



Evaluation of different serological tests for diagnosis of contagious agalactia in goats

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ABSTRACT

Contagious agalactia (CA) is an economically important disease of small ruminants caused by *Mycoplasma agalactiae* and the disease is characterized by mastitis, arthritis, keratoconjunctivitis and sometimes abortion. The diagnosis of CA in field is mainly based on serological tests with varying levels of specificity and sensitivity. It is important to compare the simple and available tests for routine diagnosis of the disease under field conditions. Therefore, the present study was designed to find out the field applicability of 3 serological test, viz. slide agglutination test (SAT) with coloured antigen, indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA). Serum samples (493) from goats of various parts of Uttar Pradesh and Utrakhnad were collected and screened for CA by SAT, IHA and ELISA. Based on this study, ELISA is found a good serological test for diagnosis of CA. However, SAT can also be used in field because of its simplicity, less time consuming and easy to perform under field conditions. Further, 74% sensitivity and 96% specificity was observed in SAT and 56% & 92% of sensitivity and specificity in IHA compared with ELISA.

Key words: Contagious agalactia, ELISA, Goats, IHA, SAT

Mycoplasma agalactiae is an important organism responsible for contagious agalactia (CA), a disease affecting sheep, goats and other small ruminants world wide including India (Gil *et al.* 1999, Ozdemir and Turkaslan 2003, Zendulkova *et al.* 2007, Cheema 2009, Zahid 2010). It causes serious economic losses in areas where the economy is largely based on shepherding (Bergonier *et al.* 1997) and the prevalence of infection is largely underestimated since very few laboratories routinely monitor the infection due to *M. agalactiae*.

The diagnosis of CA is done by using biochemical, film, spot and coagulated serum agar tests (Aloutto *et al.* 1970, Poveda 1998). However, these tests are cumbersome, time consuming and also less sensitive. Growth inhibition test, enzyme-linked immunosorbent assay (ELISA) and immunoblotting were reported for the detection of serological responses of specific antibodies to *M. agalactiae* (Clyde 1983, Levison *et al.* 1991, Tola *et al.* 1996). The serological tests are used to detect and discriminate the antibody responses elicited in both vaccinated and infected sheep (Tola *et al.* 1997, Fusco *et al.* 2007). Growth inhibition test allows discrimination of an antibody response elicited by infection from one elicited by vaccination (Tola *et al.* 1997), but it is

very cumbersome and time-consuming. The western blot assay is extremely sensitive but it does not allow the quantification of antibody level; while ELISA is the test of choice among existing serological procedures because it has the potential for high sensitivity and specificity and in addition it is simple and allows testing of a large number of samples in a short period (Fusco *et al.* 2007). However, a good ELISA should be based on antigens, which are highly immunogenic and expressed during the entire course of infection in the highest possible number of field isolates (Pepin *et al.* 2003).

Several serological tests based on indirect ELISA were proposed for the diagnosis of contagious agalactia (Lambert 1985, Levison *et al.* 1991, Cannas *et al.* 1992, Romano *et al.* 1995). Antigens using whole bacterial lysate or membrane antigens are used in epidemiological studies (Lambert 1985, Levison *et al.* 1991, Cannas *et al.* 1992, Romano *et al.* 1995). Therefore, the present study was designed to evaluate the suitability of different serological test, viz. slide agglutination test, IHA and ELISA for diagnosis of CA in Indian field condition.

MATERIALS AND METHODS

Blood samples (493), 10–15 ml, were randomly collected from female animals showing clinical signs of CA like mastitis, arthritis, keratoconjunctivitis (29) and apparently

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healthy goats (464) belonging to Uttar Pradesh and Uttarakhand. The blood samples were allowed to clot, the serum was collected, and frozen (-70°C) until use. Each sample was subjected to 3 different serodiagnostic tests.

The *M. agalactiae* strain (VP20 L15/02) was obtained from National Referral Laboratory on Mycoplasma, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar.

Slide agglutination test: The coloured antigen was prepared from *M. agalactiae* whole cells according to Srivastava *et al.* (1986). The suspension of antigen was adjusted as 5 times the opacity of tube number 10 of Brown's opacity. One drop each of antigen and unknown serum were mixed on a glass slide and results were observed. The gradation of the results as 4+, 3+, 2+ and 1+ were represented according to the size of agglutinated particles as well as time taken for agglutination. However, agglutination up to 3 min was considered as positive (Cheema 2009).

Indirect haemagglutination test (IHA): The method of micro IHA test as described by Krogsgaard-Jensen (1971) was adopted for diagnosis of CA.

Test proper: Serial 2 fold dilutions (1:10 to 1:80) of test sera were made in serum diluent (PBS, pH 7.4) in microtitre plates. To the 25 μl of each dilution of the sera, 25 μl of sensitized cells were added. The sensitized cells in serum diluents and unsensitized RBCs with the known positive serum were kept as controls. The plates were kept at 37°C and observations were recorded after 16 to 18 h. A titre of 1:20 or more was considered positive.

ELISA employing sonicated whole cell *M. agalactiae* antigen

Preparation of whole cell antigen: Sonicated whole cell antigen was prepared as per Lambert *et al.* (1998). Briefly, *M. agalactiae* from broth culture was centrifuged at 13 000g for 35 min. The pellet was washed 3 times with PBS (pH 7.4). The washed cells were pelleted, suspended in carbonate buffer (pH 9.6). The suspension was incubated on ice for 1 h, then, sonicated on ice 6 times peak to peak at 10 amplitude for 1 min each with a 1 min interval between each burst.

The ELISA was standardized by using various dilutions of the sonicated antigen (ranging from 5–80 $\mu\text{g}/\text{ml}$) and known positive serum (1:100 to 1:800). A 100 μl (30 μg) of the sonicated antigen was coated onto the ELISA plates and incubated at 37°C for 2 h. The antigen coated plates were blocked with bovine serum albumin and incubated at 4°C overnight. Subsequently, 100 μl of diluted (1:200) field serum samples were added in triplicate wells followed by incubation at 37°C for 1 h; 100 μl of the diluted (1:20,000) anti-goat IgG HRPO conjugate was added to each well and further incubated at 37°C for 1 h. Finally, 100 μl of the freshly prepared substrate solution was added into the plates, avoiding any direct exposure of the plate to light. The plates were kept at room temperature for 30 min and thereafter 50

μl stopping solution was added to the wells and then OD was measured at 490 nm using ELISA reader. Suitable antigen, serum, conjugate, substrate and plate controls were always included in the assays.

RESULTS AND DISCUSSION

The results of three serological tests on 493 field sera are presented in Table 1.

Slide agglutination test: Of 493 samples, 50 (10.4%) were detected as positive for contagious agalactia by SAT. Among the 50 positive samples, the intensity of agglutination was rated from 4+ to 1+ (4+ in 6 samples, 3+ in 13 samples, 2+ in 8 samples and 16 samples were 1+). Samples from Shahjehanpur showed more number of positive cases (12.64%) and Bareilly has least positive cases (7.8%).

Indirect haemagglutination test (IHA): Of total 493 samples tested, 30 (6%) were found positive by IHA (Table 1). As per the IHA results, Shahjehanpur samples showed comparatively more seropositivity than samples from other places.

Sonicated whole cell antigen based ELISA: For the screening field sera, 30 $\mu\text{g}/\text{ml}$ of the sonicated antigen and 1:200 dilution of serum were found optimal in checker board analysis. The cut-off OD value for interpretation of positive field sera samples was taken as double of the mean OD of known negative sera. No OD was detected in conjugate, substrate and serum controls. A total of 68 samples were detected positive, more number of positive samples from Shahjehanpur and least positive cases in Bareilly region.

To find out a suitable test screening of CA in field condition, 3 commonly used serological tests, viz. SAT, IHA and ELISA, were evaluated. The results showed that ELISA is a highly sensitive test compared to SAT and IHA in the diagnosis of CA in field conditions. ELISA based on whole-cell sonicated antigen detected 13.79% field samples as seropositive in goat compared with IHA (6%) and SAT (10.14%). Samples from Shahjehanpur showed higher

Table 1. Comparison of 3 different tests for serodiagnosis of *M. agalactiae*

Places of sample collection	Samples	SAT	IHA	ELISA
Bareilly	217 (14)*	17 (7.8%)	13 (5.99%)	21 (9.67%)
Lucknow	80 (4)*	9 (11.25%)	4 (5%)	12 (15%)
Shahjehanpur	87 (7)*	11 (12.64)	8 (9.1%)	16 (18.39%)
Pantnagar	109 (4)*	13 (11.9%)	5 (4.58%)	17 (15.59%)
Total	493 (29)*	50 (10.14%)	30 (6%)	68 (13.79%)
Sensitivity**		74%	56%	
Specificity**		96%	92%	

*Data in parenthesis is indicating number of positive cases; **sensitivity and specificity of SAT and IHA calculated based on ELISA.

positive cases invariably in all tests. Although SAT diagnosed lesser number of samples compared to ELISA, the results of SAT were comparable with ELISA. Moreover, it is simple, easy to perform less time consuming and require less technical knowledge. Therefore, SAT can be used effectively for diagnosis and monitoring of large number of animals for contagious agalactia under field conditions.

Daniz (1996) opined that sonicated antigen was less sensitive, and the results were not reproducible especially in field samples. But in the present study, the results were consistent in triplicate reaction of each sample. The 30 µg/µl of sonicated antigen was appropriate concentration to coat in plate for consistent result in ELISA.

Even though the whole cell antigens have advantage of high sensitivity, cross reactions to other mycoplasma species may increase false positive reactions (Kittelberger *et al.* 2006). Technically, the ELISA has advantage over the other 2 methods. ELISA is the test of choice among existing serological procedures because it has high sensitivity and specificity, also a number of samples can be tested in short time (Fusco *et al.* 2007).

In our study, there was no attempt made to screen the cross reactivity between different *Mycoplasma* species affecting small ruminants. In future, a study is required to explore the possibility of cross reaction between the *Mycoplasma* species affecting small ruminants most appropriately using surface-antigen based serological tests.

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