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Research Article

Comparative Potential of Traditional versus Modern Diagnostic Tests in Estimating Status of Caprine Johne's disease

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ABSTRACT

Study aimed to compare easy to perform field based conventional tests {(fecal smear examination, Delayed Type Hypersensitivity (DTH), AGID} with respect to modern laboratory tests (IS*900* PCR and Indigenous ELISA kit) in estimating the incidence of caprineJohne's disease in two important breeds of goats (Mehsani and Surti) from Gujarat (Western India) in the year 2009. A total of 219 goats screened were categorized as Group-I (123 Mehsani goats), Group-II (76 Surti goats) and Group-III, (20 Non-descript goats). Percent positivity by fecal smear examination (fecal microscopy), Delayed Type Hypersensitivity (DTH), AGID, IS900 PCR and Indigenous ELISA kit was 9.2 (7/76), 21.9 (27/123), 10.9 (24/219), 12.5% (5/40) and 43.3 (95/219), respectively. Rectal pinch smear examination was carried out in 27 DTH positive goats and all smears were negative for the presence of acid fast bacilli. Screening tests (Indigenous ELISA and Delayed Type Hypersensitivity) showed very high incidence of MAP infection in the goat population.

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INTRODUCTION

Mycobacterium avium subspecies paratuberculosis (MAP) is responsible for chronic granulomatous inflammation in animals known as Johne's disease (JD) or Paratuberculosis (Chiodini*et al.*, 1984). MAP infection leads to chronic enteritis of intestines, inflammation of lymph nodes especially mesenteric and lymph-angitis in domestic livestock and wild ruminants (Perez *et al*., 1996, Clarke, 1997, Manning and Collins, 2001, Singh et al., 2012). JD has been reported to be endemic in the animal population of world and India, where ever investigated. In India, limited studies (Pande, 1940; Singh et al., 1996, Tripathi et al., 2002, Singh et al., 2007a) reported variable incidence (2-18%) in domestic livestock. Animals may either receive infection from infected parents through semen, in-utero, milk and colostrums (Shankar et al., 2010) or pick up infection from contaminated environment soon after birth and then follows a protracted incubation period which may last few months to few years. Sharing of MAP between species (inter-species transmission) has been frequently reported (Singh et al., 2012). Clinical symptom being non-specific (weight loss and diarrhea) are not visible before disease gets fully established in the body (clinical) and progressive weakness ultimately leads to either un-timely death or culling. In small ruminants disease is clinically characterized by progressive weight loss and emaciation, soft pelleted feces in the terminal stages. Internally granulomatous enteritis leads to thickening of intestinal mucosa and regional lymphangitis and lymphadenitis (Chiodini*et al.*, 1984), however, corrugations are rare as animals goes out much before from the production system.

Johne's is spectral disease therefore presents variable bacteriological, immunological and pathological picture which is responsible for variations in efficacy of the diagnostic methods employed at different points of time for detection of infection during the course of infection (Chiodini et al., 1984). Though, culture of bacilli samples (feces and tissues from ileo-caecal lymph nodes and small intestines) is specific but is time consuming due to long and variable incubation period which may take 12 weeks (Cocito et al., 1994) but sensitivity is poor (Sockett et al., 1992). For rapid diagnosis of JD, IS*900* PCR may be used since it can detect MAP in feces, blood and tissues with sensitivity equivalent to culture (Tripathi et al., 2002, Singh et al., 2010). Animals infected with MAP elicit strong cellular immune (CMI) response in early stages of infection and in the later stages a strong humoral immune response (Clarke, 1997). Tests based on CMI response such as cutaneous testing with johnin PPD (DTH), gamma-interferon assay and lymphocyte stimulation test (LTT), though sensitive but have not been used frequently for the detection of early infection in sheep and goats (Molina et al., 1991; Storset et al., 2001; Kurade et al., 2004). Tests used for antibody detection include complement fixation test (CFT), agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) tests. ELISA has been reported to be sensitive in clinical cases of JD but performs poorly in sub-clinical stages of infection (Stewart et al., 2006). The study aimed to evaluate the efficacy of easy to perform field based conventional tests {(fecal smear examination, Delayed

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Type Hypersensitivity (DTH), AGID)} with respect to modern laboratory tests (IS900 PCR and Indigenous ELISA kit) in estimating incidence of *Mycobacterium avium subspecies paratuberculosis* (MAP) in two important breeds of goats (Mehsani and Surti) from Gujarat (Western India) in the year 2009.

MATERIALS AND METHODS

Fecal Smear

Approximately, 2 gram of fecal sample was finely ground in sterilized pestle and mortar with sterilized distilled water (10-12 ml). Finely ground samples were centrifuged at 2600 rpm for 45 min at room temperature (RT). Supernatant was discarded and from middle layer smears were prepared, stained by ZiehlNeelsen (ZN) staining and were examined under oil immersion (100X) for presence of acid-fast bacilli (AFB) indistinguishable to MAP. Positive fecal samples were stored at -20^0 C for DNA extraction and culture.

Rectal pinch smear

Rectal pinch smear were examined in goats positive in Johnin test. Rectal pinch was collected with a sterilized Artificial Insemination sheath and smears were prepared, stained by ZiehlNeelsen (ZN) staining and were observed under oil immersion (100X) for presence of acid-fast bacilli (AFB) indistinguishable to MAP.

Delayed Type Hypersensitivity (DTH) / Johnin test

Single intra-dermal (DTH) test was performed on 123 goats of group-I by inoculation of 100 µg of Johnin PPD, obtained from Division of Biological Products, IVRI, Izatnagar (India) in the form of heat concentrated synthetic medium (HCSM) of *Mycobacterium paratuberculosis*. Test was carried out as per the instructions of manufacturer on the side of neck. Skin thickness was measured with Vernier Calipers at pre, 24, 48 and 72 hours post inoculation. Animals showing hot, edematous, painful skin thickness of more than 3 mm after 48 hours were considered positive.

Post mortem and histopathology

Goats died of natural infection were subjected to post mortem examination. Tissues (Ileocaecal junction of intestine, ileum and mesenteric lymph nodes) exhibiting gross lesions were collected and stored at -20^0C without adding any preservative for DNA isolation and PCR, whereas, for histopathology tissues were stored in 10% formalin at room temperature. Formalin fixed tissues were cut into thin (2-3 mm) pieces and wash thoroughly with water for several hours before putting in ascending grades of alcohol for dehydration. Dehydrated tissues were cleared in xylene, embedded in paraffin blocks and 5 micron thick sections were prepared (Luna, 1968). Sections were stained with haematoxylin and eosin (H and E). However, smears made from tissues (Ileocaecal junction, mesenteric lymph nodes) were stained by ZiehlNeelsen (ZN) staining and were examined under oil immersion (100X) for presence of acid-fast bacilli (AFB) indistinguishable to MAP.

Agar Gel Immunodiffusion test (AGID) was perfomed as per Ferreira *et al.* (2002). Briefly, Protoplasmic Antigen (Allied Monitor, USA) was used as antigen at the concentration of 10 mg/ml. 0.75% agarose was dissolved in 0.85% NaCl solution and buffered to pH 9.0 with 0.01 moltris (hydroxymethyl)-aminomethane and 0.02% sodium azide. Gel was poured in plates on a 4-mm thick layer and wells of 5 mm diameter were made in a hexagonal pattern of six peripheral wells for serum samples and a central well for antigen. Positive serum was placed into wells adjacent to test sera and were incubated at room temperature (RT). Plates were examined after 24 and 48 h of incubation and appearance of one or more clearly definable precipitation lines before or at 48 h constituted a positive test result. Absence of any precipitation lines was recorded as a negative test result. Serum from clinically infected animals was used as positive control.

DNA Isolation

After collection of tissues (mesenteric lymph nodes and intestines) were grounded and treated with 0.9% HPC (Hexadecylpyridinium chloride) overnight. The sediment (0.5-1.0 ml) was taken in 2.0 ml capacity eppendorf tubes and washed with PBS, 3-4 times by spinning and vortexing. Pellet was subjected to DNA isolation as per van Embeden*et al.* (1993) method with some modification. Briefly, washed pellet was suspended in 1.0 ml 1X TE buffer (pH 8.0) and centrifuged at 8000 rpm for 15 minutes. Pellet was re-suspended in 450 µl 1X TE buffer and subjected to freezing and thawing (heating to boiling and snap cooling at -20^0C) repeat the process 3-4 times. Then, 40 µl of lysozyme (20 mg/ml) was added to tubes and incubated at 37^0C for 2 hours. Proteinase K (6 µl) and 10% SDS (56 µl) were added and incubated at 65^0C for 30 minutes. After that 64 µl of CTAB and 80 µl 5M NaCl were added and incubated at 65^0C for 30 minutes. Equal volume of Chloroform and Isoamyl alcohol (24:1) was added, vortexed and centrifuged at 10,000 rpm for 10 minutes. DNA was precipitated by chilled absolute ethanol and washed with 70% chilled ethanol. DNA Pellet was re-suspended in 25 µl 1X TE buffer and stored at -20^0C until further use.

Similarly, approximately, 2 gram of fecal sample was finely grounded in sterilized pestle and mortar with sterilized distilled water. Finely grounded samples were centrifuged at 2600 rpm for 45 min at room temperature (RT). Supernatant was discarded; middle layer was collected by sterilized swab and decontaminated in 0.9% HPC for 18-24 hours at room temperature. After discarding the decontaminated layer, 0.5-1.0 ml of sediment was washed with PBS and was subjected to DNA isolation as per method described by Van Embeden*et al.* (1993) with some modification.

Polymerase Chain Reaction (PCR)

DNA was isolated from decontaminated fecal and tissue samples were subjected to specific IS900 PCR using primers (Table 1)as described by Green *et al.* (1989) and Vary *et al.* (1990).

Aga test (AGID)

Table 1: Details of primers used for IS900PCR

Primer	Sequence 5' to 3'	Amplicon size	Reference
1	NT 05 (F) GAT CGC CTT GCT CAT CGC TGC CG NT 06 (R) GAT CGG AAC GTC GGC TGG TCA GG	218 bp	Green *et al.*,1989
2	(F) CCG CTA CTT GAG AGA TGC GAT TGG (R) AAT CAA CTC CAG CAG CGC GGC CTC G	229 bp	Vary *et al.*, 1990

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Briefly, PCR was set in volume of 25 µl, using 1.0-5.0 ng template DNA, 12.5 µl of 2X PCR Master Mix (Genei, Banglore) and 1.0 µl of each primer (10 pmole). Total of 37 cycles were performed in a thermocycler (MJ research) for complete amplification reaction. Thermal cycling conditions for primer 1 were: initial denaturation at 94^{0}C for 3 min, followed by 35 cycles of denaturation at 94^{0}C for 10 sec, annealing at 50^{0}C for 10sec, extension at 72^{0}C for 10 sec, and final extension at 72^{0}C for 3 min. Thermal cycling conditions for primer 2 were: initial denaturation at 94^{0}C for 4 min, followed by 35 cycles of denaturation at 94^{0}C for 30 sec, annealing at 64^{0}C for 30sec, extension at 72^{0}C for 30 sec, and final extension at 72^{0}C for 7 min. Presence and yield of specific PCR product was analyzed by 1.5% agarose gel electrophoresis.

Indigenous ELISA kit

Serum samples were screened by 'indigenous ELISA kit' as per the method of Singh *et al* (2007b). Briefly, 10µg per plate protoplasmic antigen of MAP 'Indian Bison type' was taken in 10 ml of antigen coating (carbonate-bicarbonate at pH-9.6) buffer and coated in 96 well flat bottom ELISA plates. Plates were incubated at 4^{0}C overnight, blocked with 3% skimmed milk in PBS and incubated at 37^{0}C for 1 hour. Plates were washed thrice with PBST (PBS with 0.05 % Tween 20) and stored at 4^{0}C till further use. Then100µl of 1:50 diluted serum samples added in duplicate wells and incubated for 2 hrs at 37 ^{0}C. Plate was washed three times with PBST, 100 µl of optimally diluted (1:8000). Rabbit anti-goat horse radish peroxidase conjugate (Bangalore Genei) added to all wells and incubated for one hour at 37°C. Plate was washed thrice with PBST and 200 µl of freshly prepared OPD (5 mg / plate in substrate buffer at pH-5.0) was added to each well, incubated in dark for 30 min at 37°C. Absorbance was read at 450 nm.

Data analysis

Data were managed in tables created using Microsoft Office Excel; Microsoft Corp., Redmond, Washington, USA. In ELISA, Positive and strong positive were considered as positive for MAP infection by converting OD values into sample-to-positive (S/P) ratio (using formulae; Sample OD - Negative OD / Positive OD - Negative OD in Excel cells) as per Collins (2002).

RESULTS AND DISCUSSION

In the fecal smear examination of 56 goats of Group-I, 5 (8.9%) goats were positive, whereas, in Group-III, of 20 goats, 2 (10.0%) goats were positive. Of total 76 goats screened, 7 (9.2%) were positive (Table 2). Of 5 fecal positive goats of Group-I, 3 died during study. Clinical signs were observed in all the 7 fecal smear positive goats and were also positive in ELISA and AGID. Tripathiet et al. (2006) showed that of 36 known cases of caprineparatuberculosis diagnosed by clinical and fecal smear examination, 72.2% goats were shedding MAP bacilli (Figure 1). Examination of fecal smears by ZeihlNeelsen staining exhibited more number of positive cases as compared to culture and IS*900* PCR (Munjal*et al*., 2007). Screening of 71 animals (55 goats and 16 sheep) belonging to Central Institute for Research on Goat (CIRG) located at Makhdoom (India) revealed that 40% goats and 31.2% sheep were shedding MAP in feces (Singh et al.,2007a, Singh et al., 2007b).

Table 2: Status of shedding of MAP using microscopy

Group	Tested			Positive(%)		
	Male	Female	Total	Male	Female	Total
I	4	52	56	1^D (25.0)	2^D+2*= 4 (7.6)	5 (8.9)
III	2	18	20	1 (50.0)	1 (5.5)	2 (10.0)
Total	6	70	76	2 (33.3)	5 (7.1)	7 (9.2)

^{D}Goats died during study, *Two goats found negative by PCR

Table 3: Status of MAP infection using Delayed Type Hypersensitivity (Johnin test)

Age (year)	Tested			Positive(%)		
	Male	Female	Total	Male	Female	Total
0.5-1	2	23	25	0 (0.0)	2 (8.7)	2 (8.0)
1-3	5	64	69	1 (20)	17 (26.5)	18 (26.0)
> 3	3	26	29	1 (33.3)	6 (23.0)	7 (24.1)
Total	10	113	123	2 (20)	25 (22.1)	27 (21.9)

Table 4: Sero-monitoring of MAP infection using Indigenous ELISA kit

Group	Tested			Positives (%)		
	Male	Female	Total	Male	Female	Total
I	10	113	123	4 (40)	53 (46.9)	57 (46.3)
II	5	71	76	1 (20)	29 (38.1)	30 (39.4)
III	2	18	20	1 (50)	7 (38.8)	8 (40)
Total	17	202	219	6 (35.2)	89 (44.0)	95(43.3)

Rectal pinch smear examination was carried out in 27 DTH/Johnin positive goats. All rectal pinch smears were negative for the presence of MAP (Table 2). In accordance to this it was described in Ohio State University Fact Sheet for JD in small ruminants (sheep and goats) that smears from the biopsies of rectal mucosa are difficult to get and may not be very useful since in disease process it is less likely that it will involve rectum in cases of sheep and goats (http://ohioline.osu.edu/vme-fact/0003.html). Of the 123 goats of Group-I, 27 (21.9%) were positive in DTH test. Of 5 fecal positive goats which also showed clinical signs, 2 (3.5%) goats died during study were negative by Johnin (Table 3). Similar to these findings, Paliwal and Rajya (1982) and Rajukumar (1998) stated that sensitivity of Johnin test in goats ranged between

18-30% with least specificity in both preclinical and advanced stage of disease. Tripathi*et al.* (2006) reported that of 34 cases of caprineparatuberculosis, 73.5% goats were positive for Johnin test. In the present study, of 5 infected goats, 3 (60%) were positive in Johnin test.

Of the 219 goats tested, 95 (43.3%) were positive by ELISA. Of 123 Mehsani goat breed (Group-I), positive reactors were higher 57(46.3%) and had history of recurrent diarrhea (Table 4). Sweeney et al.(1995) reported that sensitivity of ELISA is only 15.0% in animals excreting low quantities of MAP bacilli in their feces as compared to 87.0% in animals with clinical signs of Johne's disease. In this study similar results were obtained i.e. all the 7 goats showing clinical signs as well as fecal smear positivity were also positive by ELISA. Paolicchiet al.(2003) and Singh et al. (2009) observed that absorbed ELISA was useful to detect positive animals and those goats shedding MAP in feces. Similarly in the present study all animals shedding MAP in feces were positive in the ELISA test. As per OIE manual (2000), ELISA has been described as most sensitive and specific test for the detection of MAP infection however, in our study also Indigenous ELISA kit developed by CIRG, Makhdoom was found most sensitive and specific test for the detection of MAP infection.

Table 5: Status of MAP using Agar Gel Immuno Diffusion (AGID)

Group	Tested			Positive (%)		
	Male	Female	Total	Male	Female	Total
I	10	113	123	1 (10)	14 (12.3)	15 (12.2)
II	5	71	76	0 (0.0)	5 (7.0)	5 (6.5)
III	2	18	20	1 (50)	3 (16.6)	4 (20)
Total	17	202	219	2 (11.7)	22 (10.8)	24 (10.9)

Table 6: Status of MAP infection using IS900 fecal PCR

Group	Tested			Positive(%)		
	Male	Female	Total	Male	Female	Total
I	4	24	28	1 (25)	2 (8.3)	3 (10.7)
III	2	10	12	1 (50)	1 (10)	2 (16.6)
Total	6	34	40	2 (33.3)	3 (8.8)	5 (12.5)

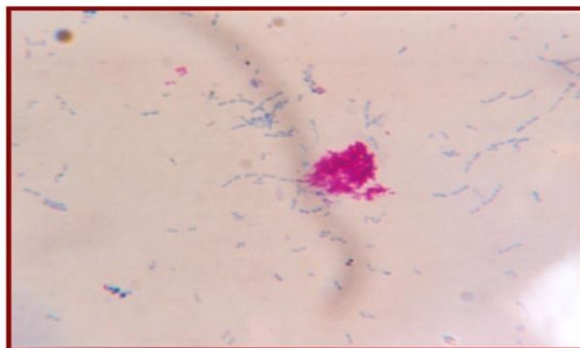

Figure 1: Microscopic view of acid fast bacilli (indistinguishable to MAP)

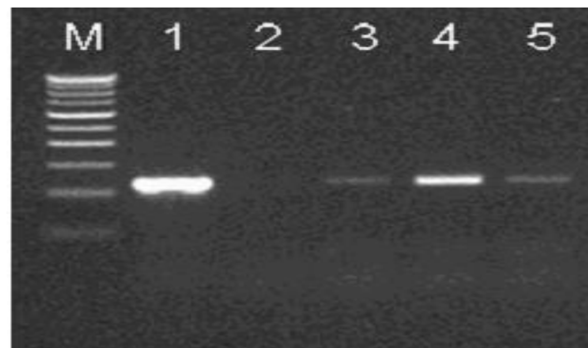

Figure 2: MAP specific amplicons (229 bp) using IS900 PCR. Lane M: 100 bp DNA marker, Lane 1: Positive control, Lane 2: Negative control (MiliQ water), Lane 3-5: samples

Of 219 goats tested, 24 (10.9%) were positive by AGID. Group-III consisting of 20 non-descript goats showed higher number (4 or 20%) of goats as positive reactors. All the 7 goats showed clinical signs and were positive in smear examination and AGID (Table 5). Similarly, Sherman et al. (1984) recorded that of 33 AGID positive cattle, 32 (96.9%) were confirmed for MAP infection by culture or necropsy. Similarly, Ferreira et al.(2002) found AGID specificity was 92.5% and sensitivity 57%. Raul et al. (1998) recorded 11.7% (64 of 546) sero-prevalence of MAP in sheep and goat flocks using AGID test.

Figure 3: Histopathological changes showing enlargement of mesentericand ileocecal lymph nodes

PCR was carried out on fecal samples of suspected animals of Group-I and III with history of recurrent diarrhoea or pasty feces and tissue samples of three goats died in Group-I. Of 40 goats, 7 showed clinical signs and were also positive by faecal smear examination (Figure 2). Among these 7 goats faecal

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PCR detected 5 goats as positive while 2 were negative (Table 6). Munjal et al. (2007) reported that examination of fecal smears with ZiehlNeelsen staining detected more number of goats as positives as compared to culture and IS*900* PCR. Some of the earlier studies have also reported higher prevalence of MAP infection in the farm herds as compared to farmer's herds (Kumar et al., 2007). Tissue samples of all 3 goats died during study were positive by PCR. Similarly, Stabel (1997) reported that IS900 based PCR could be more sensitive in tissue samples in confirming diagnosis at necropsy.

Figure 4: Histopathological changes showing corrugation andthickening of intestinal mucosa

All three goats died during study in Group-I were subjected to post mortem examination, revealed enlargement of mesenteric and ileocecal lymph nodes and thickening of ileum and ileocecal junction wall (Figure 3 and 4). In one goat slight corrugation of ileal mucosa was observed. Carcass showed depletion of fat depots. Rajukumar (1998) observed similar lesions with enlarged, edematous and congested mesenteric and ileocecal lymph nodes. Corrugations in the ileal mucosa were observed in only one goat as has been described in the Ohio State University Fact Sheet for JD that small ruminants (goats and sheep) may reveal some lesions (gross and microscopic) that are not typical as those seen in cattle. Ridges and thickening of the small intestine and cecum are not always seen as is common in cattle.

Storset*et al.* (2001) observed more number of goats with lesions in jejunum as compared to ileum while the lesions in ileum were detected in only one goat. In contrast to this lesions in jejunum were not observed in present study. Histopathological changes in goats died of JD revealed slightly congested intestinal mucosa with infiltration of mononuclear cells consisting of lymphocytes and macrophages in lamina propria. Villi were thickened and blunt at some places. Mesenteric lymph nodes exhibited infiltration of lymphocytes and macrophages. Munjal*et al.* (2005) observed similar lesions such as thickening of the intestinal villi with flat and wide tips, infiltration of lymphocytes, macrophages and epithelioid cells in lamina propria of intestine and inter-follicular area of mesenteric lymph nodes. Similar Histopathological changes observed by Paliwal and Rajya, (1982) and Rajkumar, (1998).

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CONFLICT OF INTEREST

No conflict of interest to declare.

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