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Comparative Study of Dark Field Microscopical Examination, Microscopic Agglutination Test and Polymerase Chain Reaction in the Diagnosis of Bovine Leptospirosis

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A total of 115 serum samples (33 from cattle and 82 from buffaloes) collected from different clinical cases (65 Nos) like abortion, repeat breeding, jaundice and haemorrhagic mastitis and also from apparently healthy bovines were subjected to Dark field microscopical examination, Microscopic agglutination test and Polymerase chain reaction to diagnose bovine leptospirosis. The present study showed high positivity in MAT (58.46 %) followed by DFM (38.46 %) and PCR (32.31 %). The study concluded to use MAT for serosurveillance study and MAT followed by PCR for the diagnosis of leptospirosis.

Key words: *Leptospira*, DFM, MAT, PCR, Diagnosis.

Leptospirosis is a zoonotic disease with worldwide distribution (Dey *et al*., 2007). Timely diagnosis is essential for prompt and specific treatment as early as possible to ensure a favourable clinical outcome. Eventhough several reports employ PCR (Van Eys *et al*., 1989; Merien *et al*., 2005 and Chourasia *et al*., 2006), DFM (Rao *et al*., 2003) and MAT (WHO, 1986 and OIE., 2008) for the diagnosis of leptospirosis, MAT is widely used. But MAT is difficult to perform and maintenance of cultures is a main constraint. MAT requires maintenance of a battery of serovars which is costlier too. It also requires regular testing to provide quality control. PCR has the potential to make a dramatic impact. It has high sensitivity, specificity and ability to identify early infection (Biswas *et al*., 2006). DFM is a simple method and is the only method by which the typical motility of the leptospirosis in the clinical sample could be observed. DFM may aid in early diagnosis when correlated with clinical parameters. Currently, there is no sensitive, specific, low cost, rapid and widely available diagnostic test for leptospirosis diagnosis. Hence the present study was undertaken to evaluate PCR, DFM and MAT for the diagnosis of acute case of bovine leptospirosis.

MATERIALS AND METHODS

Source of serum samples

A total of 115 serum samples (33 from cattle and 82 from buffaloes) were collected from different clinical cases (65 Nos) like abortion, repeat breeding, jaundice and haemorrhagic mastitis and also from apparently healthy bovines (50 Nos) (Table - 2). The samples were subjected to Dark field microscopical examination, Microscopic agglutination test and Polymerase chain reaction to diagnose bovine leptospirosis

Dark field microscopical examination (DFM)

Blood samples collected were processed immediately for microscopic examination. The blood was centrifuged at 2000 rpm for 10 min and the

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serum was separated. A drop (5-10 µl) of serum was placed on a clean grease-free glass slide (Blue star, 2.5 cm x 6 cm) and a cover slip (Blue star, 22 mm square) was applied. This wet mount preparation was examined under low (10x) and high (40x) power objectives of the dark field microscope. Utmost care was taken to examine as many microscopic fields as possible. The serum samples were centrifuged at 3000 rpm for 3 min, the supernatant was decanted and the sediment suspended in small volume of PBS and re-examined as described above.

Microscopic agglutination test (MAT)

Preparation of antigens for MAT

Antigens for MAT were prepared following OIE (2004). Twelve different serovars (Table - 1) of leptospires maintained in this laboratory were subcultured. Five to eight day old liquid culture of live leptospires grown at 29 ± 1° C, containing a density of $2x10^8$ leptospires per ml without any clumps was used as antigen in MAT.

Microscopic agglutination test

The test was conducted following OIE (2004) in a 96 well 'U' bottom microtitre plates (M/s. Laxbro, India). Serum dilutions were made in deep well (96 well) dilution plates (M/s. Laxbro, India). To 980 µl of PBS, 20 µl (1:50) of serum samples were added to individual wells. Serum dilutions (25 µl of 1:50) were added to each of the 12 wells in the A to G rows of 'U' bottom microplates. In the last row, only PBS 25 µl was added to all the wells which served as antigen control. Thus each row is corresponding to each sample. Twelve different antigens (25 µl) were added in wells in such a way that each column is charged with only one type of antigen (ie., antigen 1 in column 1, antigen 2 in column 2 and so on) including the respective antigen control wells, so that the final serum dilution was 1 in 100. The plates were closed with lids and incubated at 37°C for 2 h. A drop (5 µl) of mixture (final dilution of 1:100) was placed on grease-free slide and the wet preparation without cover slip was screened using 20X objective of the dark field microscope (M/s. Nikon, 200E Japan) for the presence of agglutination and/or reduction in number of organisms in comparison with the respective antigen control. A 50 per cent reduction in the number of free leptospires in the test sample comparable with the respective antigen control was considered positive with or without agglutination.

Quantitative assay

Quantitative assay was carried out in 'U' bottom microtitration plates against the reacting serovars of leptospires. All the 96 wells were added with 20 µl PBS. In the first well of each row, 20 µl of 1: 25 diluted (Initially diluted in PBS in a separate deep well dilution plate) serum samples were added and mixed well. Then equal volume (20 µl) was serially transferred upto 9 wells. From the 9^{th} well 20 µl was discarded. A constant volume of 20 µl of the respective *Leptospira* antigen (2×10^8 per ml) was added in each row and incubated at 37°C for 2 h. All the final dilution mixtures (50, 100, 200, 400, 800, 1600, 3200, 6400 and 12800) were observed under dark field microscope and the results were recorded as before. The reciprocal of the highest dilution which showed 50 per cent reduction in the number of free leptospires comparable to the respective antigen control with or without agglutination was recorded as the respective titre.

Polymerase chain reaction (PCR)

PCR was done with 115 serum samples using E1 and E2 published primers (Vitale *et al*., 2005).

Deoxyribonucleic acid isolation (DNA)

DNA isolation was done following the procedure of Boom *et al.* (1990). To 125 µl of serum samples, 900 µl of lysis buffer and 40 µl of diatom suspension were added and incubated at room temperature for 20 minutes and then centrifuged at 14000 rpm for 10 minutes. Supernatant was discarded and the pellet washed twice with wash buffer by centrifugation at 14000 rpm for 2 min. To the pellet one ml of 70 per cent cold ethanol was added and washed twice as above. Then one ml of acetone was added and centrifuged at 14000 rpm for 2 min. Later the pellet was incubated at 56°C for 15 min. To the dried pellet 5 µl of proteinase K and 125 µl of triple distilled water were added and incubated at 56°C for 15 min. Then kept in water bath at 100 °C for 15 min and centrifuged at 10000 rpm for 5 min after resuspending in TE buffer. The supernatant was collected with TE buffer and used as template DNA for PCR.

Amplification of 16s RNA

The PCR reaction was carried out as per the method described by Vitale *et al.* (2005). Amplification of DNA was performed in 25 µl reaction mixture containing 12.5 µl of super mix (Genei, Bangalore), 100 pmol (0.5 µl each) of each

primer, E1 and E2 (Sigma - Aldrich, Bangalore) and 11.5 µl of extracted DNA. For positive control 5 µl of DNA template (culture) and 6.5 µl of nuclease free water were used. The reaction components were mixed and subjected to amplification in a thermal cycler (Eppendorf, Germany). The temperature profile consisted of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute and extension at 72 °C for 1 minute. The final extension was carried out at 72 °C for 5 minutes.

Detection of PCR products

The PCR products were analysed by electrophoresis in 1.5 per cent agarose gel in TBE buffer. Agarose was dissolved in TBE buffer by heating and cooled to 50°C. To this Ethidium bromide was added to a final concentration of 0.5 µg per ml of molten gel. The PCR products (10 µl) were loaded into the different wells along with the molecular marker (100bp ladder DNA). Electrophoresis was carried out at 100 V for 20 to 30 minutes until the tracking dye (Bromophenol blue) migrated more than two third of the length of the gel. The gel was placed under the gel documentation unit (Vilber Lourmet, France) and the results were documented.

RESULTS

A total of 115 serum samples collected from clinically suspected and apparently healthy bovines were examined by Dark field Microscopy for the presence of Leptospira, MAT for leptospira specific antibody and PCR for demonstration of leptospira specific antigen. Out of the total 33 cattle serum samples collected from clinical cases of abortion (4 Nos), repeat breeding (6 Nos), jaundice (15 Nos), and haemorrhagic mastitis (8 Nos) only 16 serum samples found to have leptospires. All the cases of history of abortion (4 Nos) and haemorrhagic mastitis (8 Nos) were found to have leptospires. Out of 15 jaundice cases, only 4 were found positive for leptospires. Whereas all the six repeat breeding cases were found negative for leptospires (Table - 2).

Out of 32 buffaloes suffered from abortion (6 Nos), repeat breeding (3Nos), jaundice (10 Nos) and haemorrhagic mastitis (13 Nos), 9 buffaloes were found to be positive for leptospires. All buffaloes suffered from abortion (6Nos) and repeat breeding (3Nos) were found to be negative (Table - 2). Among the apparently healthy animals (50Nos), 10% showed positive in DFM.

Out of 33 cattle and 32 buffaloes suffered from abortion, repeat breeding, jaundice and haemorrahagic mastitis, 21 cattle and 17 buffaloes were found positive by MAT. Among the 50 apparently healthy animals, 26 were found positive. The serovars namely australis, ballum, hardjo and hebdomadis were only observed among the 115 clinically suspected and apparently healthy bovines. There is no association between clinical condition and serovar specificity in the present study.

A total of 115 serum samples collected

Table 1. Reference strains of Leptospires* used in the study

S. No	Serogroup	Serovar	Strain
1	Australis	*Australis*	Ballico
2	Autumnalis	*Rachmati*	Rachmati
3	Ballum	*Ballum*	Mus127
4	Canicola	*Canicola*	HondUtrecht IV
5	Grippotyphosa	*Grippotyphosa*	Moskva V
6	Hebdomadis	*Hebdomadis*	Hebdomadis
7	Icterohaemorrhagiae	*icterohaemorrhagiae*	RGA
8	Javanica	*Poi*	Poi
9	Pomona	*Pomona*	Pomona
10	Pyrogenes	*Pyrogenes*	Salinem
11	Sejroe	*Hardjo*	Hardjoprajitno
12	Tarassovi	*Tarassovi*	Peripellicin

*Obtained from National Reference laboratory, Indian Council of Medical Research, Andaman and Nicobar Islands, India.

Table 2. Results of DFM, PCR, MAT and Isolation from clinically suspected and apparently healthy bovines

Clinical history	Total screened	Number of samples positive							
		DFM	PCR	MAT	Serovars				
					australis	*ballum*	*hardjo*	*hebdomadis*	*pomona*
Cattle									
Abortion	4	4	3	1	-	-	1	-	-
Repeat breeder	6	-	-	3	-	-	3	-	-
Jaundice	15	4	3	9	3	3	1	1	1
Haemorrhagic Mastitis	8	8	8	8	2	-	3	2	1
Total	33	16	14	21	5	3	8	3	2
Buffaloes									
Abortion	6	-	-	3	-	-	1	1	1
Repeat breeder	3	-	-	1	-	-	1	-	-
Jaundice	10	4	3	6	2	4	-	-	-
Haemorrhagic Mastitis	13	5	4	7	4	-	-	3	-
Apparently healthy animals	50	5	3	26	10	3	4	4	5
Total	82	14	10	43	16	7	6	8	6
Cattle and Buffaloes									
Grand total	115	30	24	64	21	10	14	11	8

DFM - Dark field Microscopical examination PCR - Polymerase chain reaction MAT - Microscopic agglutination test

from clinically suspected and apparently healthy bovines were subjected to PCR using E1 and E2 published primers (Vitale *et al*., 2005). Out of 33 cattle serum samples collected from cases of abortion (4), repeat breeding (6), jaundice (15) and haemorrhagic mastitis (8), only 14 cases were found positive for pathogenic *Leptospira* as they yielded an amplicon of expected size 571bp. All the eight cattle suffered with haemorrhagic mastitis were positive by PCR, whereas all the six repeat breeding cases were negative by PCR (Table - 2).

Among the 32 buffaloes screened with the history of abortion (6), repeat breeding (3), jaundice (10) and haemorrhagic mastitis (13), only seven buffaloes were found to be positive by PCR (Table - 2). All the buffaloes which suffered with abortion and repeat breeding were found negative by PCR, whereas seven animals with history of jaundice (3) and haemorrhagic mastitis (4) were found positive. Among 50 apparently healthy buffaloes (Table - 2), three were found positive by PCR which was indicative of subclinical leptospirosis.

DISCUSSION

In the present study MAT showed high positivity (58.46 %) followed by DFM (38.46 %) and PCR (32.31 %). It is known that PCR is highly sensitive test and it can detect 5 – 10 leptospires per ml of solution (Meenambigai *et al*., 2005) whereas DFM can detect only when 10^4 leptospires are present in one ml (Turner, 1970). The primers used in PCR can detect only pathogenic leptospires (Vitale *et al*., 2005) whereas DFM can detect both pathogenic and non pathogenic leptospires, thus discrepancy in results. Both test detect leptospires present in the serum. PCR test is sensitive test but DFM test is conventionally used in many laboratories. It is suggested that both the test should be done for better understanding of the results but confirmatory diagnosis based on PCR rather than DFM.

MAT test is done for antibody detection. MAT titre of e 50 in non - endemic areas and e 100 in endemic areas are considered positive (Plank and Dean, 2000). Antibody titre develop within 5 – 7 days following infection (Smits *et al*., 2001 and Dey *et al*., 2007) and persists for seven years (Plank and Dean, 2000). In the present study more samples (64 Nos) are positive which include both recent and past infection. When paired samples are examined at 21 days intervals and if there is 2- 4 fold increase in titre that titre is considered as acute phase titre. MAT is widely used test and if clinical history is correlated with MAT result, a conclusion can be arrived. Further MAT is a preferred test for serosurveillance study to understand the changing pattern of serovars prevalent in a particular area in a different host species. It is concluded that MAT followed by PCR will be very useful in diagnosis of leptospirosis.

When different clinical cases were tested it was seen that different *Leptospira* serovars are involved in abortion, repeat breeding, jaundice and haemorrhagic mastitis. Clinical cases found were serovar specific. In the present study repeat breeding cases were positive for hardjo serovars, abortions cases were positive for serovars either hardjo, hebdomadis and Pomona. Other clinical conditions like jaundice and haemorrhagic mastitis were positive for one of the five different serovars namely australis, ballum, hardjo, hebdomadis and pomona. Some clinical cases were negative, it could be due to other etiological agents or the samples were not collected in right time. Apparently healthy animals were also found subclinically infected and they spread the infection through urine to susceptible animals. The infected animals should be treated and environment should be thoroughly disinfected to avoid the spread of infection.

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