BIO-LOAD OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN BUFFALOES

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a causative agent of Johne's disease, a disease with considerable economic impact on dairy buffalo herds. The present study was undertaken to assess the prevalence of MAP infection in buffaloes and its excretory pattern through buffalo milk. A total of 74 milk samples were collected from apparently healthy buffaloes of organized and unorganized sectors located in Tamil Nadu and subjected to Ziehl-Neelsen staining and Polymerase chain reaction (IS900 and F57 genes). Out of 74 samples, 3 (4.1%), 21 (28.4%) and 14 (18.9%) samples shed MAP organism by Ziehl-Neelsen staining, IS900 PCR and F57 PCR respectively. Besides age of the animal, stage of lactation and herd management were associated with excretion of MAP in milk. These results showed the high prevalence of MAP infection in buffaloes and warrants further studies and necessary actions to delineate the MAP infection in buffalo population.

Keywords: buffalo, *Bubalus bubalis*, *Mycobacterium avium* subsp. *paratuberculosis*, Ziehl-Neelsen staining, IS900 PCR, F57 PCR

INTRODUCTION

India ranks first in the world buffalo population with 115.4 million buffaloes (57.7 %) of the world which is primarily reared for milk, meat and draught power (FAO, 2015; Perisic *et al*., 2015). It is an integral part of agriculture sector that plays a vital role in mitigation of poverty, providing livelihood security as well as employment among farming community living in developing countries (Warriach *et al*., 2016). Though India globally ranks first in total milk production, per animal productivity remains low and production losses were unnoticed and under estimated in India (Barburah and Joseph, 2008). This might be due to chronic infection like Johne's disease (Audarya *et al*., 2016).

Paratuberculosis (Johne's disease) is an incurable chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis*

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(MAP) (Sweeney *et al*., 2012). In recent times, the zoonotic importance of MAP is also alarming one due to the similarities that appear to exist between bovine paratuberculosis and human Crohn's disease (Liverani *et al*., 2014; Cossu *et al*., 2017). Consumption of unpasteurized milk or milk products harboring MAP from paratuberculosis infected animals was speculated as a potential source of MAP infection in humans (Pierce, 2010; Patel and Shah, 2011). Hence, the present study was designed to assess the current status MAP infection in buffaloes and to ascertain the influence of age, stage of lactation and herd management in the lactogenic excretory pattern of MAP.

MATERIALS AND METHODS

Collection of sample

A total of 74 milk samples were collected from apparently healthy buffaloes of organized and unorganized dairy sectors located in Tamil Nadu, India. Prior to collection of samples, udder was disinfected with 70% alcohol samples and about 15 to 20 ml of milk was collected from four quarters of udder after discarding the initial few strips. The samples were transported to the laboratory in igloo boxes and stored at -20°C until further processing. At the time of sample collection various factors like age, stage of lactation and herd management also were recorded.

Sample processing

About 10 ml of each milk sample was centrifuged at 3000 rpm for 15 minutes and the supernatant (including fat and cream layer) was discarded. The smears were made from sediment pellet and stained with commercial Ziehl-Neelsen staining kit (Hi-Media, Mumbai) as per the manufacturer's instructions. Intensity of MAP shedding in milk sample were categorized as +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) and +4 (10 bacilli or one bunch in each field) (Singh *et al*., 2014). Remaining sediment pellet was washed thrice with sterile phosphate buffer saline solution (pH 7.2) and DNA was extracted by using commercial DNA extraction kit (Qiagen Biotech, Germany) as per the manufacturer's instructions.

Polymerase chain reaction (PCR)

The DNA was amplified by PCR using specific IS900 primers (Forward: 5'- CGT CGT TAA TAA CAA TGC AG -3'and Reverse: 5'- GGC CGT GCG TTA GGC TTC GA -3') (Giese and Ahrens, 2000) and F57 specific primers (F57 Forward: 5'- CCT GTC TAA TTC GAT CAC GGA CTAG A -3' ; F57 Reverse: 5'- TCA GCT ATT GGT GTA CCG AAT GT -3' and F57 Nested Reverse: 5'- TGG TGT ACC GAA TGT TGT TGT CAC -3') (Vansnick *et al*., 2004). Amplified products were subjected to electrophoresis in 2% agarose gel photographed with a gel documentation system (Bio-Rad, USA).

Statistical analysis

Agreement and association between Ziehl-Neelsen staining and PCR assays in detection of MAP infection were evaluated by Kappa index (*K*) and Chi-square test ($\chi 2$) with a confidence interval of 95% (Martin *et al*., 1987) respectively. Furthermore, relative sensitivity and specificity were also calculated (Martin *et al*., 1987).

RESULTS AND DISCUSSION

Out of the 74 milk samples screened, 3 (4.1%) showed the presence of acid fast bacilli among that 2 (66.7%) and 1 (33.3%) were categorized as +2 and +3 respectively. Polymerase chain reaction assays revealed that 21 (28.4%) and 14 (18.9%) samples were positive by IS900 and F57 PCR, respectively. The presence of MAP in milk could be due to udder invasion through the teat channel or as a result of systemic dissemination (Ayele *et al*., 2004). The true prevalence of MAP could be even higher due to irregular and intermittent shedding of MAP organisms in milk (Nielsen and Toft, 2008).

Paratuberculosis is endemic in the cattle population of India and its sero-prevalence varies from 13.3 to 41.0% in the different parts of India (Singh *et al*., 2008; Trangadia *et al*., 2012; Gupta *et al*., 2012; Audarya *et al*., 2016; Bhutediya *et al*., 2017; Bharathy *et al*., 2017). The present study revealed the quite high prevalence of MAP infection in buffaloes might be due to collection of samples from known positive herds and also attributable to diversity in topography and environment, animal rearing system, and husbandry practices followed in the study area.

Comparative evaluation of Z-N staining with IS900 PCR showed a low sensitivity of 14.3% and specificity of 100%. The kappa value between these tests showed poor agreement (K=0.19) and chi-square test indicated a significant difference between the tests ($\chi 2$=14.32; P<0.95). Low sensitivity of Z-N staining could be due to prerequisite of $>10^3$ to 10^4 organisms per gram for positive microscopy as well as shedding low quantity of organisms by apparently healthy animals (Chiodini *et al*., 1984; Pillai and Jayarao, 2002).

On comparison of IS900 PCR with F57 PCR, the latter showed a sensitivity and specificity of 66.7 and 100 %, respectively. Kappa value showed substantial agreement (K=0.19) and chi-square test indicated a significant difference between the tests ($\chi 2$=39.37; P<0.95). Similar studies by Youssef *et al*. (2014); El-Malek *et al*. (2015) were also found F57 PCR had sensitivity of 71.42% and 55% respectively with IS900 PCR. It could be attributed to the greater number of IS900 copies in the MAP genome whereas F57 sequence presents as a single copy which may reduce the sensitivity of the F57 PCR in this study (Tasara and Stephen, 2005).

Influence of age, stage of lactation and herd management in the lactogenic excretion pattern of MAP is presented in Table 1. The present study showed that the early stages of lactation had more shedding of MAP (35.7 and 25.0%) followed by late (25.8 and 16.12%) and mid (20.0 and 13.33%) stages of lactation by using IS900 and F57 PCR respectively. Our results were similar to findings of Bradner *et al.* (2013); Prabu (2015); Laurin *et al.* (2017) who reported that MAP primarily shed into milk and colostrum in early lactation while mid and late lactation sheds the organism less frequently. The dissimilarity of MAP infection in different stages of lactation could be due to high levels of stress associated with parturition, pregnancy and peak milk production in dairy cows (Chiodini *et al*., 1984; Norton *et al*., 2010; Sweeney, 2011).

The prevalence of MAP infection among the buffaloes aged above five years showed comparatively higher prevalence (33.9 and 24.5%) than the animals aged less than five years (14.3 and 4.8%) using the IS900 and F57 PCR respectively. Our results are in agreement with Prabu, (2015) and Carvalho *et al.* (2009) who recorded higher MAP infection in older cows than younger

animals. This could be due to chronic nature of the disease that entails the late clinical manifestation of paratuberculosis as late as 3 to 5 years after infection (Chiodini *et al*., 1984) and clinical disease is not usually apparent in cattle until 2 to 5 years of age (Olsen *et al*., 2002).

In addition to that, unorganized dairy herds (19.6 and 10.9%) showed less positivity than organized herds (42.9 and 32.1%) using the IS900 and F57 PCR respectively. Our results are in agreement with Mohan *et al.* (2009); Bhutediya *et al.* (2017) which found that the prevalence of paratuberculosis in unorganized rural dairy farms was lower than organized dairy farms.

The present study signified the prevalence of MAP infection among the buffalo population of Tamil Nadu. However, a comprehensive study with of a large number of samples is required to find the prevalence pattern of paratuberculosis in Tamil Nadu. In addition the present study also revealed the influence of herd management, age and lactation stage of animals on shedding of MAP in milk.

CONCLUSION

Though India leads first in milk production, average productivity per animal still remains very low. *Mycobacterium avium* subsp. *paratuberculosis* is one of established pathogen in Indian livestock industry that leads to reduced productivity of domestic livestock with significant consequences for farming economy. The present study revealed that MAP infection among the buffalo population was quite high which warrants suitable control measures to put off further spread MAP infection to other animals as well as humans.

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Table 1. Molecular prevalence of map in buffalo milk samples.

BUFFALO (N=74)		IS900 PCR		F57 PCR	
		Prevalence = 21/74 = 28.4%		Prevalence = 14/74 = 18.9%	
		Positive	Prevalence	Positive	Prevalence
Herd management	Organized	12/28	42.9%	9/28	32.1%
	Unorganized	9/46	19.6%	5/46	10.9%
Age	<5 years	3/21	14.3%	1/21	4.8%
	>5 years	18/53	33.9%	13/53	24.5%
Stage of lactation	Early	10/28	35.7%	7/28	25.0%
	Middle	3/15	20.0%	2/15	13.33%
	Late	8/31	25.8%	5/31	16.12%

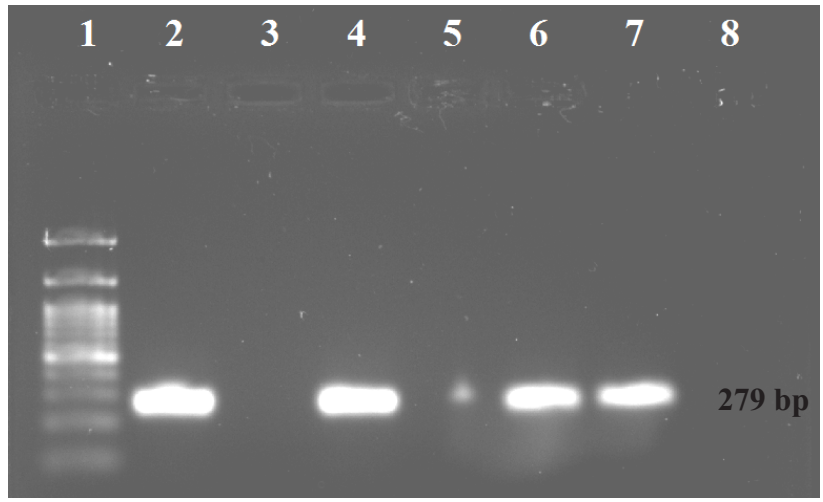

Figure 1. PCR showing band at 279 bp using IS900 primer for MAP.
Lane 1: Ladder (100bp),
Lane 2: Positive control,
Lane 3: Negative control,
Lane 4-8: Samples

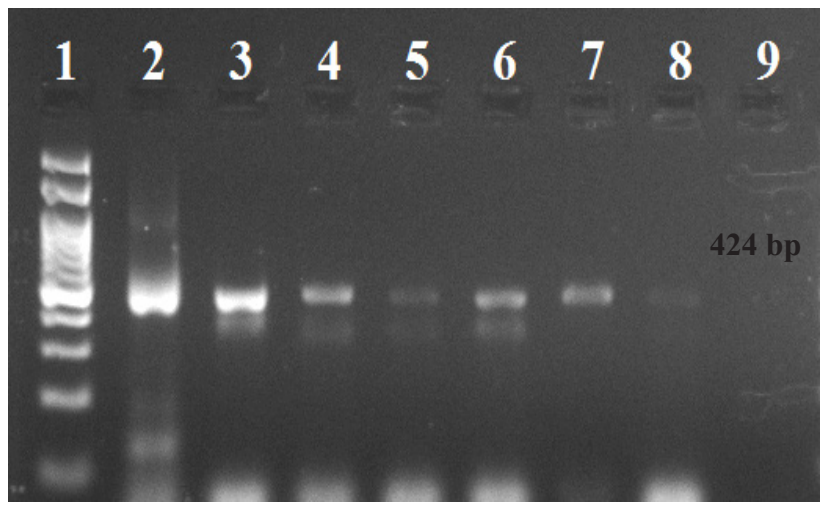

Figure 2. PCR showing band at 424 bp using F57 primer for MAP.
Lane 1: Ladder (100bp),
Lane 2: Positive control,
Lane 3-8: Samples,
Lane 9: Negative control

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