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Short Paper

Comparison of microscopy and blood-PCR for the diagnosis of clinical Johne's disease in domestic ruminants

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Summary

Microscopy as "field based test" was compared with IS*900* blood PCR for the diagnosis and estimation of prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in domestic livestock with clinical Johne's disease (JD). Of 252 animals screened, 39.3 and 13.1% were positive by faecal microscopy and blood PCR, respectively. Proportional agreement between "microscopy" and blood PCR was substantial. 1262 faecal samples from animals suspected of clinical JD were screened by microscopy in 2009-2010 and 41.3% were positive. Prevalence of MAP was highest in cattle (61.8%), followed by goats (41.3%), sheep (33.4%) and buffaloes (15.7%). Faecal microscopy was cost effective, easy to adopt and repeatable for the screening of domestic ruminant population against MAP infection.

Key words: *Paratuberculosis*, Ziehl Neelsen staining, IS*900* PCR, India

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) has emerged as a major animal pathogen. Moderate to high prevalence of MAP has been reported in the domestic livestock population of the country. Increased prevalence of clinical Johne's disease (JD) in domestic ruminants is directly correlated with increased population of low productive animals (Vinodhkumar *et al*., 2013). Animals (goats, sheep and buffaloes) which can be salvaged are preferred and their population showed an increasing trend in the last 50 years. Cattle population has shown decreasing trends in the last decade due to a ban on cow slaughter. Johne's disease is primarily responsible for low per animal productivity in domestic livestock and is a major concern plaguing the Indian dairy industry (Singh *et al*., 2008). *Mycobacterium avium* subspecies *paratuberculosis* is continually entering in the human food chain since MAP is not inactivated during pasteurization (Grant *et al*., 2002). Recently MAP has been associated with Inflammatory Bowel Disease (IBD) or Crohn's Disease (CD) in human beings. Presence of MAP in human gut (Feller *et al*., 2007) and recovery of MAP from human breast milk (Naser *et al*., 2000), has increased concern for the control of MAP in animals.

Critical issues in the diagnosis of MAP are: endemicity of MAP infection, low sensitivity of diagnostic tests, infrequent shedding of MAP, fastidious nature and high cost of imported kits. Johnin (field test) lacks sensitivity and specificity and is known for false positive reactions (Kalis *et*

al., 2003). Cellular immune response is stronger in early infection, which diminishes with progression of disease and animals may be completely non-reactive to PPD (Johnin) at later stages of infection (Perez *et al*., 1996). IFN-γ assay though sensitive require a sophisticated laboratory besides being costly, better in early infection and has low utility for routine diagnosis (Kalis *et al*., 2003). ELISA, agar-gel immuno-diffusion and CF tests offer rapid detection of antibodies in clinical cases. Tests have varying sensitivity and specificity, particularly in sub-clinical JD. Culture though "Gold standard", need long incubation and is not popular. In PCR, isolation of DNA from low shedders is difficult, presence of MAP in blood is transient, high cost of DNA kits, and PCR reactions do not favour it as a routine screening test. In absence of a "field test", information on national prevalence of MAP is limited, therefore disease lacks priority for control in domestic livestock. This study evaluated efficacy of microscopy with blood PCR and for estimation of prevalence of MAP in the domestic livestock suspected for Johne's disease.

Materials and Methods

Animals

Faecal and blood samples of 252 animals (39 goats, 133 sheep and 80 cattle) were screened for MAP infection using microscopy and IS*900* PCR. Faecal samples from small (281 goats and 602 sheep) and large (309 cattle and 70 buffaloes) ruminants, from different geographical regions (Mathura, Agra, Palampur, Bikaner, Bhopal, Raibareilly, Mannavanur) were submitted to Animal Health Division of CIRG, Makhdoom from 2009 to 2010 for screening against MAP infection using microscopy.

Microscopy

Two grams of faecal sample was finely ground in pestle and mortar with distilled water and centrifuged at 4000 rpm for 45 min at room temperature (RT). Supernatant was discarded, smears were prepared from middle layer, stained by Ziehl Neelsen (ZN) and examined under oil immersion (×100) for acid-fast bacilli (AFB) indistinguishable to MAP (Fig. 1). Nearly 10 fields were screened and samples were categorised as pauci and multi-bacillary (+1, 10 bacilli or one bunch; +2, 10 bacilli or one bunch in alternate of 2, 3 or 4 field; +3, 10 bacilli or one bunch in each alternate field; +4, 10 bacilli or one bunch in each field). The +4, +3 to +2 and +1 were taken as super, moderate and low shedders, respectively.

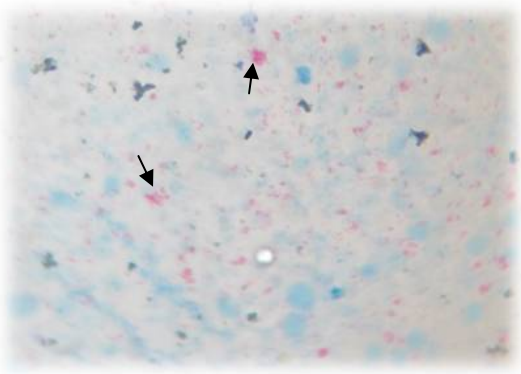

Fig. 1: Microscopic view of acid fast bacilli (indistinguishable to MAP under oil immersion ×100)

Blood PCR

Animals positive in microscopy were processed for DNA extraction and IS*900* PCR. DNA extracted from blood samples was subjected to specific IS*900* PCR as per Singh *et al*. (2010), Briefly, PCR was set in 50 µl, using 1.0-5.0 ng template DNA, 5 µl of 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer and 5 U Taq. Cyclic conditions were: initial denaturation at 94°C for 3 min, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 7 min. Amplicon sizes of 413 bp were considered positive, after separation on 1.5% agarose gel stained with ethidium bromide (Fig. 2). MAP IS*900* primer sequences used:

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(i) forward primer- P90B 5′- GAA GGG
TGT TCG GGG CCG TCG CTT AGG -3′
(ii) reverse primer- P91B 5′- GGC
GTT GAG GTC GAT CGC CCA CGT GAC -3′
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Results

Of 252 animals (39 goats, 133 sheep, 80

cattle) screened, 99 (39.3%) were positive in faecal microscopy and 20, 10, 3 and 66 were +4, +3, +2 and +1 level shedders, respectively. Prevalence of MAP was 3 times higher (68.8%) in cattle as compared to goats and sheep (25.5-25.6%). Screening of blood samples of 99 animals, 33 (33.3%) were positive in blood PCR (Table 1). PCR detected more animals from super (+4) and moderate (+2 to +3) shedders and none from low (+1) shedders (Table 2). Sensitivity of blood PCR to detect positive cases from low shedders was low as compared to faecal microscopy. Proportional agreement between "microscopy" and blood PCR was substantial (72.0%). Of 1262 animals screened, 41.3% were positive in microscopy. Prevalence of MAP was highest in cattle (61.8%), followed by goats (41.3%), sheep (33.4%) and buffaloes (15.7%) (Table 3).

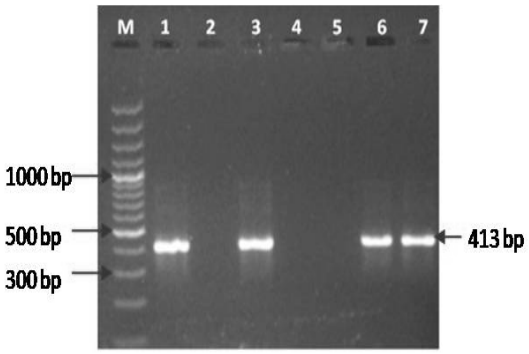

Fig. 2: MAP specific amplicons (413 bp) by PCR using IS*900* specific primers. Lane M: 100 bp ladder. Lane 1: Positive control (MAP DNA), Lane 2: Negative control (miliQ water), and Lane 3-7: DNA samples isolated from blood

Discussion

Due to poor sensitivity, specificity and reproducibility of Johnin, there is no genuine field-test for diagnosis of MAP infection. Present study detected higher number of animals positive in faecal microscopy (39.3%) as compared to blood PCR (33.3%). Earlier studies reported higher sensitivity of microscopy as compared to ELISA, faecal and blood PCR, higher detection of MAP by faecal microscopy may be attributed to screening of animals with clinical JD. In the present study, prevalence of MAP was highest in cattle (61.8%) followed by goats (41.3%), sheep (33.4%) and buffaloes (15.7%), which correlate with slaughter rate of these livestock species. Due to a ban on cow slaughter, the number of low and unproductive cows has increased in the country (Fig. 3), therefore prevalence of MAP was significantly higher in cattle as

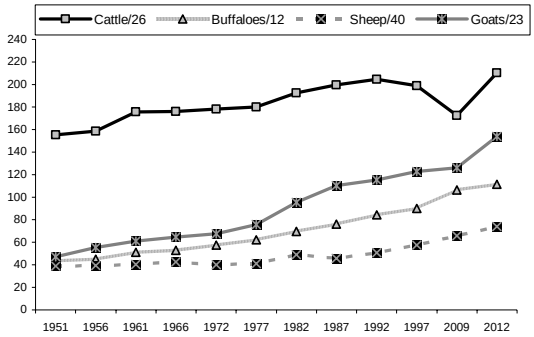

Fig. 3: Population of livestock in India (in Millions) (Source: FAO, 2012)

Table 1: Comparative evaluation of microscopy and blood PCR for the screening of MAP infection in domestic livestock

Species	Microscopy		Blood PCR	
	Animals (n)	Positive (n) (%)	Animals (n)	Positive (n) (%)
Goats	39	10 (25.6)	10	0 (0.0)
Sheep	133	34 (25.5)	34	11 (32.3)
Cattle	80	55 (68.8)	55	22 (40.0)
Total	252	99 (39.3)	99	33 (33.3)

Table 2: Comparative evaluation between faecal microscopy and blood PCR for detection of MAP

Animals	Faecal microscopy		Blood PCR
	Rate of shedding	Positive (n) (%)	Positive (n) (%)
99	+1	66 (66.6)	0 (00.0)
	+2	3 (3.0)	3 (100)
	+3	10 (10.1)	10 (100)
	+4	20 (20.2)	20 (100)

Table 3: Prevalence of MAP infection in the domestic livestock by faecal microscopy (2009-2010)

Place	Source of sample (organised farm/farmer)	Species	Animals (n)	Positives (n) (%)
Mathura (UP), Raibareilly (UP), Dantiwada* (Guj.), Bhopal* (MP), Palampur* (HP)	Both	Goats	281	116 (41.3)
Mannavanur* (TN), Bikaner* (Raj.)	Both	Sheep	602	201 (33.4)
Agra (UP), Mathura (UP), Dantiwada* (Guj.), Palampur* (HP)	Both	Cattle	309	191 (61.8)
Mathura (UP), Mannavanur (TN)	Farmer	Buffaloes	70	11 (15.7)
Grand total			1262	519 (41.1)

* Animals from organized farm. UP-Uttar Pradesh, Guj.-Gujarat, MP-Madhya Pradesh, Raj.-Rajasthan, HP-Himanchal Pradesh, TN-Tamil Nadu

compared to goats, sheep and buffaloes.

Despite high slaughter rate, prevalence of MAP was higher in goats due to higher susceptibility to MAP. Despite high slaughter rate in goats, endemicity of MAP has been high due to poor thermo-regulation and poor ability to drive nutrition in the grazing area deficient of green shrubs and grasses. Sheep had better resistance to MAP due better conservation of energy due to wool and lower effect of temperature stress and ability to graze in degraded pasture. High to very high slaughter rate of low productive buffaloes and better thermo-regulation of energy due to black colour was directly related to low prevalence of MAP in this species.

Presence of MAP in blood PCR indicated dissemination of MAP to other organs than intestines (Hines *et al*., 1987) through blood cells. MAP being an intracellular organism is disseminated by blood phagocytes to extra intestinal sites (Zurbrick and Czuprynski, 1987). Information on MAP bacterimia and time of dissemination in blood stream needs to be further explored for proper sampling time.

Faecal microscopy detected 2-3 times higher animals for MAP infection as compared to blood PCR. However, specificity of microscopy depends largely on experience of user. Blood PCR detected more cases from heavy MAP shedders in microscopy. Low positivity in blood PCR may be due to presence of MAP in blood stream for a limited period. Prevalence of MAP was very high (41.1%) in domestic ruminant population which may be due to screening of animals with suspected and clinical JD, since JD is endemic in native herds and flocks.

Study concluded that in absence of "field test" faecal microscopy can be adopted as "field based test", since faecal microscopy is cost effective, easy to adopt and repeatable in mass screening of animals to estimate national prevalence of JD in countries with large population of domestic livestock and limited resources. This study exhibited increased bio-load of MAP in the domestic livestock population of the country and need for implementation of control programmes at national level.

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