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Current scenario and future prospectus in serodiagnosis of *Theileria annulata* in bovines

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Abstract
Tropical bovine theileriosis is an important tick borne haemoprotozoan parasitic disease of ruminants particularly in bovines. *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum* are the two host tick responsible for transmission of this disease trans-stadially in India. It may be highly prevalent disease in upcoming days. Screening of animals for theileriosis by ELISA with various antigens such as crude antigen, recombinant (r) antigens, Tams1, TaSP, Spm2 and COCKTAIL-ELISA were used for diagnosis of theileriosis in bovines with high sensitivity of the test.

Keywords: *Theileria annulata*, tick borne disease, diagnosis, ELISA

Introduction
Tropical bovine theileriosis is an intracellular haemoprotozoan disease of cattle caused by *Theileria annulata.* It is transmitted trans-stadially through the Ixodid tick of the genus *Hyalomma*. The parasite reproduces asexually (schizogony and merogony) in cattle and reproduces sexually (gametogony and sporogony) in vector ticks.
It cause fever, peripheral lymph node enlargement, progressive anaemia. It is a serious bovid disease in India, the Middle East, Asia, North Africa and Southern Europe. The infection primarily impacts exotic and crossed cattle and significantly affects livestock output and breeding programs.
Tropical theileriosis risks roughly 25 crores bovid globally (Bishop *et al*., 2004) [6], comprising (approximately) 3 crores 90 lakh half-blood cattle in India (Kolte *et al*., 2017) [21]. Tropical theileriosis is predicted to cost India US$ 1.295 billion per year in lost revenue (Narladkar, 2018) [29]. Further, the reduction in milk production per cow was also calculated as 2.76 L/day, or 31.92 percent of the overall milk production (Ayadi *et al*., 2017) [2]. Long-term carrier states occur in cattle that overcome the acute infection, resulting in considerable productivity and monetary losses, and the carrier state is critical for parasite transmission.
The most common ways of diagnosing *T. annulata* infection in cattle include microscopy, serological techniques and molecular approaches. Microscopically, nevertheless, distinguishing *Theileria* spp. is challenging. Sensitive and specific molecular techniques including PCR, LAMP, or reverse line blotting (RLB) could be utilised for detecting
T. annulata infection in both the cattle and the ticks. Nonetheless, low quantities of pathogen DNA in carriers (Santos *et al*., 2013) [36] and competitive pressure among primers targeting species-specific conserved sequences over specific genes, namely the 18S rRNA (Edwards and Gibbs, 1994), lowers the assay sensitivity. Serological assays are still the best choice for large-scale investigations aiming at detecting disease carriers and evaluating disease dissemination. It is attributed to the fact that during infection, the cattle immune system is encountered a range of *T. annulata*-antigens (Boulter *et al*., 1998) [7], causing antibodies to be produced that react with distinct stages of the parasite's life cycle. This article would reveal the various antigens used for the serodiagnosis of *T. annulata* in bovines with special reference to ELISA.

Serological detection of *T. annulata* infection in bovines
Serological detection with crude antigens
Serological assays, which monitor the antibody responses of livestock to measure the responsiveness to spontaneous infection as well as immunisation for disease prevention, can be used to dependably detect low-grade or past infections. Different serological assays could be used to identify antibodies, but the IFAT assay, which is approved by the OIE for the majority of economically relevant parasites, including *T. annulata*, remains the gold standard. This

technique was found to be very effective in detecting immune responses to piroplasm and schizont antigens in *T. annulata* infected bovines (Pipano and Cahana, 1968; Burridge, 1971; Burridge and Kimber, 1974, 1973) [30, 9, 8, 10]. For decades, the assay has been used effectively to identify immunoglobulins to *T. annulata* in bovines. It is more sensitive than microscopic inspection of blood smears in the field. Despite this, because of its labour-intensive aspect and the length of time it takes for greater sample screening, it is questionable whether the assay is adequate for statistically valid sero studies. Further, the most serious issue with the IFAT, on the other hand, is the high level of cross-reactivity reported among similar species (Morzaria *et al*., 1993) [27].

ELISA, rather than the IFAT, would be more suitable for epidemiological surveys because of its simplicity of standardization and ease of application, as opposed to the IFAT (Bakheit *et al*., 2007) [4]. ELISA assays incorporating soluble antigens from different stages of the parasite have been developed to identify *T. annulata* infection in bovids. A crude infected erythrocyte extract was utilised by Kachani *et al.* (1992) [16] to detect antibodies in *T. annulata* infected bovids on day thirteen post-infection. Using ELISA, Kachani and colleagues (1992) [16] evaluated the antigenicity of piroplasms, schizonts and sporozoites stages of *T. annulata*. The investigation revealed that the antigen from the piroplasm stage was the best appropriate for use in an ELISA. In addition, it was observed that the schizont or piroplasms antigen-based ELISA was better than IFAT in terms of identifying immunoglobulin response more than six months following immunisation (Kachani *et al*., 1996) [17].

According to Deshpande (1989) [11], when assessing the immune response against *T. annulata* in sera of bovids, the schizont antigen displayed better ELISA reactivity to the piroplasm antigen. Later, Thimma Reddy and co-workers employed schizont antigen-based avidin-biotin ELISA to test the carriers (Thimma Reddy *et al*., 1991) [42]. Later, Ray and Bansal (1993) [32], in a follow-up study, tested the efficiency of freeze-dried and fresh schizont antigen for identifying antibodies against *T. annulata* using an ELISA. According to the observations, the schizont antigen in freeze-dried form has the extra edge of maintaining at four degrees Celsius of prolonged duration without losing its effectiveness. In a cattle experimentally infected with the *T. annulata*, Sundar and co-workers (1993) showed that the immunoglobulins produced by the parasite could be identified for about 42 Weeks, 6 Days utilizing piroplasm infected red blood cell homogenate-based ELISA. *Theileria annulata* immunological responses, as per Bansal and Ray (1998) [5], are stage-specific, and the antibody-mediated response to piroplasm antigen remains significantly longer compared to antigens of schizont/sporozoite stages. A micro ELISA was developed by Dutta and colleagues that recognized antibody responses down to 815 days post-exposure to infection with antigen fractions made from purified piroplasms Dutta *et al*. (2000) [12].

Piroplasm antigen-based Dot-ELISA was established and assessed by Sharma and his associates in 1995 for sero-detection of *T. annulata* infection in experimental bovids. Subsequently, the technology got adapted to detect antibodies in bovines and water buffaloes Soundararajan *et al*. (2000) [39]. Further, the efficacy of the assay was compared with the CFT and revealed that Dot-ELISA was better in diagnosing the theileriosis in animals (Sharma *et al*., 2000) [23].

Serological detection with recombinant (r) antigens

Despite the fact that an ELISA employing native piroplasms/schizont antigen was developed for sero-detection of theilerial infections with differing sensitivity, their standardization has proven to be a difficult task (Manuja *et al*., 2000) [23]. In addition, the conflict of utilizing animal models to serve as continual parasitic suppliers may well be addressed by applying recombinant proteins to serve as detecting antigens in immunoassays. Indirect-ELISAs that use r-proteins to detect *Theileria* infection in animals have been established (Gubbels *et al*., 2000; Bakheit *et al.*, 2004) [15, 3]. An increasing number of epidemiological surveys are now being conducted using these assays.

***Theileria annulata* merozoite surface antigen (Tams1)**

Studies on the TAMS gene revealed that it expresses extremely varied molecules, notably in a domain containing many putative N-linked glycosylation sites, which has been demonstrated through sequence analysis (Katzer *et al*., 1998) [18]. Subsequently, the two allelic forms were expressed as r-proteins in E. coli. Later, Tams1 based ELISA was developed and evaluated utilising four unique r- proteins derived from two distinct Tams1 alleles, all in truncated construct. Further, the Tams1 in the developed assay cross-reacted solely to antisera against *T. parva* (Gubbels *et al*., 2000) [15]. It was also determined that sera acquired 90 days or above the following infection had the highest ELISA response to Tams1. IFAT was more dependable if samples were taken within 90 days of post-infection.

Several studies have shown that Tams1 is a highly varying antigen with inadequate antigenic cross-reactions among them. It was observed that infections caused by *T. annulata* that lack the particular Tams1 allele could be misread by the ELISA developed with a different allele (Katzer *et al*., 1998) [18]. For a Tams1-ELISA test to be effective, a minimum of certain epitopes needs to be conserved among all variants of Tams1 protein (Katzer *et al*., 1998) [18].

Rajendran and Ray developed and deployed a rTams1-ELISA in 2014 for detecting tropical bovine theileriosis, with the ELISA based on soluble piroplasm antigen-ELISA serving as a standard.

***Theileria annulata* surface protein (TaSP)**

Schnittger and colleagues detected the TaSP, a single-copy gene, in 2002, which is only transcribed during the parasite's sporozoite and schizont phases. The gene produces a TaSP, a membrane protein having 315 amino acids with a predicted molecular weight of 36 kDa. The amino and carboxyl portions of the protein are conserved, whereas the central part is variable. Some *Theileria* species, namely the *T. lestoquardi* and *T. parva*, have been reported to contain TaSP homologues (Schnittger *et al*., 2002) [37]. The schizont cytoplasm is confronted by the minor component of the protein, whilst the more significant portion, which contains a variable area, is pointed towards the host's cytoplasm. The amino and carboxyl terminals of the protein are exposed toward the cytoplasmic part of the parasite & host separately. The presentation of the protein draws attention to the capacity of the host cell to process the TaSP antigen before presenting it to the MHC molecules, thereby clearing the route for the development of anti-TaSP antibodies. The polymorphic part of the TaSP antigen contains extremely immunogenic epitopes, and these epitopes are conserved across various strains of *T. annulata*. Consequently, the protein is a better acceptable diagnosing

antigen for tropical theileriosis surveillance in diverse parts of the world (Schnittger *et al*., 2002) [37].

Using the rTaSP protein encoded by the middle polymorphic sequence of the gene, Schnittger and colleagues (2002) [37] developed a rTaSP-ELISA. A positive response in the ELISA was observed with sera of cattle from two diverse locations that had been infected by *T. annulata*. The results of the study showed that epitopes located inside its polymorphic section of *T. annulata* are conserved across a wide range of stocks.

Using the variable domain of rTaSP protein, Bakheit and collaborators (2004) [3] established an indirect ELISA that exhibited 90.5 percent specificity and 99.1 percent sensitivity. The polymorphic domain of the r-antigen was also detected by *T. annulata*-infected bovid sera sampled from various geographical locations.

Mousa and co-workers (2005) [28] reported sequences of the TaSP gene from Sudan to be exceedingly varied, yet it did not affect the ability of r-protein to recognise the sera from cattle infected with different field strains. As part of the validation process, Salih and colleagues (2005b) [35] showed that the rTaSP-ELISA was capable of detecting tropical bovine theileriosis under field settings. The following serosurvey, which included 2661 sera collected from various locations throughout Sudan, demonstrated that *T. annulata* infection was prevalent across the nation. The dispersion of tick vectors was shown to be closely correlated with these findings.

The development and validation of a competitive ELISA utilizing rTaSP protein were later carried out in order to improve the specificity of the test for identifying circulating immunoglobulins (Renneker *et al*., 2008; 2009) [33, 34]. Vanlalhmuaka and colleagues (2010) [44] developed and assessed the rTaSP-ELISA. The standardized assay was reported to be capable of detecting antibodies in four experimentally infected bovid calves from seven to fourteen days after the infection. Furthermore, there was no evidence of cross-reactivity toward other haemoprotozoans being found.

Using rTaSP antigen and colloidal gold, Abdo and colleagues (2010) [1] established a lateral flow apparatus for the sero-detection of *T. annulata* infection. The assay did not cross-react with related haemoparasites. The test makes it possible to check the results with an unsupported eye in 10 min. and is appropriate for usage in the field. Most recently, Mohmad and collaborators (2018) [25] established rTaSP-Dot-ELISA for sero-detecting *T. annulata* infection in bovids. It was determined that the developed assay could be deployed in the field because it was relatively inexpensive and simple to implement.

(Sporozoite and macroschizont antigen) Spm2

Knight and co-workers, in 1998 [20], found and characterised the *T. annulata's* Spm2 protein. Spm2 is a single-copy gene transcribed at different phases, *viz*. piroplasm, schizont and sporozoite, of the parasite's life cycle. Immunoglobulins of all the three life stages of the *Theileria* parasite reacted strongly with the rSpm2 antigen. Tian and associates (2018) [43] demonstrated the usefulness of rSpm2-indirect ELISA for the identification of *T. annulata*, with 98.7 percent sensitivity and 98.4 percent specificity. A bigger segment of the rSpm2 was subsequently expressed by Prabhakaran and associates (2021) [31] to include three hundred sixty-eight amino acids and established rSpm2-ELISA for diagnosing tropical theileriosis that was 100 percent sensitive and 90.9 percent specific.

Cocktail-Elisa

In recent decades, the cocktail-ELISA assay has been developed, and it has shown to be an exceptionally sensitive diagnostic tool (Moendeg *et al*., 2015) [24]. There are several advantages of using a cocktail mix rather than individual antigens. Antigen cocktails are more effective since they require less antigen concentration than individual antigens. In addition to this, no single r-ELISA that included only a particular antigen has yet been demonstrated to be sensitive enough to detect all IgG- or IgM-positive samples tested throughout an infection (El-Sayed *et al*., 2015) [14]. Different justifications have been put forward to explain why this would be the situation. The antibody-mediated immune system reacts in several different ways depending on the stage of infection (Moir *et al.*, 1991) [26]. Consequently, in order to assess antibodies circulating at different stages of the infection, it is necessary to employ several epitopes from various antigens in an ELISA (El-Sayed *et al*., 2015) [14]. Furthermore, the expressing vectors and antigen purification methods used affect the capacity of r-proteins produced in *E. coli* to configure into native epitopes form. Because of this, incorporating two or many r-antigens together will boost the sensitivities of r-ELISAs (El-Sayed *et al*., 2015) [14]. Compared to single-specified antigens, multiple antigens have been demonstrated to increase diagnostic capacity in parasitic diseases such as malaria (Kim *et al*., 2003) [19], schistosomiasis (Moendeg*et al*., 2015) [24], clonorchiasis (Li *et al*., 2011) [22], toxoplasmosis (Velmurugan *et al*., 2008) [45], and equine theileriosis (El-Sayed *et al*., 2015) [14].

Conclusion

Diagnosis of *T. annulata* by the commonly used serological test, ELISA with different antigens were discussed in this review. Among these, cocktail-ELISA could be the one highly recommended by the researchers which is more effective since they require less antigen concentration than individual antigens. Also it is possible that some specific antibodies that are detectable at one stage of the disease will not be detected during another stage, and vice versa. The cocktail ELISA could assess antibodies circulating at different stages of the infection by employing several epitopes from various antigens.

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