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REVIEW ARTICLE

Uncovering Strategies for the Detection of Babesia Species

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ABSTRACT

Babesia belongs to the phylum Apicomplexa and its order is Piroplasmida. Babesia mainly spread through Ixodid ticks where the sexual stages of its life cycle are completed by sporogony. It is a Protozoan parasite that can infect the red blood cells of vertebrate hosts including man and animals. Sporozoites of Babesia multiply in the saliva of ticks during the blood meal and infect RBCs, where they replicate asexually and develop into merozoites. There are a variety of ways for the detection of Babesia infection i.e. blood smear (detection under the microscope), serology (detection of antibodies against *Babesia* spp. in plasma or serum), and molecular detection (DNA isolation including PCR) and animal inoculation method. Modern molecular studies involving gene sequencing and phylogenetic analysis are required for diversity detection among Babesia species. However, phylogenetic analysis has been done for a variety of pathogens but needs much improvement. Newer immunological methods are more reliable and more precise than conventional approaches.

Key words: Babesia, Analysis, PCR, Culture.

INTRODUCTION

Babesia (*B.*) belongs to the phylum Apicomplexa and its order is Piroplasmida. It is a tick-borne protozoan disease that can infect erythrocytes of vertebrate hosts of animals and humans. Mainly *B. bovis*, *B. bigemina*, *B. equine*, *B. canis*, *B. gibsoni*, *B. microti,* and *B. divergens* have been involved in bovine babesiosis, equine babesiosis, canine babesiosis, and human babesiosis, respectively (Table. 01). More than 100 species of Babesia have been recognized and spread by different tick vectors. Babesiosis is characterized by respiratory distress, severe hemolysis, intravascular clotting, kidney dysfunction, liver damage, heart attack, and mortality. Trans-placental transmission has also been described (Villatoro & Karp, 2019; Atif et al., 2021).

Firstly, Babesia was recognized in 1957. An animal owner who had a history of visiting a tick-infested pasture suffered from high body temperature, loss of blood in the body, and hemoglobinuria. His death occurred due to renal failure after the second week of the disease. *B. microti* is a very common cause of babesiosis in humans. *Ixodes (I.) scapularis is the main vector of babesiosis* (Bonnet et al., 2014; Atif et al., 2022). Babesiosis and its association with disease

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Table 1: Distribution of important Babesia species and their vectors

Species	Vector	Distribution	Special Features	References
B. microti	*Ixodes scapularis*	USA	Rodents	(Bussieras & Chermette, 1992)
B. gibsoni	*Haemopysalis longicornis*	Global (Asia, Australia, Africa)	Anemia	(Ueti et al., 2003)
B. conradae	*Rhipicephalus (R.) sanguineous*	America (Western USA)	Harmful in dogs	(Chae et al., 1998)
B. bigemina	*Boophilus annulatus*	Southern Europe, Central America	Cattle and Buffalo	(Edwards et al., 2009)
B. trautmanni	*Rhipicephalus* spp.	Former USSR	Pig	(El-Sayed et al., 2023)
B. *bovis*	*Boophilus microplus*	Spain, UK, Africa	Cattle	(Galon et al., 2022)
B. vogeli	*R. sanguineous*	Tropic, sub-tropic	Colder zones	(Gubbels et al., 1999)
B. canis	*Dermacenter reticulatus*	Europe	Haemolytic anemia	(Halos et al., 2004)
B. rossi	*Haemaphysalis elliptica*	South Africa	Haemolysis	(Herwaldt et al., 2003)
B. cabelli	*Dermacenter reticulatus*	Croatia	Molecular detection only	(Galon et al., 2022)

severity, spread, and therapeutic effectiveness are linked with its causal factors (Boom et al., 1990; Bussieras & Chermette, 1992). This review focuses on the methods currently used for the diagnostic and epidemiological studies of human and animal babesiosis. Consequently, the findings of clinical babesiosis are based on diagnostic testing.

SYMPTOMS OF BABESIA SPECIES

Symptoms caused by Babesia vary from species to species. Thus, babesiosis has been categorized as bovine Babesiosis which includes symptoms such as fever, sudden decrease in the number of RBCs, lack of coordination, and teeth crushing (Halos et al., 2004; Fatima et al., 2019; Zaman et al., 2022). When the nervous signs of cerebral babesiosis progress, it leads to death. Dark-colored urine is seen along with anorexia (Fig. 01). In equine babesiosis severe clinical signs occur such as high body temperature, unable to eat, anxiety, anemia, icterus, hemoglobinuria, and colic. Chronic babesia can be typically seen after an acute phase, clinical signs are not specific and include loss of condition (Bussieras & Chermette 1992; Zaman et al., 2022). There are very less chances of gastro-enteritis, bronchopneumonia, and abortions. In ovine and caprine Babesiosis chronic form, the affected animal shows fever, anemia, hemoglobinuria, icterus, and 30-40% of the morbid animals usually expire. The infected sheep are asymptomatic (Holman et al., 1998). Furthermore, canine babesiosis includes a decrease in feed intake, high body temperature, pale gums and tongue, orange or red-colored urine, pale stool, decrease in the body weight, distended lymph nodes, an inflamed spleen, yellowing of the eyes and skin. Infection can affect many parts of the body along-with lungs, GI tract, kidneys, and nervous system (Atif et al., 2021; Aslam et al., 2023). In cats, the signs of feline babesiosis are anorexia, weakness, and an uneven hair coat. However, in dogs, fever and icterus are infrequent. Human babesiosis includes running nose, high body temperature, cold, sweats, head and body pains, lack of appetite, nausea, or fatigue (Holman et al., 1994; Chae et al., 1998).

DETECTION THROUGH MICROSCOPE

Isolation of Piriform Bodies

Examination for the identification of parasites in the thin blood film of red blood cells is the most used procedure. The blood smears are stained with Wright's or Giemsa stain (Holman et al., 1993). Though detection of babesia in a blood smear is an easy and cheap method that is present in almost all laboratories, and it is feasible in the farms to check severe cases. For preparing the blood smear slide, the slides are made as thin smears of blood and are dried in the air, then fixed with the help of absolute methanol for 2 min, and staining is performed (Edwards et al., 2009; Javeed et al., 2022). Some stains like Romanowsky, Diff-Quik, and Wright's stains can also be used for staining purposes. We can also check sensitivity through a thick smear of blood (Krause, 2019; El-Sayed et al., 2023). Many Babesia spp. stick within capillaries and tissues of the body, like *B. bovis* are more in the blood (the blood which is taken from the tips of the body) or from different body organs, for example, the head (specifically, the gray matter of the brain), spleen, liver, or kidneys (Kjemtrup et al., 2006). If we look at human infections, it is difficult to differentiate between Babesia species and plasmodium in the blood (Meredith et al., 2021; Galon et al., 2022; Holman et al., 1993; Kjemtrup & Conrad, 2000).

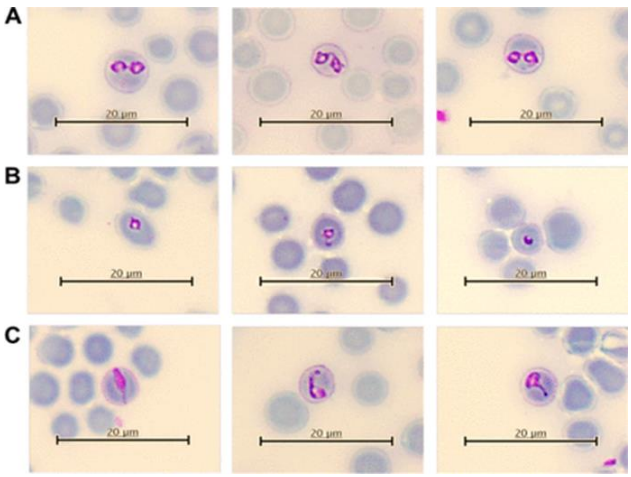

Fig. 1: Morphology of Babesia parasite in Giemsa stained smear under light microscope. Paired pyriform (A), ring form (B), Elongated and irregular form (C) (Morrison 2015)

Advantage

Microscopic detection is advantageous because we can detect the number of parasites along with the ratio of RBCs that are infected with parasites (Schnittger et al., 2012; Krause et al., 1994; Hurtado, 2015).

Disadvantage

The results of microscopy are important until laboratories complete tests. In routine, this test is not performed by a laboratorian. It is possible that it cannot uphold optimal proficiency (Schnittger et al., 2004; Javeed et al., 2022).

Limitation

Limitations involve the use of manual diluting pipettes, necessary to prepare diluting solutions and to distinguish between small lymphocytes and platelets. Due to limitations on attaining total cell counts in blood, especially leukocyte counts, the evaluation of cell morphologic features is an important part of the assessment of the hemogram (Katzer et al., 2006; Krause, 2019; Scott et al., 2021).

Identification of Babesia at Molecular Level

Preservation of Samples

For the detection of Babesia species at the molecular level in animal and human hosts, samples should be taken from the spleen and veins. There should be proper storage of samples from -20 degrees Celsius and they should not be preserved for more than 72 hours. 70% ethanol can also be used for the preservation of spleen samples (Tretina et al., 2015; Meredith et al., 2021). In field studies, blood samples could be preserved on FTA cards, which permits transportation smoothly with maximum storage (Rahikainen et al., 2016). We can preserve ticks in 70% ethanol and for long-term usage we can store and freeze them at -20℃ or -80℃ (Ueti et al., 2003; Morrison, 2015).

Separation of Genomic Material and Protein

Before starting the DNA extraction, hold the scalpel blade into your hand and cut the tick into two longitudinal halves. Separate both the sections of ticks; one section of the tick sample will be used promptly and the other half can be saved for later use (Uilenberg et al., 2006). The length-wise cutting is recommended for fully fed ticks (Morrison, 2015; Yasin et al., 2023). We can separate salivary glands of ticks, ovaries and mid-gut by cutting tick into two halves. Gut is present as dark red, spider-shaped. When we remove gut and ovaries there is a folded U-shaped arrangement at the lower side of sac in rectum (Edwards et al., 2009). Before separation, tissue extraction can be done with the help of Qiagen DNA extraction kit. Although many technologies have been developed for DNA extraction from proteinase K but through the use of commercial DNA extraction kit we can separate whole DNA from blood or tissue very easily (Boom et al., 1990). After extraction DNA samples can be preserved at 4°C for short period or -20°C for long term preservation until needed. Separated DNA can be checked through gel electrophoresis, spectrophotometry, and fractional amplification of the gene (18S, b-actin, ITS etc.) (Vega et al., 1985; Morrison, 2015).

Polymerase Chain Reaction

Variety of polymerase chain reaction separation techniques are designated to uncover Babesial parasite species in vertebrate as well as tick hosts. PCR gives more accurate results as compared to other microscopic methods (Wang et al., 2015). Different types of techniques are developed to check the status of different piroplasm species (Herwaldt et al., 2003, Hunfeld et al., 2008, Bhoora et al., 2009). Different types of PCRs such as q PCR, nested PCR, real time PCR and multiplex pcr techniques can be applied for the detection of pathogen.

Nucleic Acid Amplification and Sequencing (NAAS)

NAAS is developed for the detection of foreign particles in the body. It gives an accuracy for certain viruses and pathogens present in an organism. This NAAS or nucleic acid amplification test varies according to variety of tests (Wilson et al., 2015; Rahikainen et al., 2016). The identification of nucleic acid permits the initial detection of pathogens. The quantity of many nucleic acids is frequently very minute, nucleic acid amplification test gives increased wavelength results (Rahikainen et al., 2016). There are several ways to increase or decrease the wavelength some of them are; polymerase chain reaction (PCR), strand displacement assay (SDA), or transcription mediated assay (TMA). Pathogens that makes DNA base with diversity of human disorders are frequently somewhat unlike as compared to ordinary genome. Recurrently, they can only be altered in one base, for example, additions, deletions, and single-nucleotide polymorphisms (SNPs) (Wilson et al., 2015). Defective probe-target can occur which results into negative results or sometimes it can show some false positive results. The confirmation of novel strains of *Babesia* spp. causing human infections have also been possible due to the genome sequencing approaches used for phylogenetic analysis (Zahle et al., 2000; Rahikainen et al., 2016).

The *B. duncani* was discovered from a patient in Washington, and the patient expresses malaria-like illness. Microscopic examination showed that the morphology is similar to *B. microti*. *B. duncani* was formerly labeled as WA-1 strain. For *B. duncani* the phylogenetic study revealed it to be distinct from *B. duncani* (WA-1) and *B. gibsoni*. While *B. microti*, WA-1, and WA-like were the species identified for causing human infections in the Unites States, a new strain from Missouri not reacting with any of these antigens in IFA but phylogenetically related to *B. divergens* was

reported. This new strain is now referred as "*B. divergens*-like" (previously MO-1). Similarly, "*B. microti*-like" strains were described from Japan and Taiwan, and another novel strain reported from Korea, labeled as KO-1 strain.

Multiplex PCR Amplification

There is a gene known as dystrophin gene. First time in history in 1988 a process was developed to check changes in that gene and the process is now known as multiplex-PCR. It has also been taken with the steroid sulfatase gene. In 2008, multiplex-PCR was brought for enquiry of microsatellites and SNPs (Chae et al., 1998; Schnittger et al., 2012). In multiplex PCR many PCR primers are used (for example, variety of polymerase chain reactions, are composed of a single preparation). The said procedure heat up DNA in blood or tissue by using a number of primers and a thermal cycler machine is used to heat the DNA (Boom et al., 1990; Rahikainen et al., 2016).

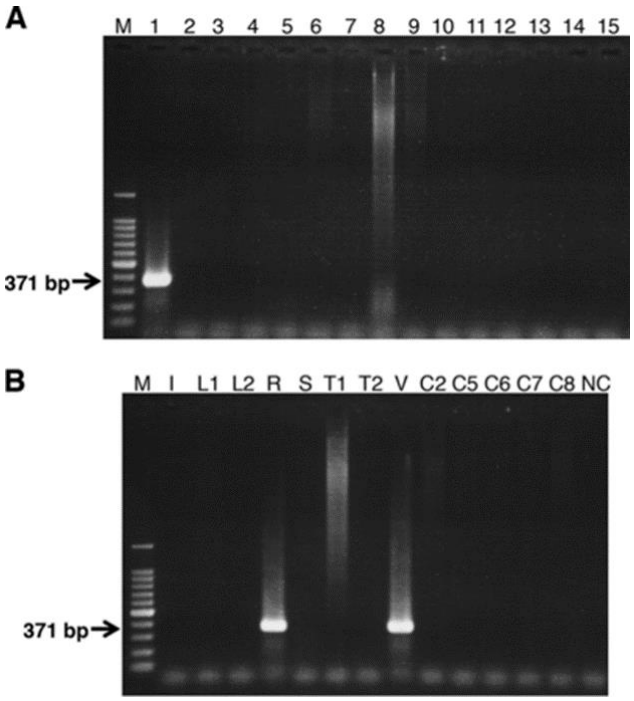

Fig. 2: PCR assay of Babesia specie (Edwards et al. 2009)

Multiplex-PCR uses different groups of primers into one set of polymerase chain reactions and make them of variable lengths which varies as dissimilar genomic types. To work suitably in one reaction there is need to increase strengthening temperatures of each primer for example to make different variable bands during gel process we should increase the length of different base pair. On other side, the amplicons of large size can be seen through fluorescent dyes. There are different salable preparations for PCR available in the market. These kits are taken for forensic purposes by different labs to increase degraded genomic matrix (Schnittger et al., 2012; El-Sayed et al., 2023).

Multiplex PCR for babesia species is done into liquid encompassing 5 µl of extracted DNA and 45 µl of 0.4 pmol for every single primer, 300 µ M of dNTP (QIAGEN Germany), 4 units of Hot Star Taq DNA Polymerase (QIAGEN Germany), 1xPCR buffer (20 mM Tris–Hydrochloric acid) pH 8.4, 50 mM potassium chloride, 1.5 mM $MgCl_2$ and nuclease-free water. The whole procedure is done in a thermercycler (PTC-200, MJ Research, Water Town, MA). The band length is disconnected by electrophoresis in 2.5% agarose gel in 40 mM Tris-acetic acetate pH 8.4, 1 mM EDTA that can be visualized with ethidium bromide (0.5 µ g / ml) and observed under UV light. Table. 2 (Scott et al., 2021).

Advantages

Multiplex PCR is more effective (less dNTPs, enzymes, and other consumables), time saver, less chemical material is required. In this procedure less pipetting errors occur, which can lead to less contamination during sample handling (Scott et al., 2021).

Disadvantages

The main disadvantage is the setting of the annealing temperature which is difficult to set for the required samples (Holman et al., 1994; Sunaga et al., 2002).

Loop-mediated Isothermal Amplification (LAMP)

For differentiation of different babesia species, a Loop-Mediated Isothermal Amplification (LAMP) stage which shows specific arrangements in a gene called as ITS gene. The sensitivity of LAMP process is roughly 0.1pg DNA for both *B. bovis* and B. *bigemina,* that is more with standard polymerase chain reaction. The procedure starts by taking samples from infected cattle. LAMP method is good for identification of Babesia species, principally in those areas where babesiosis is endemic (Timms et al., 1983; Mori et al., 2001).

Advantage

Due to the potential to strengthen genomic material in isothermal conditions in the range of 65°C. This method is simple and rate effective. The amplification of genomic material through LAMP is checked through the naked eye by checking the ty resulting from the precipitate (Tretina et al., 2015; Javeed et al., 2022).

Disadvantage

It is not as much adaptable as PCR. LAMP cannot be used for cloning purposes. Multiplex method for loop-mediated isothermal amplification are less than polymerase chain reactions. Primers are targeted singly in loop-mediated isothermal and the amplification enhances primer-primer exchange (Krause, 2019; Tonnetti et al., 2019).

Table 2: Detection of different pathogens using multiplex PCR

Organisms	Target genes	Primer sequences	Reference
A. marginale	*Msp1b*	CCATCTCGGCCGTATTCCAGCGCA	(Kjemtrup et al., 2006)
B. ovis	*Bbo- F*	*TGGGCAGGACCTTGGTTCTTCT*	(Javeed et al., 2022)
B. motasi	*P1*	CACAGGGAGGTAGTGACAAG	(Meredith et al., 2021)
Theleria spp	*1F*	GTGAAACTGCGAATGGCTCATTAC	(Krause, 2019)
A.centrale	*Msp 2*	TTGTGGCTCTAGTCCCCCGGGGAG	(Kjemtrup et al., 2006)

Serology

The present serological "gold standard" to detect *Babesia microti* antibody is an indirect immunofluorescent antibody (IFA) assay specifically for chronic infections was done firstly, in 1978. Whereas IFA assay adept for checking immunoglobulin M and immunoglobulin G antibodies to *Babesia microti*, is noticeably used to find the latter, which may stay for months to years (Wiegmann et al., 2015; Meredith et al., 2021). Ab are generally seen in animals in which first infection of *Babesia microti is present*. After infection of parasitic disease Ab titers remain high for 1 year of infections.

Summary

Last five decades showed exposure in many ways for the uncovering of Babesia parasites in vertebrate and tick hosts. Sample, research facilities, infrastructure, worker experience and ability to work can help for more precise detection. Blood smear tests are generally performed for detection of pathogens but, serology and molecular tests normally shows greater positivity. For confirming pathogen, PCR is most reliable method followed by sequencing for strain/varient detection. Nevertheless, we would like to ask researchers to consider by use of at least 2 methods, as this will enhance our understanding of the biological characters of many "new" species, based on molecular procedures.

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