Assessment of Ovine Johne’s disease in the Mandya sheep breed in South India using multiple diagnostic tests and bio-typing of *Mycobacterium avium* subspecies *paratuberculosis* infection

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**Abstract:** Johne’s disease is major infectious disease of domestic livestock in India. Despite low per animal productivity, the country is yet to initiate plan for the survey and control of disease at National scale. A total of 81 clinical samples from Mandya breed of sheep were collected from an organised farm (Livestock Research and Information Centre, Nagmangala) and farmer’s flocks suspected for Johne’s disease.

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**PUBLIC INTEREST STATEMENT**
The present study give the information about the high incidence of Ovine Johne’s disease in the Mandya sheep breed in South India. The prime interests of the authors were to sensitize the farmers and policy makers regarding there is immediate need for a Nationwide program for the control of Ovine Johne’s disease in the country in order to reduce the production losses and frequent passage of MAP in the domestic livestock species (inter-species transmission) and to human population through food chain, specially milk and milk products. As conclusion the authors suggested screening of all the animals before introducing in to the flock with multiple tests should be mandatory. Utility of multiple diagnostic tests is suggested for confirmatory detection and epidemiological diseases investigations of Johne’s disease in animals.
on the basis of clinical symptoms (weakness, emaciation, diarrhea and alopecia). Only 10.0% samples from two farms were screened for bio-load of Mycobacterium avium subspecies paratuberculosis (MAP) using multiple tests (microscopy, indigenous ELISA, IS900 PCR and culture). Results showed that 100.0% samples were positive for MAP infection both in microscopy and “Indigenous ELISA”. IS1311 PCR-REA bio-typed 60.0% (feces) and 100.0% (blood and tissue) isolates as “Indian Bison Type”. Typing investigated for the first time that biotype was of Indian origin and present in sheep flocks of South India having wide geographical distribution and broad host range. Study reported high bio-load of MAP in Mandya breed of sheep in Nagamangala, Mandya district and Bilagi taluk of Bagalkot district in South India. Being Johne’s disease a spectral disease, multiple tests were useful for the screening of MAP infection in sheep farms. Study emphasised the need for initiation of short and long term control programs to reduce production losses and sharing of MAP strains by other domestic livestock species and prevent contamination of human food chain and reduce risk to human population.

**Subjects:** Microbiology; General Science; Epidemiology

**Keywords:** Mycobacterium avium subspecies paratuberculosis; Ovine Johne’s disease; Mandya breed; Indigenous ELISA; IS900 PCR; culture

1. Introduction

Ovine Johne’s disease is a chronic incurable inflammation of intestines caused by Mycobacterium avium subspecies paratuberculosis (MAP). Though considered primarily a disease of domestic ruminants, MAP infection has been reported from wide range of host species such as wild ruminants, primates and human (Singh, Kumar et al., 2014; Singh, Singh, Singh, et al., 2013; Singh, Singh, Singh, & Sohal, 2010; Singh, Singh et al., 2014). Disease leads to significant economic losses to the dairy, meat and wool industry world-wide (Bauman, Jones, Menzies, Jansen, & Kelton, 2014; Chiodini, VanKruiningen, & Merkal, 1984; Norton, Johnson, Jones, & Heuer, 2010; Stau, Seelig, Walter, Schroeder, & Gaunter, 2012) including India (Rawat, et al., 2014; Sonawane & Tripathi, 2013; VinodhKumar, Gunaseelan, Ronald, & Sakthivelan, 2013).

Variable prevalence (15.1–36.0%) of Ovine Johne’s disease has been reported using traditional tests for diagnosis of Johne’s disease (Gupta, Rani, Agrawal, & Kumar Gupta, 2012; Singh et al., 2009; VinodhKumar et al., 2013). However, Collins et al. (2005) suggested that no single diagnostic test has been recommended as a confirmatory test for the screening of herds. Based on IS1311 PCR-REA, MAP strains have been genotyped as “Cattle type”, “Sheep type” and “Bison type” (Sevilla, et al., 2005; Whittington, Marsh, & Whitlock, 2001). There is only one report of “Bison type” strain isolated from bison in Montana, USA (Whitlock, Sweeney, Fyock, & Smith, 2005). From India a new biotype, “Indian Bison type” has been reported by Sevilla et al. (2005) from goats and sheep. Later this new bio-type has been found to be highly pathogenic to native livestock population and has widest host range (Singh, Singh, Singh, et al., 2010; Singh, Singh et al., 2014). It has been shown that virulence of MAP strains was ‘bio-type dependent’ (Gollnick et al., 2007; Hajra, Singh, Srivastava, Chakraborty, & Dhama, 2005).

Sharing of MAP bio-types between species (inter-species transmission) has been frequently reported (Sohal et al., 2008). Earlier studies in India, invariably reported endemicity of Johne’s disease in the livestock population and in established sheep farms primarily located in North India (Singh, Singh, Singh, et al., 2013; Singh, Singh et al., 2014).
Data presented in this study is not based on random sampling of the domestic livestock species; therefore, the term “Prevalence” was not used. Since the information is based on screening of animals suspected of suffering from JD in naturally infected research farm and farmer’s flocks, therefore, to represent the probable level of disease in animals, the term “Bio-load” was used. The present study was undertaken to estimate bio-load of Johne’s disease in Mandya breed of sheep at Livestock Research and Information Centre (LRIC), Karnataka Veterinary and Fisheries Science University (KVAFSU) Nagmangala farm and local breed of sheep in Bagalkot region of Karnataka state in South India. This is first comprehensive study to screen the clinical samples (tissue, blood, faeces, serum and milk) from suspected population of Mandya breed of sheep of Mandya district, Karnataka in South India, for the diagnosis of MAP infection using standard diagnostic tests (Microscopy, culture, PCR and “Indigenous ELISA kit”).

2. Material and methods

2.1. Collection of samples

During April 2015 a total of 81 clinical samples (10% samples of total farms) including feces (25), blood (2), serum (52), milk (1) and tissue samples (1) were randomly collected from suspected animals showing clinical symptoms of Johne’s disease (weakness, emaciation, diarrhea and alopecia) from two farms, an research farm organised farm (LRIC, KVAFSU, Nagmangala) and farmer’s sheep flocks (Bilagi taluk, Bagalkot) in India. Samples were sent to Microbiology laboratory at CIRG, Makhdoom and stored at −20°C till further processing.

2.2. Culture

Herrold’s egg yolk medium (HEYM) with mycobactin J was used for the culture of MAP from tissues as per Whipple, Callihan, and Jarnagin (1991), with some modifications (Singh, 1998). Approximately 2 g of fecal and tissue sample was finely grounded in sterilized pestle and mortar in 12 ml normal saline solution (NSS). Grounded material (12 ml) was centrifuged at 4,500 rpm for 45 min at room temperature. Supernatant was discarded and middle layer was decontaminated in 40 ml of 0.9% hexadecyl pyridinium chloride (HPC) for 18–24 h at room temperature. After decontamination and sedimentation, supernatant was removed slowly and the sediment was inoculated on the slants of HEYM with mycobactin J slants were incubated at 37°C and screened for the appearance of colonies every 2 weeks.

2.3. Fecal microscopy

Shedding of MAP in fecal samples was monitored by microscopy as per Singh, Singh, Gupta et al. (2013). Shedding of MAP was measured as quantitative (+1 as low shedders, +2, +3 as moderate shedders and +4 as heavy shedders) and any animal that was found positive (+1 to +4 scale) was taken as positive for MAP infection.

2.4. Molecular characterization and genotyping

2.4.1. IS900 PCR

DNA was isolated from the feces, blood, tissue and milk samples and subjected to specific IS900 PCR as per Singh, Singh, Kumar, Sohal, and Singh (2010) using primers used by Vary, Andersen, Green, Hermon-Taylor, and McFadden (1990). Presence and yield of the specific PCR product (229 bp) was considered as positive for MAP infection.

2.4.2. IS1311 PCR_REA

IS1311 PCR was carried out using M56 and M119 primers as per Singh, Singh, Kumar, et al. (2010). Restriction digestion (IS1311 PCR_REA) reaction was carried out using endonucleases HinfI and MseI as per Sevilla et al. (2005). Genotype profiles were interpreted as per Whittington et al. (2001).
2.5. Indigenous ELISA

Serum samples collected during the study were screened by using “Indigenous ELISA kit”, developed for screening of goats and sheep against Johne’s disease infection. Indigenous ELISA kit uses semi-purified protoplasmic antigen (PPA) from the highly virulent native isolate of MAP “Indian Bison type” biotype of goat origin. The test was originally developed and standardized in goats and sheep (Singh, Singh, Singh, Sohal, & Singh, 2007) and was also standardized in bovines (Singh, Singh, et al., 2015). Optical density values were transformed to Sample to Positive (S/P) ratio and animals in positive and strong positive categories in S/P ratio were considered as positive for MAP infection or sero-converts (Collins, 2002).

2.5.1. Analysis of OD (absorbance) values

Sample to Positive (S/P) ratio was calculated as follows (Table 1):

\[
S/P \text{ ratio value} = \frac{(\text{Sample optical density} - \text{Negative optical density})}{(\text{Positive optical density} - \text{Negative optical density})}
\]

3. Results

Out of the 52 serum samples screened, all (100.0%) sheep were positive for MAP antibodies using goat based “Indigenous ELISA kit”. In Indigenous ELISA kit, 41 (78.8%) and 11 (21.2%) sheep were in positive and strong positive categories, respectively (Table 2). Screening of fecal samples showed that all (100.0%) of the sheep were shedding MAP bacilli as revealed by microscopy (Table 3). Fecal, blood, milk and tissue samples were subjected to screening by IS900 PCR and 100.0% were positive for MAP infection (Table 3). Bio-typing of positive isolates by IS1311 PCR-REA categorized 60.0% (fecal samples) and 100.0% (tissue and blood samples) as “Indian Bison type”, bio-types (Table 3). In three tests combinations; 16 (59.2%) sheep were positive in all the three tests (IS900 PCR, Microscopy and IS1311 PCR-REA), 11 (40.7%) were positive in IS900 PCR and Microscopy (Table 4). Typical MAP colonies (multi-bacillary) started appearing from 60 days onwards and maximum colonies appeared around 90 days post inoculation on HEYM (Figure 1). Initially, colonies were very small pinpoint size and were white, rounded in appearance (Figure 1).

| S. No | Calculated value of S/P ratio | Johne’s disease status in animal |
|-------|-------------------------------|---------------------------------|
| 1     | 0.00–0.09                     | Negative                        |
| 2     | 0.10–0.24                     | Suspected or borderline          |
| 3     | 0.25–0.39                     | Low positive                    |
| 4     | 0.4–0.99                      | Positive                        |
| 5     | 1.0–10.0                      | Strong positive                 |

Note: Serum samples in positive and strong positive categories were taken as positive.

| ELISA test | Positive (P) | Strong Positive (SP) |
|------------|--------------|----------------------|
| Categories |              |                      |
| Sub-total  | 41 (78.8)    | 11 (21.2)            |
| Total      | 52 (100%)    |                      |

Note: Serum samples in positive (P) and strong positive (SP) categories were taken as positive.
4. Discussion

Like many other developing and un-developed countries, in India also, sampling of desired animals based on random table number is one of the biggest constrains. Therefore, information on the National prevalence of Ovine Johne’s disease and losses caused by disease, as available in developed countries, is extremely limited. In these conditions it is not easy to present easy picture of diagnosis especially in chronic infections like Johne’s disease, where clinical symptoms are also not specific; therefore it is prudent to use multiple screening tests.

In the present study, fecal microscopy and “Indigenous ELISA kit” were used as screening tests. IS900 PCR and IS1311 PCR were used as confirmatory tests. Limited numbers of samples were bio-typed (IS1311 PCR_RE) to know MAP bio-type infecting Mandya sheep. This study reports high bio-load of MAP infection in the suspected population of Mandya breed of sheep belonging to Nagmangala KVAFSU farm and Bagalkot region of South India. Higher bio-load of MAP directly correlated with low

| Table 3. Screening of clinical and necropsy samples for the diagnosis of Mycobacterium avium subspecies paratuberculosis infection in the Mandya breed of sheep from Livestock Research Information Centre, Karnataka Veterinary Animal Fisheries Sciences University, Nagmangala farm and Bagalkot region (India) |
|---|---|---|
| **Samples** | **Positives n (%)** | **Microscopy** | **IS900 PCR** | **IS1311 PCR-REA** |
| Faeces (n = 25) | 25 (100.0) | 25 (100.0) | 15 (60.0) |
| Tissue (n = 1) | 1 (100.0) | 1 (100.0) | 1 (100.0) |
| Milk (n = 1) | 1 (100.0) | 1 (100.0) | 0 (0.0) |
| Blood (n = 2) | ND | 2 (100.0) | 2 (100.0) |
| Total (n = 29) | 27 (100.0) | 29 (100.0) | 18 (62.0) |

Note: 27/27 = 100.0%, because two blood microscopy samples were not done

| Table 4. Comparative study of different tests for the detection of Mycobacterium avium subspecies paratuberculosis in 27 samples (faeces, milk and tissue) of sheep from Livestock Research Information Centre, Karnataka Veterinary Animal Fisheries Sciences University, Nagmangala farm and Bagalkot region (India) |
|---|---|---|---|---|---|---|---|---|
| **Diagnostic tests** | **Test combinations** | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| **Microscopy** | + | − | + | − | + | + | − | − |
| **IS900 PCR** | + | − | + | + | − | − | + | − |
| **IS1311 PCR-REA** | + | − | − | + | + | − | − | + |
| **Total (n = 27)** | 16 (59.2) | 0 (0.0) | 11 (40.7) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

Notes: Figures in parenthesis are percentages. + = positive in test, − = Negative in test.
1 to 8 are maximum permutation and combinations possible in 3 test regimen.

![Figure 1. Mycobacterium avium subspecies paratuberculosis colonies on HEY medium.](image-url)
per animal productivity and increased risk of human exposure (VinodhKumar et al., 2013). Present study reported 100.0% bio-load of MAP infection in suspected sheep population (farm and farmer’s flocks) by microscopy, “Indigenous ELISA kit” and IS900 PCR. High incidence of MAP as reported in the present study may be due to high endemicity of disease and screening of limited number of suspected sheep. In bio-typing using IS1311-REA, “Indian Bison Type” strain of MAP was found the dominant biotype in the flocks studied. Singh, Singh et al. (2014) also reported high (32.7%) bio-load of MAP in sheep population in the last 28 years (1985 to 2013) of survey in India. “Indian Bison Type” was also reported earlier as the dominant biotype, irrespective of domestic livestock species screened and the geographical region surveyed (Singh, Chauhan et al., 2015). Recent study by Sonawane and Tripathi (2013) reported 100.0% sensitivity for detection of MAP in sheep with the help of quantitative real-time PCR (qPCR) assay employing IS900 gene specific primers of MAP. Earlier, in sheep, prevalence of MAP was found to be moderate in sheep from Tamil Nadu as compared to Rajasthan (Singh, Singh, Gupta et al., 2013). Unlike holy cows, buffaloes, goats and sheep undergo high rate of slaughter in order to meet ever growing demand for meat. This high rate of slaughter indirectly result in “natural selection” in these three livestock species by regular removal of low productive animals (personal experience of corresponding author). Moreover, similar findings related to higher incidence of MAP have been reported in the four4 domestic livestock species in different regions of the country using multiple screening tests (Barad et al., 2013; Gupta et al., 2012; Shroff et al., 2013; Singh, Singh, Singh, et al., 2013; Singh, et al., 2008). In the four diagnostic tests used for the screening of sheep against MAP infection, microscopy was found to be more sensitive because shedding was often seen prior to peripheral immune response (Lybeck, Storset, Djønne, Valheim, & Olsen, 2011). However, multiple tests were found to be useful for the screening of MAP infection in domestic livestock of the country. High bio-load of MAP in livestock was directly associated with low productivity of domestic livestock in India, which in turn increased the chances of spread to human population (Singh, Singh, Gupta et al., 2013). Hence, there is immediate need for a Nationwide program for the control of Ovine Johne’s disease in the country in order to reduce the production losses and frequent passage of MAP in the domestic livestock species (inter-species transmission) and to human population through food chain, specially milk and milk products. This is the first exploratory study reporting high bio-load of MAP infection in the Mandya breed of sheep in South India. Similar to the findings in North India by Singh, Singh et al. (2014) “Indian Bison Type” bio-type was the dominant bio-type infecting Mandya breed of sheep flocks in South India.

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Competing Interests
The authors declare no competing interest.

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