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Bio-prevalence and molecular diagnosis of *Mycobacterium avium* Subsp *paratuberculosis* infection in small ruminant population of Ganderbal district of Kashmir valley

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Abstract

Present study first time determined bio-prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in sheep population of Ganderbal district of Kashmir valley. A total of 288 faecal samples (260 sheep and 28 goats) collected from 23 small ruminant farms were screened using a multi-stage simple random sampling technique. A total of 40 faecal samples positive on microscopy were subjected to DNA isolation and IS900 PCR to confirm the presence of MAP. Bio-prevalence of animals shedding acid fast bacilli (AFB), indistinguishable to MAP was 32.9% (92). Block-wise bio-prevalence of AFB shedders in small ruminants was: Ganderbal (29.4%), Lar (35.2%), Wakura (32.5%) and Kangan (30.88%). Of the 40 faecal samples subjected to DNA isolation and amplification by IS900 PCR, 8 (20.0%) samples were positive for MAP. The study reported moderate bio-prevalence of MAP infection in small ruminant population of Ganderbal district of Kashmir valley.

Keywords: Kashmir, *Mycobacterium avium* subspecies *paratuberculosis*, bio-prevalence, IS900 PCR

Introduction

Small ruminants play important role in the lives of poor people and in the rural economy in developing countries like India. Their importance is even greater in hill and mountain areas like Jammu and Kashmir (J&K), which are not suitable for agriculture crops. Both sheep and goat provide means for sustainable production and food security. Ganderbal district of Kashmir valley by virtue of its vast lush green pasture areas and orchard land, offers great scope for sheep and goat rearing, not only on marginal scale but on entrepreneur scale as well. The district has the small ruminant population of 2.62 lakhs (2.34 lakhs sheep, and 0.28 lakh goat). *Mycobacterium avium* subsp *paratuberculosis* (MAP), is the causative agent of Johne's disease in ruminants that causes chronic granulomatous enteritis leading to emaciation and occasional diarrhoea in sheep and goats [1]. Disease has worldwide distribution in small ruminants, including India [2, 3, 4, 5] and diagnosis in small ruminants is difficult due to absence of characteristic symptoms exhibited by large ruminants. Diagnosis mainly depends on the shedding of acid fast bacilli by animals in its faeces and immune response mounted by it. Shedding of MAP bacilli can be detected by Ziehl-Neelsen staining of faecal smears and by isolation of MAP DNA from faecal samples and subsequent amplification by IS900 PCR [6]. Though bio-prevalence of Johne's disease has been reported in goats in the region [7], however a comprehensive study on the bio-prevalence in small ruminant population has not been carried out. Bio-prevalence of the disease in the temperate climate of the region holds continuous threat both to the small ruminant population and human population and contaminate the environment. Present study determined the bio-prevalence of MAP infection in the small ruminant population of Ganderbal district and confirmation of the MAP by molecular diagnosis of disease.

Materials and Methods

Study design

For the bio-prevalence, multi-stage simple random sampling technique was used with single animal as the epidemiological unit of concern, to draw a simple random sample from small ruminant population of Ganderbal district.

Total sample size was calculated as per Thrusfield [8]. Sample size was based on the following parameters: 95.0% level of confidence, $\pm 5\%$ desired level of precision and the expected bio-prevalence of Johne's disease in ruminants of 26.8% [6] by using following formula:

$$n = Z^2 \times p(1-p) / d^2$$

Where: n = required sample size, p = expected prevalence (25%), d = desired absolute precision (5%) and for 95% confidence interval Z will be taken as 1.96, the sample size required to determine the prevalence was 288 animals.

Selection of animals

A total of 288 faecal samples (260 sheep and 28 goats) were collected from 23 small ruminant farms of the Ganderbal district. The study was conducted from October, 2016 to October, 2017. Samples were collected from four administrative blocks of Ganderbal: Lar, Ganderbal, Wakura and Kangan. Sample collection was done in proportion to the small ruminant population of individual blocks (Table 1). Sheep breed reared in the region was indigenously developed Kashmir merino (cross of delaine merino and local poonchi, gaddi and bhakerwal). Animals were reared in the intensive management in winter months and transhumance takes places in months of June to October.

Table 1: Number of faecal samples collected from individual blocks of Ganderbal

S. No	Blocks	Faecal samples	No. of farms
1.	Ganderbal	95	8
2.	Lar	85	6
3.	Zazna	40	4
4.	Kangan	68	5

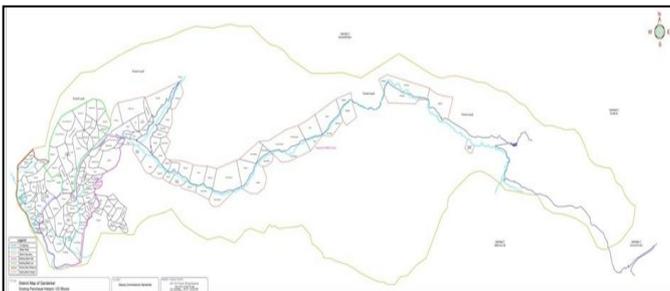


Fig 1: Map of ganderbal district

Collection of faecal samples and ZN staining

About 2-3 gram of faecal sample of sheep and goats were collected in polythene bag directly from the rectum of animals. The samples were homogenized and concentrated by centrifugation at 4500 rpm for 30 min at room temperature. Supernatant was discarded and from middle layer smears were prepared, stained by Ziehl Neelsen (ZN) staining and were examined under oil immersion (100X) for presence of acid-fast bacilli (AFB) [9].

Isolation of DNA and IS900 PCR

A total of 40 faecal samples positive on microscopy were processed for DNA isolation as per Van Embden *et al* [10]. and Singh *et al* [11]. Samples were screened for the presence of MAP in faecal samples using IS900 PCR to obtain the frequency of distribution of MAP. DNA samples were amplified using specific IS900 (P90 and P91) primers published by Millar *et al* [12]. Briefly, in a volume of 12.5 μ l of 2X master mix, 0.5 μ l forward primer (10 pmole/ μ l) and 0.5 μ l

reverse primer (10 pmole/ μ l), 9.5 μ l of nuclease free water and 2 μ l of template DNA was added (total volume 25 μ l). Total of thirty seven cycles were performed in a thermal cycler for complete amplification reaction. Thermal cycling conditions were initial denaturation at 94 $^{\circ}$ C for 5 min (1 cycle), followed by 37 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 64 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 30 s and final extension at 72 $^{\circ}$ C for 7 min. The specific amplicon of 413bp product was analyzed by 1.5% agarose ethidium bromide gel electrophoresis and were taken as positives.

Results

Prevalence of MAP infection

Of the 288 faecal samples collected from small ruminant farms, 32.9% (92) were positive for acid fast bacilli (AFB), indistinguishable from MAP. Block wise bio-prevalence of AFB shedders in small ruminants was Ganderbal (29.4%), Lar (35.2%), Wakura (32.5%) and Kangan (30.8%). Bio-prevalence based on species was; Goats (35.7%) and Sheep (31.5%) (Table 2). The age wise bio-prevalence of AFB shedders, indistinguishable from MAP, on ZN staining was found to be 20%, 31.11% and 27% in age group of animals less than 2 years, 2-5 years and more than 5 years, respectively (Table 3).

Table 2: Block-wise bio-prevalence of MAP infection in Ganderbal district

S.no	Blocks	Faecal Samples	Percent positive
1.	Ganderbal	95	29.74
2.	Lar	85	35.29
3.	Wakura	40	32.5
4.	Kangan	68	30.88

Table 3: Bio-prevalence of MAP infection with respect to age of animals

S. no		Age (in years)		
		Less than 2 Years	2-5 Years	More than 5 years
1.				
2.	Positive/examined	10/50	61/196	21/78
3	% positive	20	31.11	27

PCR Results

Of the 92 samples positive on faecal microscopy, 40 faecal samples (+3 and +4 positives in microscopy) were subjected to DNA isolation and amplification using IS900 PCR. A total of 8 (20.0%) faecal samples were positive for MAP (Fig.2).

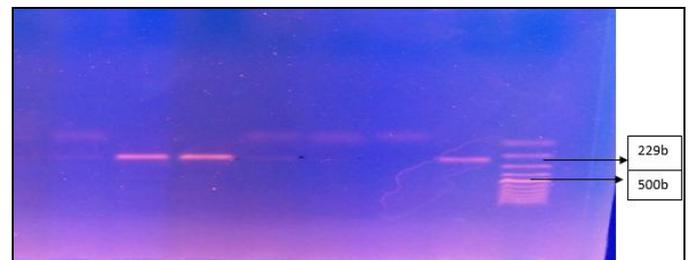


Fig 2: Results of faecal IS900 PCR (Lane 1: 100 bp DNA ladder, Lane 2: positive control, 6 and 7: positive faecal sample, Lane 3, 4 and 5: negative faecal samples)

Discussion

Johne's disease causes serious economic losses to farmers rearing sheep and goat. Effective control of Johne's disease is hampered due to the lack of information on the bio-prevalence of MAP infection using rapid and accurate diagnostic tests. Diagnosis of the disease in sub-clinically

infected animals is extremely challenging, since disease gets transmitted prior to the development of clinical signs^[13, 14], therefore control of disease becomes very difficult. Designing any 'strategy' for 'disease control' in small ruminant flocks, it is essential to know the frequency and distribution of MAP infection.

This is the first study on bio-prevalence of MAP infection in small ruminants in the Ganderbal district of Kashmir valley. ZN staining provides information about the shedding pattern and shedding load of AFB by the animals and is helpful in diagnosing the disease in those animals that are in the shedding stage (sub-clinically or clinically infected). The prevalence of MAP infection by ZN staining of faecal smears was found to be 31.5% in Kashmir Merino sheep and 35.7% in goats, reared in the region. Bio-prevalence was uniform in all the four administrative blocks of Ganderbal district: Ganderbal (29.4%), Lar (35.2%), Wakura (32.5%) and Kangan (30.8%). Shah *et al*^[7], reported 34.0% prevalence by faecal smear examination in goats. Singh *et al*^[6], reported the prevalence of MAP infection by microscopy in sheep (32.0%) and goats (21.6%) of eight Indian states by ZN staining of faecal smears. Singh *et al*^[11], have reported higher prevalence (77.5%) in goats at CIRG, Makhdoom. Coelho *et al.* (15) also reported the higher prevalence of 20.7% and 16.7% from apparently healthy and suspected sheep of Trás-os-Montes e Alto Douro (TMAD, Portugal) respectively. Mukartal *et al.*^[16], reported the prevalence of 35.0% in two farms of Karnataka where Mandya breed of sheep is reared.

Faecal IS900 PCR results revealed 20.0% of animals positive for MAP infection. Detection of MAP by IS900 PCR in faecal samples was rapid, but has low through-put as MAP shedding at early and sub-clinical stages is low and intermittent. Shah *et al*^[7], reported the prevalence of 8% in goats of Kashmir based on IS900 PCR. Barad^[17] found 12.5% faecal PCR positive cases of 40 JD suspected goats in Gujarat. Manning *et al*^[18], opined that even though sensitivity and specificity of microscopy was low, it helped in estimating rate of shedding of MAP in faeces of infected animals. Present study reported moderate bio-prevalence of MAP infection in small ruminant population of Ganderbal district of Kashmir valley. The disease requires immediate attention given its high prevalence, effect on growth and reproductive traits of animals and as continued source of MAP to naive animals, new flocks (by movement of animals) and regions and spread to wild ruminants^[5] and also to the human population^[5, 19].

Conclusion

The study concluded moderate prevalence of AFB shedders in small ruminant population of Ganderbal district of Kashmir Valley.

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