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Statistical evaluation of modalities for diagnosis of Anaplasmosis in dairy cattle

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ABSTRACT

Anaplasmosis is an important tick borne rickettsial disease affecting bovines. Disease diagnosis plays an important role in limiting the spread of infection because of persistence of its carrier state. Conventional microscopy of stained blood smear, serological assay and molecular tests are commonly used for the diagnosis of anaplasmosis. The present study was planned to compare different diagnostic methods for detection of *Anaplasma* spp. in blood samples of cattle. A total of 72 blood and serum samples were collected from cattle suspected to be suffering from anaplasmosis. Paired stained blood smear was examined for characteristic dot shaped intraerythrocytic form of organism. Serum samples were processed for competitive ELISA for the presence of antibodies to *Anaplasma* spp. DNA was extracted and subjected to PCR assay for detection of *Anaplasma marginale* targeting *msp4* gene. Out of 72 samples, 91.66% of cases were found to be positive by cELISA, followed by 56.94% by PCR assay and only 25% by microscopy. Microscopy was found to be 100% specific whereas ELISA was found to be 100% sensitive with microscopy showing 100% positive predictive value and ELISA showing 100% negative predictive value. For early and rapid detection, PCR assay should be employed, while for rapid screening of herd and control strategies cELISA may be used but in routine practice, microscopic examination can suffice.

Keywords: Anaplasmosis, cELISA, Microscopy, msp4 protein, msp5 gene, PCR assay

Anaplasmosis is a rickettsial disease caused by an intra-erythrocytic organism transmitted by ticks. The prevalence of anaplasmosis in cattle in India ranges from 5.1 to 38.2%, depending on the region and the season (Patel *et al.* 2019). The disease is clinically characterized by fever, jaundice and anemia. Microscopic examination of stained blood smears looking for the characteristic dot shaped organism inside the RBCs is considered as gold standard test but it suffers limitation of false negativity in cases with low parasitemia and non-detectable carrier state.

For herd screening, serological assay targeting major surface protein has been used in past. A variety of serological tests, including the Complement Fixation Test, Card Agglutination Test, Indirect Fluorescent Antibody test, as well as different types of Enzyme-Linked Immunosorbent Assays (ELISA) such as Competitive Enzyme Linked Immunosorbent Assay (cELISA), indirect ELISA and dot ELISA have been used for epidemiological studies. Their results reflect the collective exposure history indicating from the late acute infection period to convalescence (Schotthoefer *et al.* 2013).

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Molecular diagnosis and prevalence studies of *Anaplasma* have utilized various target genes such as *16S rRNA* by Reinbold *et al.* 2010, major surface proteins (*msp1α, msp1β, msp2, msp3, msp4, and msp5*) by De La Fuente *et al. 2007*, the heat-shock gene *groEL* by Park *et al. 2005*, and the *23S rRNA* by Dahmani *et al.* 2015. Out of above mentioned methods, polymerase chain reaction (PCR) assay is more advantageous due to high sensitivity and effectiveness in diagnosing active and carrier state infection (Ghosh and Nagar, 2014). The present study was planned to compare the three diagnostic modalities i.e microscopic examination, serological test and molecular test for diagnosis of anaplasmosis in cross bred cattle suspected to be suffering from anaplasmosis.

MATERIALS AND METHODS

Samples collection and sample area: A total of 72 blood samples from suspected cattle were collected in EDTA containing vials and serum vials. Among them, twenty one samples were collected from cattle presented to University Veterinary Hospital and suspected for anaplasmosis on the basis of clinical signs while fifty one samples were collected randomly from cattle from various gaushalas for screening of anaplasmosis. Among the samples collected from gaushalas, 20 were collected from Rohtak Pashu Gaushala, Rohtak, 16 from Shree Vileshwar gaushala, Chinder, Agroha and 15 from Shree Ladwa gaushala, Hisar.

Screening of blood samples by conventional microscopic examination for Anaplasma spp.: A small drop of blood was placed on the cleaned and grease free slide. Another slide was used as spreader for making thin blood smears by keeping at 30-45º angle on first glass slide. The smears were labeled and air-dried for 10-15 minutes. The dried blood smears were immersed in absolute methanol for 5 to 10 minutes, completely air-dried, then stained with 10% Giemsa's stain (Himedia Laboratories Pvt. Ltd, Mumbai, India) for 30 minutes followed by rinsing with distilled water and air drying. Blood smears were examined for dot-shaped organisms on the margins of erythrocytes under oil immersion objective (100x) of microscope**.** A single parasitic form found on slide smear was considered as a positive sample.

Serological examination of blood samples for diagnosis of anaplasmosis by competitive ELISA: Blood samples collected in serum vials from the suspected cases were assessed for the presence of antibodies to *Anaplasma* spp. using a commercial competitive ELISA test kit (VMRD, Inc., Pullman, USA) following the manufacturer's instructions. This serological analysis identifies serum antibodies against *msp5* of *Anaplasma* spp.

In plate A coated with antigen, 50 µl of both load controls and serum samples were added. The plate was tapped repeatedly to ensure that the samples had coated the bottom of the wells, and incubated for one hour at room temperature (23 ± 2°C). After incubation, the plate was washed two times with 1X wash solution using a multichannel filling device. The wash solution was then removed from the plate, and the plate was inverted and sharply struck on a clean paper towel. In each well, 50 µl of antibody-peroxidase conjugate, diluted at 1X, was added and incubated for 20 minutes at room temperature (23 ± 2°C). Following the incubation, the plate was washed four times. For each well, 50 µl of substrate solution was added and tapped repeatedly on the side of the assay plate to ensure the substrate had coated the bottom of the wells. The plate was then incubated for 20 minutes at room temperature (23 ± 2°C). To each well, 50 µl of stop solution was added to stop the reaction, and the plate was tapped repeatedly on the side to mix the substrate solution and the stop solution. Immediately after adding the stop solution, the microplate absorbance spectrophotometer's optical density reading was taken at a wavelength of 620 nm. The optical density values of the controls and samples were measured at 620 nm wave length using an automatic UV max kinetic microplate reader (Agilent Biotek, USA) and the percentage of inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = 100 - [1 - (\text{Sample OD}/\text{Negative Control OD})].$$

Serum samples with ≥30% inhibition were considered positive and samples with <30% inhibition were considered negative as recommended by the manufacturer.

Molecular detection of Anaplasma marginale using PCR assay: DNA was extracted from blood samples collected in EDTA vials using QIAamp DNA blood mini kit (Qiagen, Germany). Manufacturer's instruction were followed for extraction of DNA. Positive control and negative control were used in each reaction. Primer set (Forward: 5'-ATCTTTCGACGGCGCTGTG3' /Reverse: 5'ATGTCC TTGTAAGACTCATCAAATAGC-3') from published source (M'ghirbi *et al*. 2016) was used for targeting the *msp4 gene.* A 25 µl PCR reaction mixture was prepared containing 12.5 µl Dream Taq Green PCR Master Mix (2X) (Fermentas, USA), 1 µl of forward and 1 µl reverse primer (10µM concentration), 7.5 µl NFW and 3 µl of DNA extracted from samples to be tested. Each PCR tube was gently vortexed for 10 seconds. PCR amplification was performed using thermocycler (Bio-Rad, USA). Nuclease free water was taken as negative control and PCR mixture without template was taken as PCR control to check the possibility of contamination. PCR product with a sharp band at 420bp was considered as positive.

Statistical analysis: Sample wise results of microscopic examination, ELISA and PCR assay were tabulated. Analysis of degree of agreement between the sample wise results of microscopic examination, ELISA and PCR assay were determined using Kappa Statistics using MedCalc online tool(https://www.medcalc.org/calc/kappa.php). Specificity, sensitivity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value, negative accuracy value and accuracy was calculated, using online tool MedCalc Statistical Software version 22.023 (https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS AND DISCUSSION

Out of 72 blood samples, 91.66% of cases were found to be positive by cELISA (Fig.1), followed by 56.94% by PCR assay (Fig.2) and only 25 % by microscopy (Fig. 3). Results of the present study indicated that cELISA is the most sensitive diagnostic method for the diagnosis of anaplasmosis in cattle as it showed a positivity rate of 91.66% with a cutoff value of 30% inhibition and sensitivity of 100% (Fig. 4). Comparable accuracy was obtained

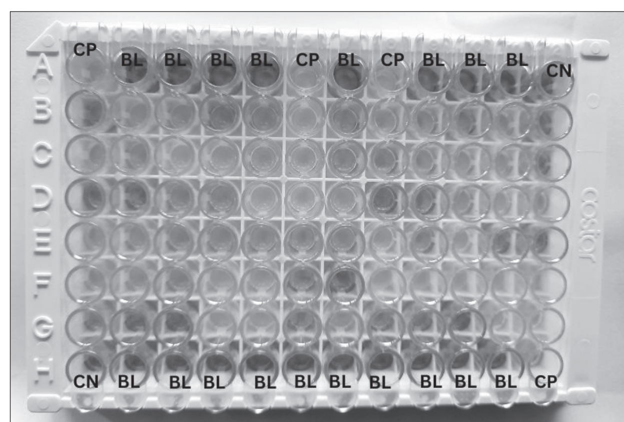

Fig. 1 Result of competitive ELISA showing inhibition of reaction in control positive(CP- Control positive), no inhibition in blank (BL-blank), and variable degree of inhibition in test samples from B1 to G1.

for microscopic examination and cELISA. In contrast to ELISA, the microscopic examination had the lowest sensitivity with a positivity rate of only 25%, which may be attributed to low parasitemia and carrier state. However, microscopic identification is a definitive diagnostic and relationship of clinical signs with microscopic findings can easily help in identification on ongoing infection. The cELISA test is based on the inhibition of monoclonal

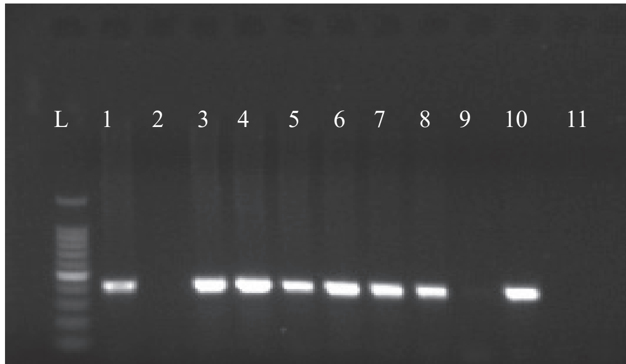

Fig. 2 Result of Agarose gel electrophoresis of PCR products for diagnosis of Anaplama marginale showing 420bp product size in positive samples and control with no non-specific amplication with no band in negative control. L:100bp ladder;1,3,5,6,7,8 positive samples; 2, 9 negative samples; 10 positive control and 11 negative control.

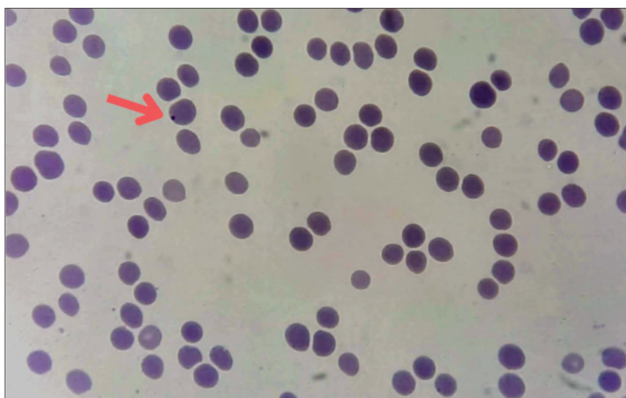

Fig. 3 Picture of stained blood smear showing characteristic dot shaped parasitic form highlighted by arrow at the margin typical of Anaplasma marginale

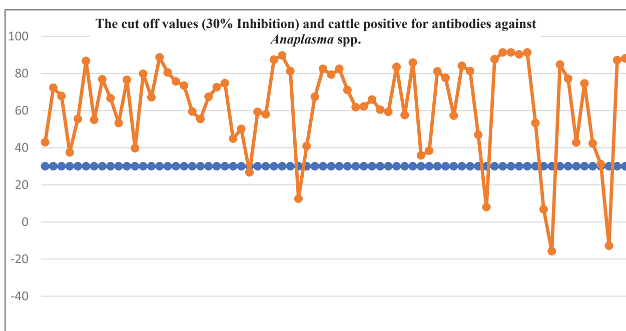

Fig. 4 Graph showing the cut off values and percent inhibition observed in different cattle samples tested for antibodies against *Anaplasma* spp.

Table 1. Sensitivity, specificity, positive likelihood ratio, negative likelihood ratio and accuracy of microscopic examination and cELISA for the detection of Anaplasma infection in cattle blood samples

	Microscopy	cELISA
Sensitivity	40.91% (26.34-56.75)	100% (91.96-100%)
Specificity	100% (87.66-100)	21.43% (8.30-40.95%)
Positive Likelihood ratio	-	1.27 (1.05-1.54)
Negative likelihood ratio	0.59 (0.46-0.76)	0.00
Positive predictive value	100% (81.47-100%)	62.73% (58.11-67.13)
Negative predictive value	56.14% (50.02% - 62.07%)	100% (54.07-100)
Accuracy	66.35% (54.25-77.07%)	66.17% (54.09-76.90%)

Values shown in parenthesis are 95%CI values

antibody binding to rMSP-5 by the test serum antibodies. The test kit used in present study uses recombinant *msp5* (r*msp5*) fused to maltose binding protein as antigen and is detected by the monoclonal antibody AnaF16C1 as conjugate (Knowles *et al.*1996). The msp5 targeted in present study is a surface exposed protein that plays a crucial role in the pathogenesis of *A. marginale* infection by aiding in the adherence and invasion of erythrocytes by the bacteria. Its high antigenicity and specificity make it an ideal target for antibody detection assays such as ELISA. However, the epitope recognized by the monoclonal antibody is common among *Anaplasma* spp., so the cELISA test can detect antibodies to *A. bovis, A. centrale* and *A. phagocytophilum* in addition to the targeted *A. marginale* strains (Dreher *et al.* 2005). In India, vaccination for control of bovine anaplasmosis is not practiced and therefore, the seropositivity recorded in the study could be due to exposure to the *Anaplasma* spp only. It is important to clarify that high seropositivity in this study does not necessarily imply acquisition of new infections or incidence of disease due to *A. marginale.* Conversely, cattle that survive *Anaplasma* infection are persistently infected because *A. marginale* antibodies can be identified for long period of time after the initial infection.

Among the twenty-one samples collected from university veterinary hospital 28.57%, 85.71% and 57.14% samples were positive by microscopy, competitive ELISA and PCR assay, respectively. From samples collected from gaushalas, 23.52% were positive by microscopy, 94.11% by cELISA and 56.86% by PCR assay. A characteristic dot shaped parasite was seen in positive microscopy sample as shown in fig.3 and a characteristic band of 420bp in PCR assay comparable to positive control (Fig. 2). Similar to present study, Selim *et al*. 2021 examined 650 samples

using same cELISA kit as used in the present study and found 20% positive cases. They further had confirmed the samples to be positive by PCR assay targeting msp4 gene. PCR-based detection targeting the msp4 gene has been extensively applied to study the prevalence and molecular epidemiology of *A marginale* across different regions. In Sicily, *A. marginale* was detected in 47% of cattle using an msp4 based PCR assay, confirming its widespread endemicity in Mediterranean herds (de la Fuente *et al.* 2005). Similarly, studies in Egypt reported a prevalence of 32% among cattle, indicating active transmission and carrier states in endemic zones (Abdelaal *et al.* 2018). In Brazil, msp4 PCR assays identified *A. marginale* DNA in 28.6% of cattle samples, demonstrating both infection and genetic diversity among field isolates (da Silva *et al.* 2018). A study from India also reported an msp4 PCR positivity rate of 35.5%, highlighting the utility of this gene target for surveillance and molecular characterization of *A. marginale* in tropical regions (Khan *et al.* 2021). These findings collectively indicate that msp4 based PCR is a robust diagnostic and epidemiological tool, consistently revealing moderate to high prevalence of *A. marginale* in cattle worldwide.

Statistical analysis results have been shown in Table 1. Specificity, sensitivity and accuracy of microscopy and ELISA were calculated considering the PCR as reference method. Microscopic examination was found to be 100% specific with a sensitivity of 40.91%, accuracy of 66.35%, NLR of 0.59 and positive predictive value of 100%. ELISA was found to be 100% sensitive with a specificity of 21.43%, accuracy of 66.17%, PLR of 1.27 and negative predictive value of 100%. PLR for microscopic examination could not be calculated as the denominator was zero which indicates the test as good as reference test.

Degree of agreement between different diagnostic modalities was evaluated using Kappa statistics test. Analysis revealed no agreement between results of microscopy with ELISA with a kappa value of 0.0588, however a fair agreement was found between microscopy with PCR assay and ELISA with PCR assay with a kappa value of 0.350 and 0.250, respectively. Considering PCR assay as a reference test for comparison, PCR assay revealed 56.94% samples positive which was more as compared to microscopy and less when compared with ELISA. On kappa correlation test, fair agreement was found between microscopy and PCR assay but no agreement exist between result of ELISA and PCR assay. Lack of agreement between ELISA and PCR assay has been observed as many samples that were found negative by PCR assay were found positive by ELISA. It supports the possibility of past infections which could not be diagnosed by PCR assay and ELISA detecting antibodies against other *Anaplasma* spp. A negative PCR result and a positive ELISA result indicated the presence of antibodies against the *Anaplasma* organism in the sample but absence of DNA specific to *Anaplasma marginale* in blood samples. Alongwith this, disease features existence of carrier status and infected cattle will be having a mounted immune response persisting for longer period.

Our results were in contraindication with the results of Sharma *et al.* 2015 showing high positivity with PCR than ELISA. This can be due to area difference, stage of ongoing infection in the sampled cattle. Overall, findings suggest that ELISA and PCR assay are more effective and reliable methods for the detection of anaplasmosis in cattle compared to microscopy.

In conclusion, it is important to select appropriate diagnostic methods for the accurate detection of anaplasmosis in cattle, which is crucial for effective control and management of the disease. For early detection, PCR assay should be employed, for herd screening and control strategies cELISA and in clinical set up microscopic examination can be used.

AUTHORS CONTRIBUTION

Conceptualization was carried out by Ravina and GC. Methodology was finalized by GC and YS. Project administration was done by Ravina under the supervision of YR and GC. Data analysis was done by GC, JK and RJ. Original draft and Writing done by Ravina and GC. Finalization and edition was done by YR.

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