and shipped at room temperature. Alternatively, stool specimens can be collected in a clean vial and kept unpreserved; however, these specimens must be stored cold or frozen and shipped either refrigerated (4°C) or frozen (shipped with dry ice). Trichrome stained smears (for E. histolytica/E. dispar) or acid-fast smears (for C. parvum or C. cayetanensis) should accompany the stool specimen when requesting PCR for any of these protozoa. All stained smears will be read first and if an identification of the parasite can be made, PCR will not be performedFixatives/preservatives that are not recommended for molecular detection include formalin, SAF, LV-PVA, and Protofix. For specific applications or when commercial fixatives are not an option, the stool can be mixed in

potassium dichromate 2.5% (1:1 dilution) or in absolute ethanol (1:1 dilution) and shipped refrigerated. Loop-Mediated Isothermal Amplification (LAMP).

The Luminex xTAG gastrointestinal pathogen panel has received FDA approval and can simultaneously detect 14 enteropathogens, including Giardia and Cryptosporidium spp. This assay is the first molecular method approved by the FDA for the detection of pathogenic protozoa (*Navidad J et al f,2013)*.

DIAGNOSIS OF HAEMOPROTOZOA

Different techniques have been implemented to diagnose bovine babesiosis, usually the first choice is the thin or thick blood Giemsa-stained smear to microscopically demonstrate the presence of parasites as etiology of the clinical symptoms. To probe cattle exposure to Babesia or the passive transfer of antibodies by colostrum in calves, serological assays have been utilized, such as the Indirect Fluorescent Antibody Test (IFAT), Enzyme-Linked Immunosorbent Assay (ELISA) and Immunochromatography Test (ICT), as these tests provide information on the humoral immune response. LAMP is a commonly used technique, it is a rapid, cheap, highly sensitive and specific assay, which relies on the auto-cycling strand displacement synthesis of target DNA by Bst DNA polymerase under isothermal conditions. This assay allows detection and discriminates between B. bovis and B. bigemina species. The analytical sensitivity of LAM is 0.1 pg DNA for both species' assays. A multiplex loop-mediated isothermal amplification (mLAMP) was developed; in this assay, primers for rhoptry-associated protein-1 genes of B. bovis and B. bigemina mprovements made for to this method, include the avoidance of gel electrophoresis for visualization of LAMP products, as the chromatographic lateral flow dipstick (LAMP-LFP) format has been applied to reveal products in a simpler and faster way. LAMP-LFP included a set of four primers targeting and amplifying six distinct regions of the *Theileria* sp. cytochrome b gene under isothermal conditions.

Parasite detection in infected animals having low parasitemia can also be made by xenodiagnoses.

- Severe Combined Immunodeficiency (SCID) Mice
- An interesting SCID mice model harboring circulating bovine red blood cells has been used for Babesia parasites isolation.
- Conventional PCR
- DNA Extraction
- Real-Time PCR
- PCR Analysis
- Sub inoculation

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