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Detection of *Mycobacterium avium* subspecies *paratuberculosis* by conventional and real time PCR in goats of Punjab, India

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Abstract
Caprine paratuberculosis is a mycobacterial, chronic, debilitating, progressive, granulomatous, intestinal infectious disease of goats. The aim of this study was to detect *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in blood and fecal samples of goats. Blood and fecal samples were collected from goats (n=200). Acid fast staining was done in fecal samples. The IS*900* PCR assay targeting MAP with amplicons of 413bp from both blood and fecal samples and TaqMan real time PCR to further confirm the disease was performed. Nine (4.5%) animals were positive for *M. avium* subspecies *paratuberculosis* by conventional PCR. Out of these 9 animals, four were positive for MAP in both fecal and blood samples. However by TaqMan real time PCR, thirteen (6.5%) animals were positive for MAP. Thus the present study reports the prevalence of caprine paratuberculosis in Punjab.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*, conventional, real time PCR

1. Introduction

Paratuberculosis (PTB) or Johne's disease is an OIE listed chronic, debilitating, progressive, granulomatous,mycobacterial disease affecting the intestine in both domestic and wild ruminants [1, 2] characterized by diarrhea, weight loss and eventually death [3, 4]. It is caused by an acid fast bacillus, *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Besides cattle, sheep and goats may also be susceptible to MAP infection. Unlike cattle which showed clinical signs of profuse watery diarrhea, sheep and goat show no clinical signs except for weight loss in the face of a good appetite [2]. Caprine PTB not only hamper the eradication campaigns against bovine PTB in affected areas but may be also responsible for cases of TB [5-8] and Crohn's disease in humans [9].

In Indian scenario, goat (*Capra hircus*) is considered as poor man's cow and contributes greatly to the economy of small, marginal farmers in rural area. Diagnosis of caprine paratuberculosis is difficult, and it cannot be done based on clinical symptoms alone. Diseases like chronic intestinal parasitic infestation, internal abscess caused by *Corynebacterium pseudotuberculosis*, chronic hepatic infection and chronic malnutrition also showed similar clinical signs [10].

Diagnosis of PTB includes conventional methods (isolation of MAP, serological test, histopathology and detection of acid fast bacilli by Ziehl's- Neelsen stain) and molecular methods like PCR and real time PCR. Though fecal isolation of MAP is considered asa gold standard for PTB diagnosis, it is time consuming (upto four months) to produce visible colonies [11]. These conventional methods have been used for diagnosis of animals with latent infection, however due to the slow-growing nature of MAP; in the early stage/sub-clinical infection diagnosis is very difficult. Many molecular methods have been developed that can potentially diminish the diagnostic period from weeks to days. Polymerase chain reaction (PCR) is a rapid method that is sensitive, reliable and easy to perform, requires less time and less amount of sample (fecal, milk, tissue and blood). This technique is based on the amplification of highly specific DNA sequences present in the agent of interest.

Few reports are available on caprine tuberculosis from Punjab and thus the study was conducted with the aim to detect MAP in blood and fecal samples of goats from Punjab, India.

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2. Materials and Methods
2.1 Animals
A total of 200 animals above 2 years of age were included in the study during period from 2016-2017. Goats from one organized and four unorganized farms located in and around Ludhiana district of Punjab, India were randomly selected for the study. One hundred animals were from an organized farm while one hundred animals were from four unorganized farms. Fecal and blood samples of 200 animals were collected for the study.

2.2 Acid fast staining
Two grams of fecal sample was finely ground in pestle and mortar with distilled water and centrifuged @ 4000 rpm for 45 min at room temperature (RT). Supernatant was discarded; smears were prepared from middle layer, stained by Ziehl-Neelsen's (ZN) and examined under oil immersion (×100) for acid-fast bacilli (AFB) indistinguishable to MAP.

2.3 Polymerase Chain Reaction
Isolation of DNA: DNA was isolated from 200 blood samples using QIAamp Blood & Tissue DNA kit (Qiagen) and fecal samples by conventional method [12]. DNA extracted from fecal and blood samples were amplified by PCR using primers based on IS900 sequence (Forward - P90B 5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3' and Reverse - P91B 5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3'). PCR was performed as per Millar *et al.* [13] with some modifications. Ready to use Taq master mix (Qiagen) was optimized in the present study for IS900 PCR. PCR reaction was performed in a total 25 µl volume, using 12.5 µl Taq master mix, 0.5 µl nuclease free water, 1 µl each primer (10 pmole/µl) and 10 µl DNA template. Along with the DNA of the test samples the known positive control DNA and negative control were also amplified.

Thermal cycling were performed in Biometra T-Gradient thermal cycler and cycling conditions includes an initial denaturation at 94 ℃ for 3 min, followed by 37 cycles for denaturation at 94 ℃ for 30 sec, annealing of primers at 62 ℃ for 30 sec, extension at 72 ℃ for 1 min and final extension at 72 ℃ for 10 min. PCR products were analyzed by agarose gel electrophoresis. Amplicon sizes of 413 bp were considered positive, after separation on 1.5% agarose gel stained with ethidium bromide.

2.4 IS900 MAP TaqMan Real-time PCR
TaqMan real-time PCR assay for this study was done as per the method of Kim *et al.* [14] with minor modifications for the detection of MAP. The MAP specific sequence IS900 was targeted as this sequence is having the highest copy number. Primer and probe sequences are Forward5'- AATGA CGGTT ACGGA GGTGG T- 3', Reverse 5'- GCAGT AATGG TCGGC CTTAC C- 3' and probe 5'- TCCAC GCCCG CCCAG ACAGG- 3'. The probe was labeled with the fluorescent reporter dye 5-carboxyfluoroscein (FAM) on the 5' end and the quencher dye N_-,N_-,N_-,N_--tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. Primers and probes specific for MAP IS900 sequence DNA were obtained from Applied Biosystem. TaqMan Real-time assay was performed with the Applied Biosystems (ABI) step one plus RT-PCR. The reaction mixture (20 µl) prepared for the assay includes 10µl 2X TaqMan Master Mix,1µl20X Primer-Probe Mix, 2 µl DNA template and 7µl Nuclease free water. Amplification and detection were performed on the ABI step one plus system with the cycling conditions as Initial denaturation at 95 °C for 10 min, followed by a 40 cycles of denaturation at 95 °C for 15 sec andannealing and extension at 60 °C for 1 min and final extension at 60 °C for 30 sec. Dilutions of the standard DNA (30 ng/µl, D3, GENEKAM, Germany) were made and the standard curve was plotted against CT values. CT based quantification of the MAP IS900 gene was recorded from the software.

3. Results and Discussion
3.1 Microscopic examination of fecal samples by Ziehl-Neelsen's (ZN) staining
In the present study, out of 200 animals, 15 (7.5%) were positive by acid fast staining.The success of microscopic examination of fecal matter depends on the number of acid-fast bacteria present in the sample [15]. Kaur *et al.* [16] detected 17 (68%) fecal samples out of 25 samples in Ziehl-Neelsen's staining for acid-fast bacilli in goats from Punjab. Rawther *et al.* [17] found that out of 200 goats, six goats (3%) were found positive for paratuberculosis. Lilenbaum *et al.* [18] stated that though direct examination of fecal sample is cheap, it is not recommended for diagnosis as in low shedders it could lead to false-negative results.

3.2 Detection of MAP by IS900PCR and IS900 MAP specific q RT-PCR TaqMan Assay
In the present study, IS900 PCR was employed directly on DNA extracted from fecal and blood samples. A total of 9 animals were positive for MAP in blood, out of which 4 animals were also positive in fecal samples (Table 1 and Figure 1).

Table 1: Detection of *M. avium* subspecies *paratuberculosis*

Farm	No. of animals	Acid fast staining	PCR		Real time PCR
			Fecal	Blood	
1.	100	0 (0%)	0 (0%)	0 (0%)	0
2.	20	2 (10%)	0 (0%)	1 (5%)	1 (5%)
3.	30	5 (16.66%)	1 (3.33%)	3 (10%)	3 (10%)
4.	40	7 (17.5%)	3 (7.5%)	5 (12.5%)	9 (4.5%)
5.	10	1(10%)	0 (0%)	0 (0%)	0
Total	200	15 (7.5%)	4 (2%)	9 (4.5%)	13 (6.5%)

Fig 1: Agarose gel electrophoresis of the amplified IS*900* gene

[L1 –L13: Ladder Marker (GeneRuler 100 bp plus DNA ladder), L2: Positive control, L3: Negative control, L4, L5, L7, L9 & L12: Sample positive for MAP, L6, L8, L10 & L11: Negative sample]

DNA based detection of MAP is more sensitive than the bacterial culture [19]. IS*900* PCR is more sensitive than bacterial culture in detecting subclinical paratuberculosis with sensitivity of 70% in PCR and only 30% in bacterial culture [20]. Soumya *et al.* [21] found that sensitivity of fecal culture was 52.5% where as that of PCR assay was 90%. Every culture positive fecal sample yielded PCR positive but not every PCR positive yielded culture positive [22]. Bauman *et al.* [23] found that 38/241 goats (15.8%) samples were positive on the direct PCR. Also conducted RT-PCR on FPCR-positive samples and found that 16.6% (96/580) were positive in dairy goats. The Ct values for FPCR-positive samples ranged from 23.96–41.66 (mean positive Ct =36.04; median Ct =36.63) for goats.

Singh *et al.* [24] detected 1 (7.14%) and 6 (12.0%) samples positive out of 14 fecal and 50 blood samples, in fecal and blood PCR, respectively. Rawther *et al.* [17] found that out of 200 goats, 42 goats (21%) were found positive for *Mycobacterium avium* subspecies *paratuberculosis* with IS*900* fecal PCR. Barad *et al.* [25] found 12.5% fecal PCR positive cases of 40 JD suspected goats in Gujarat. Bhat *et al.* [26] reported 8 (20.0%) samples were positive for MAP out of 40 fecal samples by IS*900* PCR. In the present study, only 4 fecal samples were positive for MAP by PCR. This may be due to low shedding of MAP which is usually seen in early stage of infection or sub-clinical stages.

Further, IS*900* TaqMan Real-time PCR was performed to confirm the detection of MAP. The sensitivity of IS*900* TaqMan was assessed by doing serial dilutions of the known concentration (30 ng/μl) of the standard genomic DNA and the sensitivity was upto 3 fg/μl of genomic MAP DNA. Only MAP showed amplification which indicates the specificity of IS*900* TaqMan real-time PCR. Real time PCR using primers for MAP and β actin were performed on each sample in duplicate. β actin served as a control to detect inhibition of PCR reaction. A sample was considered positive when the PCR product was detected in both duplicates from β actin and IS*900*. In the present study, among all the samples, Ct values of the samples were greater than 40 which considered negative based upon our results of standardization of q-PCR protocol. Thirteen samples (6.5%) whose CT ranges from (18.59-35.28) were considered positive by real-time PCR (Table 2 & Figure 2).

Thus by IS*900* TaqMan real-time PCR, 13 (6.5%) animals were positive for MAP as compared to that of conventional PCR which detected MAP from only 9 animals. IS*900* MAP specific q RT-PCR TaqMan assay measures the amount of target DNA produced during each cycle of an amplification reaction in a real time format. Thus, the system is able to quantify the amount of target DNA in contrast to the conventional PCR, which measures only the end point values with qualitative results.
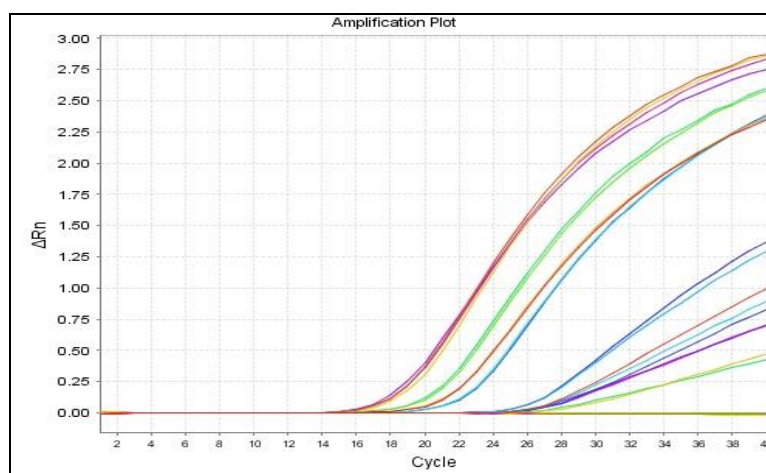
Fig 2: Amplification Plot of *M. avium* subspecies *paratuberculosis* DNA in clinical samples using qRT-PCR TaqMan Assay

Table 2: Ct values of samples obtained by IS*900* real-time PCR

Animal no.	C$_t$ value obtained by IS*900* qRT-PCR
11	33.24
12	22.92
16	30.32
17	29.35
20	20.86
32	29.68
701	18.59
1226	18.85
51	21.54
45	34.98
48	32.49
163	31.56
189	35.28

Many researchers have used real time PCR for diagnosis of PTB in goats [27-29]. Real time PCR is more rapid, sensitive, has high resolution and more reproducible than PCR [30]. Real-time PCR is also a particularly attractive alternative to conventional PCR for the study of microbial load because of its low inter-assay and intra-assay variability [31-33].

Further in the present study, there were differences in detection of acid fast bacilli by ZN staining in 15 samples as compared to 13 animals for MAP by real time PCR. This may due to the fact that acid fast staining technique has low specificity as it can detect *Mycobacterium* other than MAP or sample contamination with other acid-fast bacteria. Acid fast staining does not provide any information on the species of Mycobacteria causing the infection or differentiate between viable and non-viable cells [34].

Moreover, the present study also showed that blood can also be a good sample source for detection of MAP by PCR. Blood PCR was rapid, sensitive, and specific assay for detection of MAP in any stage (early, subclinical, and clinical) and age (kids, young, and adult) of goats [28]. PCR using blood as the source sample reduced the possibilities of detecting passive infection. The test raised hopes for detecting subclinical MAP infection. Thus screening of animals for MAP can be done at any stage in live animals so that quick segregation of infected animals; restrict transmission and rapid eradication of caprine JD from goat herds and country as well could be endorsed.

4. Conclusion
The present study reports the prevalence of caprine paratuberculosis in Punjab, India. This study may provide an additional knowledge in the field of mycobacterial disease occurring in the country. The presence of MAP in animal may raise concerns regarding the zoonotic risk for humans, especially those living at the animal-human interface. Further screening of MAP are required in large sample size so that animals which are either in early and subclinical stages may be diagnosed and thus may help in the control of the disease.

5. Acknowledgement

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