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Serological, Microscopical and Molecular Examinations of Cow Milk Samples for Diagnosis of Paratuberculosis at Mhow of Madhya Pradesh in India

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A B S T R A C T

Mycobacterium avium subspecies paratuberculosis (MAP) is a Gram positive, acid-fast bacterium which causes Johne's disease or Paratuberculosis in domestic livestock species. For screening of cows for MAP infection a total of 103 milk samples from individual cows were collected from organized (57) and unorganized (46) farms. Presence of anti-MAP antibodies in milk were screened by indirect enzyme linked immunosorbent assays (iELISA) test, 72 (69.90%) cow milk samples were found positives (66.07% from organized farms and 74.46% from unorganized farms). Positivity percentages were 62.50 in Gir cows and 70.52 in non-descriptive and crossbred cows irrespective of the farming system for the presence of MAP antibodies. Milk samples from 13 clinically suspected cows were used to prepare smears on glass slides and stained by Ziehl-Neelsen method. Microscopically 2 cow milk samples were found positive for presence of acid-fast organisms. Extracted nucleic acid, deoxyribose nucleic acid (DNA) from strong positive milk sample in microscopy was further tested in polymerase chain reaction (PCR) to amplify MAP DNA using insertion sequence 900 (IS900) specific primers. Except in positive control, specific amplifications were not visualized for test samples after agar gel electrophoresis. The present study indicates circulation of *Mycobacterium avium subspecies paratuberculosis* in cattle population of Mhow of Indore in Madhya Pradesh.

Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) is an intracellular pathogen, responsible for an infectious and contagious disease, Johne's disease or Paratuberculosis in domestic livestock species (cattle, sheep, goats, buffalo, camels) (Chaubey *et al.,* 2017)

as well as long range of wild animals, (antelope, deer, rabbits, blue bull, monkeys as well as human being (Gumussoy *et al.,* 2015). Primary route of MAP infection is ingestion of the bacterium in fecal-contaminated feed, milk, colostrum and water. Presence of MAP also reported from soil, river water, pastures, environment as well as in biofilms present in watering troughs (Chaubey *et al.,* 2017).

The disease is transmitted *in utero* and via milk and colostrums to calves, and fecal-orally to all age classes (Robins *et al.,* 2015). MAP has been recognized as an important animal pathogen with significant zoonotic and public health concerns (Sonawane and Tripathi, 2016).

It causes huge production losses and has high impact on animal industry (Deb *et al.,* 2011; Rawat *et al.,* 2014). The disease can cause substantial economic losses to the cattle industry hence utmost attention is imparted to the control (Rawat *et al.,* 2014; Garcia and Shalloo, 2015). MAP infected animals shed viable MAP in their faeces and milk. MAP may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products.

The current methods of milk pasteurization are not sufficient to kill all MAP cells present in milk, and MAP has been cultured from raw and pasteurized milk and isolated from cheese.

The presence of MAP in milk samples can be detected via culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) (Gumussoy *et al.,* 2015). The present study reports serological, microscopical and molecular investigations of milk samples from cows at Mhow of Indore in Madhya Pradesh for the diagnosis of Paratuberculosis.

Materials and Methods

Livestock

Cows of Gir breed and non-descriptive and crossbred adult (unvaccinated for protection against Paratuberculosis) from Mhow were used in the present study. The cows suffering from chronic diarrhea and emaciation are grouped as clinically suspected cows.

Collection of milk samples

About 3 ml of milk was collected in sterile container from individual cows. Milk samples were collected aseptically (before collection washed the udder and discarded first few stripes of milk) in sterile containers. A total of 103 milk samples were collected from individual cow from organized (57 milk samples) and unorganized (46 milk samples) farms. Milk samples were collected during the year 2017.

Storage and transportation of samples

Milk samples after the collection immediately brought to the Veterinary Microbiology laboratory of the college and kept at frozen conditions at -20°C. These milk samples were transported under cold chain conditions to Animal Health Laboratory of Central Institute for Research on Goats (CIRG), Makhdoom, Farah-281 122 and also kept at -20°C till its use in indirect enzyme linked immunosorbent assay (iELISA) test, an indigenously developed test by CIRG.

Indirect enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA), an immunological test is most widely available and commonly used to detect *Mycobacterium avium subspecies paratuberculosis* (MAP) antibody for

diagnosis of Paratuberculosis (Slana *et al.*, 2008). In the present investigation, indirect ELISA (iELISA) test was employed to detect presence of antibodies against Paratuberculosis in milk samples. Indigenous ELISA kit developed for goats has been previously standardized for screening of cattle in India (Singh *et al.*, 2007; Sharma *et al.*, 2008). Antigen coated flat bottom 96 well ELISA plates were used in the study. Plates were washed thrice with washing buffer composed of phosphate buffer saline (PBS) and 0.05% Tween-20, pH 7.4 (PBST) and blocked by using blocking buffer (3% skimmed milk powder in PBS). These plates were incubated at 37°C for 1 hr. Plates were washed thrice with PBST. Now a volume of 100 µl of 1:2 diluted test whole milk samples in duplicates (diluted using buffer containing 1x PBST with 1% bovine serum albumin, BSA) was added to the wells of these plates and incubated for 2 hr at 37°C. These plates were washed thrice with 1x PBST. Then in the wells of the plates 100 µl of diluted conjugate in ratio 1:5000 (anti-bovine) in 1x PBS was added and incubated for 1 hr at 37°C. The plates were washed 4 times with 1x PBST. Finally, 100 µl of freshly prepared substrate o-phenylenediamine dihydrochloride (OPD) was added at the concentration of 5 mg per plate in substrate buffer (pH 5.0) and incubated (in the dark) for 3-5 min at room temperature. The absorbance at 450 nm in ELISA reader (Bio-Rad) after adding stop solution (5N Sulfuric acid, H_2SO_4) was recorded. The results were noted after ensuring to run blank, positive and negative controls with test milk samples in each plate.

Analysis of absorbance values

Optical density (OD) value of samples at 450 nm were converted to S/P (sample to positive) ratio by using following formula: SP ratio value = OD at 450 nm of test milk sample - OD at 450 nm of negative control / OD at 450 nm of positive control - OD at 450 nm of negative control.

Microscopy

For microscopic examination, a total of 13 milk samples from clinically suspected cows were randomly selected. Milk smears were prepared on glass slides from these milk samples and stained by Ziehl-Neelsen staining method and examined for presence of acid-fast organisms microscopically with light microscopy (Nikon) (Singh *et al.*, 2009; Jatav *et al.*, 2018).

Nucleic acid extraction

Deoxyribonucleic acid (DNA) was extracted from microscopically acid-fast organisms positive milk samples (Singh *et al.*, 2009). A total volume of 500 µl of milk sample was added into 100 µl of milk lysis buffer (50 mM Sodium chloride (NaCl), 125 mM ethylenediaminetetra acetic acid (EDTA), 50 mM Tris-hydrochloride (HCl); pH 7.6) and incubated at room temperature for 15 min.

Thereafter 100 µl of 24% Sodium dodecyl sulfate (SDS) was added and it was incubated at room temperature for 10 min and heated at 80°C for 10 min. Now 20 µl of proteinase K (10 mg/ml) was added to the above mixture and incubated at 56°C for 2 hrs. 100 µl of 5M NaCl and 64 µl Cetyl trimethylammonium bromide (CTAB)-NaCl were added and mixed to incubate at 65°C for 30 min.

After adding equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1), the mixture was centrifuged at 10000 revolutions per minute (rpm) for 15 min at 4°C. This step was repeated once again and the aqueous phase was transferred to sterilized Eppendorf tube. DNA was precipitated by adding 0.6 volume of chilled isopropanol and the tube was kept at -20°C for overnight. DNA was

pelleted by centrifuging at 10000 rpm for 20 min at 4°C. The pellet was washed with 1 ml of 70% ethanol and re-suspended in 30 µl Tris- ethylenediaminetetra acetic acid (TE) buffer. DNA was stored at 4°C for overnight duration to completely dissolve and then finally it was stored at -20°C.

Polymerase chain reaction

Deoxyribonucleic acid (DNA) extracted from milk samples positive for acid-fast organisms in microscopy were used in polymerase chain reaction (PCR) test. It was employed to amplify insertion sequence 900 (IS 900) gene which is specific for Paratuberculosis to confirm presence of *Mycobacterium avium subspecies paratuberculosis* (MAP) organisms in milk samples.

DNA extracted from individual milk sample was screened by using P90 and P91 primers specific for IS900 (Millar *et al.*, 1996) to amplify product of 413 bp. PCR test was performed in a total volume of 30 µl in 200 µl separate PCR tubes containing 5 µl extracted DNA (template) from individual milk samples, positive and negative controls, 3 µl each of forward and reverse primers, 1 µl of Taq DNA polymerase (5U/ µl), 2.4 µl of Magnesium chloride (25 mM), 2.5 µl of 2 mM deoxyribonucleotide phosphates (dNTPs), 8.12 µl of high performance liquid chromatography grade water, 3 µl of 10x buffer, 1.5 µl of dimethyl sulfoxide (DMSO), 0.48 µl of 10 mg/ml bovine serum albumin (BSA).

Cyclic conditions used to amplify Paratuberculosis specific product were initial denaturation at 95°C for 15 min. followed by 37 cycles of denaturation at 95°C for 15 sec., annealing at 58°C for 20 sec., extension at 72°C for 30 sec. and final extension at 72°C for 7 min. Amplification cycles were carried out in a thermocycler machine (Techne).

Agarose gel electrophoresis

The amplicons after completion of polymerase chain reaction were run using submersive agarose gel electrophoresis (2% agarose gel containing ethidium bromide) (Singh *et al.*, 2009). The resolved amplicons were visualized using ultraviolet transilluminator (Bio-Rad) and photographed.

Results and Discussion

Paratuberculosis (Johne's disease) is a chronic and infectious mycobacterial infection of gastrointestinal tract. The disease is characterized by chronic granulomatous inflammatory changes in the intestine of bovines (Slana *et al.*, 2008). Paratuberculosis is one of the very serious diseases of bovines because of *Mycobacterium avium subspecies paratuberculosis* (MAP) organisms are not easily destroyed by heat, freezing or desiccation, acid or alkaline compounds or chemical agents and hence easily transmitted to susceptible livestock population (Lavers, 2013). The MAP organisms have been found in biofilms, including those present in watering troughs. MAP is recognized as an important animal pathogen with zoonotic significance (Sonawane and Tripathi, 2016). It causes huge production losses and has high impact on animal industry (loss in milk yield, decline in body weight, reduce fertility, increase in mastitis cases and emaciation) (Deb *et al.*, 2011). Paratuberculosis affected animal shed viable MAP agents, especially in their milk. The current methods of milk pasteurization are not sufficient to kill all MAP cells present in milk, and MAP has been cultured from raw and pasteurized milk and isolated from cheese. MAP may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products. The presence of MAP in milk samples can be detected via culture, polymerase chain reaction (PCR), and enzyme

linked immunosorbent assay (ELISA). Long incubation (12-16 weeks) and low sensitivity limits the use of culture in prompt and fast diagnosis of MAP infection (Gumussoy *et al.,* 2015). Milk is the main source of transmission of MAP to human beings and animals. Milk samples are easily collected during the time of milking of cows without any special requirement to restrain the animals by the researcher or addition of any chemicals while collection of milk samples. An immunological test that is widely available and commonly used is ELISA (Singh *et al.,* 2007; Audarya *et al.,* 2013; Audarya *et al.,* 2016). Detection of antibody using ELISA test is the most frequently used methods for diagnosis of Paratuberculosis. Indirect milk ELISA has been reported to be most sensitive, fast and inexpensive test for large scale screening as compared to milk microscopy and milk PCR. ELISA kit is rapid, economic and sensitive test for large-scale screening of cattle population against incurable paratuberculosis. Milk microscopy and ELISA can also be a good combination to detect MAP in clinical specimen. So, in the present investigation cow milk samples were collected and screened for presence of antibodies to MAP infection in indirect ELISA (iELISA) test. Milk samples from suspected animals were also used to prepare milk smears to examine microscopically for acid-fast organisms after acid-fast staining. Microscopically acid-fast organisms positive and also iELISA positive milk samples were used to extract nucleic acid. Extracted nucleic acid was used in Paratuberculosis specific PCR.

A total of 103 milk samples were collected in sterile container from individual cow of organized and unorganized farming systems (Table 1). All the collected milk samples were stored in the refrigerator at - 20°C. These milk samples were transported to Animal Health laboratory of Central Institute for Research on Goats (CIRG) at Makhdoom. Milk samples were screened by using iELISA, an indigenously developed test by CIRG (Fig. 1). ELISA showed high capabilities, relatively low price, rapidity and accurate results for screening large quantity of milk samples in field condition hence it is more frequently being used. The results of indirect ELISA are presented in Table 1. Out of 103 cow milk samples tested in the study, 72 (69.90%) were positive for presence of antibodies against MAP. A range of percent positivity from 18.33% to 70% was reported from milk samples in ELISA used to detect MAP specific antibodies (Nielsen *et al.,* 2000; Gupta *et al.,* 2012; Singh *et al.,* 2016). Findings of the present study reports higher level of percent positivity (69.90%) for presence of antibodies to MAP infection in cow milk samples.

This higher percentage may be due to exposure of Paratuberculosis susceptible healthy animals to persistently infected animals kept in organized farms as well as those reared in the unorganized way. However total percent positivity for antibodies to MAP infection in milk samples recorded was higher in unorganized farming system (74.46%) when compared to organized farms (66.07%). In case of Gir cows, non-descriptive and crossbred cows from organized and unorganized farms 62.5%, 66.66% and 74.46% milk samples were found positive for presence of MAP antibodies. For microscopic examination milk smears were prepared from clinically suspected 13 milk samples (7 from organized and 6 from unorganized farms) and stained by Ziehl-Neelsen (ZN) method of staining and examined for presence of acid-fast organisms microscopically (Table 2). ZN staining has advantages of being simple, fast and inexpensive but it can only detect the acid-fast organisms and not specifically MAP (Fig. 2). In the study, 2 milk samples found positive for the presence of acid-fast organisms (1 each from organized and unorganized farms).

Table.1 Detection of *Mycobacterium avium subspecies paratuberculosis* antibodies in cow milk samples from organized and unorganized farms at Mhow of Indore in Madhya Pradesh by indirect enzyme linked immunosorbent assay

Cow	Description/Breed	Samples tested	Positive	Negative	Per cent positivity
Organized farms	Gir	8	5	3	62.50
	Non-descriptive and crossbred	48	32	16	66.66
	Total	56	37	20	66.07
Unorganized farms	Non-descriptive and crossbred	47	35	11	74.46
	Total	47	35	11	74.46
	Grand total	103	72	31	69.90

Table.2 Microscopical detection of acid-fast organisms in cow milk samples from organized and unorganized farms at Mhow of Indore in Madhya Pradesh

Cow	Samples tested	Positive	Negative	Per cent positivity
Organized farms	7	1	6	14.28%
Unorganized farms	6	1	5	16.66%
Total	13	2	11	15.38%

Table.3 *Mycobacterium avium subspecies paratuberculosis* specific primers used in polymerase chain reaction

Primer	Primer sequence for amplification of insertion sequence 900 (IS900)	Size	Product size
Forward primer: P90	5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3'	27 mer	413bp
Reverse primer: P91	5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3'	27 mer	

Fig.1 Detection of antibodies against *Mycobacterium avium subspecies paratuberculosis* in cow milk samples from Mhow of Indore in Madhya Pradesh by indirect enzyme linked immunosorbent assay

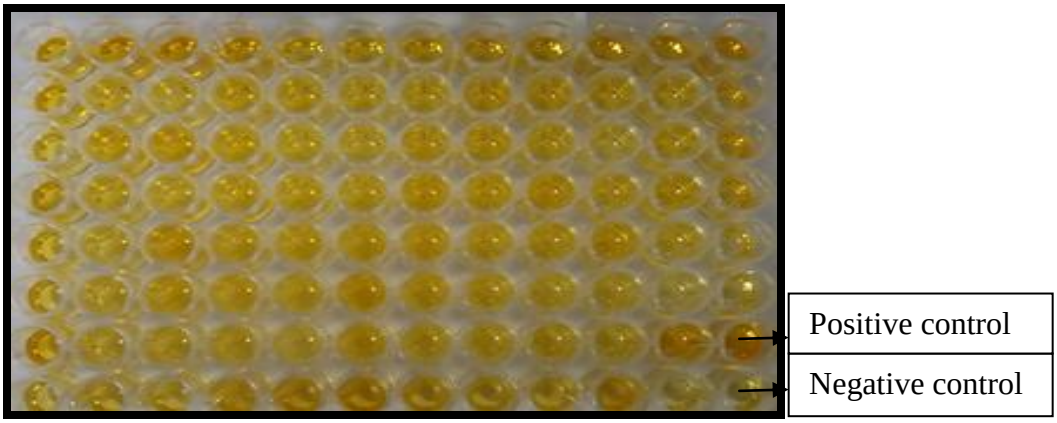

Fig.2 Presence of cluster of acid-fast organisms in cow milk smear (1000x)

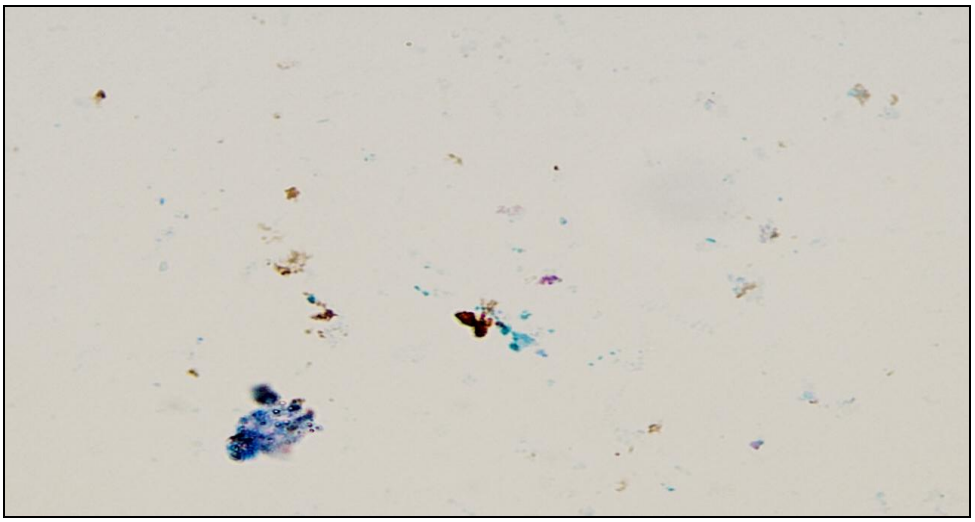

Fig.3 Agarose gel electrophoresis of amplicons (Lanes: 1-1 kb DNA ladder, 2-Positive control (413 bp), 3-Blank well, 4-Negative control, 7, 8-Cow samples)

Polymerase chain reaction is used as confirmatory test for detection of MAP in milk samples. A total of 33% positivity was reported for *Mycobacterium avium subspecies paratuberculosis* by insertion sequence 900 gene (IS900) PCR from milk samples (Pillai and Jayarao, 2002). In one of the other studies, a total of 8% paratuberculosis positivity in bovine milk samples by using PCR was reported (Franco *et al.*, 2013). Milk samples detected positive for acid-fast organisms in microscopy were used to extract nucleic acid (deoxyribonucleic acid, DNA) for its use in Paratuberculosis specific PCR for amplification of 413 bp product of MAP DNA (Table 3). Positive and negative controls were also kept in the study. Except in positive control, amplification of IS900 gene was unsuccessful (Fig. 3). MAP infection was detected in raw milk by PCR but cultivation of milk for MAP was found more sensitive (Giese and Ahrens, 2000). MAP DNA was detected in 13.61% milk samples (Gumussoy *et al.*, 2015). Difficulty in detection of MAP in milk samples from asymptomatic animals by performing PCR was reported (Ozpinar *et al.*, 2015). MAP in milk samples are not detected by PCR because in case of bovines 50 ml of milk sample has only about 2-8 colony forming units (CFU) titer of MAP agent (Narang *et al.*, 2017). This extremely low amount reduces the chance of isolating the agent in milk. Quantitative PCR (qPCR) technique is more sensitive than conventional PCR with respect to detection of MAP in milk samples.

In the study 69.90% of screened milk samples tested positive for the presence of MAP antibodies indicating higher level of MAP infection in cow population at Mhow area of Indore in Madhya Pradesh. The study will help in implementing control strategies and management of animals. The outcome of the present study warrants thorough investigations of livestock population of Madhya Pradesh for Paratuberculosis (Johne's disease) infection to evaluate its severity and economic impact. It also recommends immunization of susceptible livestock population of the region by administration of recently launched vaccine for therapeutic and preventive purposes against Paratuberculosis.

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