OPEN ACCESS
Journal of Animal Health and Production

Research Article

Isolation of *Mycobacterium bovis* from Milk and Nasal Discharge Samples of Cattle from Hyderabad and Tando Allahyar Districts

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Abstract | *Mycobacterium bovis* is known to cause significant economic losses to livestock industry in developing countries including Pakistan. Carrier animals with asymptomatic state shed pathogens through many ways as in saliva, nasal or oral secretions, milk, urine etc. In present study, milk and nasal discharge samples were used for the isolation of *M. bovis* bacterium, in order to explore the most contaminant carrier agent for the spread of bovine tuberculosis and prevalence of disease in the study area. A total of 160 cattle were selected randomly from Hyderabad and Tando Allahyar districts of Sindh province to collect nasal discharge (n= 160) and milk (n= 120) samples that were used for the isolation of *M. bovis* using Lowenstein-Jensen medium. Risk factors like age, breed, sex, type of farming, pregnancy status, parity, milk production and stage of lactation, associated with bovine tuberculosis were considered as epidemiological risk factors and the data regarding was collected on predesigned proforma. Results showed that, incidence of *M. bovis* was found higher ($p < 0.05$) in nasal discharge (1.875%) as compared to milk samples (0.833%). An overall prevalence of 1.42% was found in both types of samples/districts. Individually a higher ($p < 0.05$) number of *M. bovis* isolates were recovered from Hyderabad (2.143%) than Tando Allahyar district (0.714%). In Hyderabad district, no any isolate found from female whereas unlike Hyderabad district, no any sample taken from male cattle of Tando Allahyar district found positive. The role of risk factors analyzed during the study were found statistically non-significant ($p > 0.05$) for both districts. In brief, this study indicated that apparently healthy cattle shed more *M. bovis* pathogens in nasal secretions as compared to milk. Moreover, both Hyderabad and Tando Allahyar districts, prevailing bovine tuberculosis which is relatively higher in district Hyderabad than district Tando Allahyar.

Keywords | *Mycobacterium bovis*, Cattle, Culture, Bovine Tuberculosis, Hyderabad, Tando Allahyar

Editor | Sanjay Kumar Singh, Animal Reproduction Division, Indian Veterinary Research Institute, Izatnagar, Bareilly (UP), India.
Received | September 12, 2016; **Accepted** | September 27, 2016; **Published** | October 17, 2016
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Citation | Leghari A, Kamboh AA, Dewani P, Abro SH, Umrani AP, Malhi KK, Rajput ZI, Lakho SA, Bano I, Shah JM. (2016). Isolation of *Mycobacterium bovis* from milk and nasal discharge samples of cattle from Hyderabad and Tando Allahyar districts. J. Anim. Health Prod. 4(4): 105-110.
DOI | http://dx.doi.org/10.14737/journal.jahp/2016/4.4.105.110
ISSN | 2308–2801

INTRODUCTION

The causative agent of bovine tuberculosis, *Mycobacterium bovis*, is an aerobic, acid fast and slow growing bacterium. Genus mycobacterium also contains many other species as *Mycobacterium tuberculosis* that causes pulmonary tuberculosis in human that may also infect other animals (Grange et al., 1996). This pathogen is distributed worldwide, hence considered as socio-economical disease, as it is the major cause of disease in cattle and other mammals (Carslake et al., 2011; Mathews et al., 2006; Pollock and Niel, 2002; Cousins et al., 2001).

The pathogen may shed through many ways as in saliva,

nasal/oral discharges, urine, feces, milk *etc.* (Neil et al., 1991). Inhalation is considered as a major root of transmission of *M. bovis*. In case of prolong close contact of healthy animals with infected once, the chances of spread are more and vulnerable (Neil et al., 1994). The infection could easily occurs at certain gathering places as water points, dipping tanks and intense farming of livestock, where pathogen has the chance to transmit easily from infected to healthy animals (Ayele et al., 2004; Menzies and Neil, 2000).

Bovine tuberculosis is a zoonotic disease that can infect many animal species and can be transmitted from animals to humans. Raw milk and direct contact with infected animals were recognized as a major cause of its human transmission. The people deal with pasteurization of milk and work at slaughter houses have also been observed as a great risk of tuberculosis (Khattak et al., 2016).

It is reviewed by many studies that age is one of the main risk factors. Tuberculosis is a latent infection, mostly the signs and symptoms of the disease appears when the animal become older even when it got infection in early age as the exposure of disease is more to old aged animals as compare to younger. In Africa, the breed is also found as a risk factor for tuberculosis identified through tuberculin test. Immuno- suppressant also considered a major factor associated with bovine tuberculosis in many countries (Menzies and Neil, 2000; De la Rau et al., 2006).

In Punjab Province (Pakistan), bovine tuberculosis has been reported from 0.51 to 12.7% in different areas (Qamar and Azhar, 2013). However, to the best of our knowledge, no any study has been carried out before in Sindh province for the epidemiological status of bovine tuberculosis in cattle population. Therefore, the present study was designed to record the prevalence of bovine tuberculosis in cattle of Hyderabad and Tando Allahyar districts using culture technique.

MATERIAL AND METHODS

Animals

For present study, 160 cattle (40♂ and 120♀) were selected randomly, 80 from Hyderabad and 80 from Tando Allahyar district. From these cattle, a total of 280 samples (160 nasal secretion and 120 milk samples) were collected aseptically. For nasal secretions, sterilized swabs were used, whereas for milk, sterilized bijoux bottles were used to collect 5ml milk from each lactating cattle. Before collection, the teats were cleaned with antiseptic agent and first few drops of milk were discarded (Durrani et al., 2015). Data regarding epidemiological risk factors such as sex, age, breed, type of farming, parity, pregnancy status, stage of lactation and milk production was also collected in pre-designed questionnaire.

Bacteriological Culture

Homogenization and decontamination: Prior to culture, nasal secretions and milk samples were homogenized and decontaminated with 4% NaOH using Petorof's method (Brasil, 1994). Sodium hydroxide and N- acetyl was added into the bottles having the nasal sample, bottles were incubated for 30 min at 37°C. The content was centrifuged for 15 min at 3000 rpm, and sediment was used for culture while supernatant was discarded. For milk samples, 10 ml of milk sample was centrifuged at 3000 rpm for 15 minutes. Supernatant was discarded, sediment was suspended in 2 ml sterilized physiological saline solution, and the suspension was added with equal volume of 4-N sodium hydroxide and one drop of 0.05% phenol red indicator. The suspension was incubated at 37°C for 30 minutes, then 4-N hydrochloric acid was added equally to neutralize the solution, solution was again centrifuged like nasal sample, and sediment was used for culture.

Media preparation: Medium was prepared according to manufacturer instructions. In brief, a 37.3g of Lowenstein-Jensen medium (Difco Laboratories, Detroit, Michigan, USA), 600 ml distilled water and 12 ml of sodium pyruvate (Sigma-Aldrich, Co., USA) was measured and dissolved using magnetic stirrer. Then the medium was sterilized at 121 °C for 15 min at 15 lbs pressure and cooled to 50 - 60°C. Aseptically 600 ml of Lowenstein-Jensen medium was mixed with 1000 ml of egg suspension and placed in a slanting position at 85°C for 45 min in drying oven, then dispensed into sterile screw-cap test tubes.

Culture conditions: All the samples were cultured on the *Mycobacterium* specific medium (Lowenstein Jensen). Thick inoculums of decontaminated sample sediments were smeared on the surface of medium slopes and the cultured tubes were incubated at 37 °C for six to eight weeks (Buxton and Fraser, 1971).

Bacteriological Smear and Staining

A colony was picked with the help of loop and spread on the slide having a drop of distill water and air dried slide was fixed over the flame. Basic dye (carbol fuschin) was poured on the whole fixed slide, slide was heated with the help of spirit lamp till the fumes appeared and left the slide for 5 min, washed the slide thoroughly with tape water, decolourizer (20% sulphuric acid) was applied on the slide and washed immediately. This step was repeated till the slide became light pink, methylene blue was poured on the slide and left for two minutes. Finally slide was washed and air dried, smears were examined under 100x power lens for Acid fast bacilli (AFB).

Biochemical Tests

Growth after incubation was confirmed through nitrate

reduction and niacin test for characterization and identification of mycobacteria as described previously (Palomino et al., 2007). Niacin test is used to check the medium having nicotinic acid. *M. tuberculosis* does not excrete nicotinic acid in the cultured medium while *M. bovis* excreted it in the medium. This test can differentiate *M. bovis* form *M. tuberculosis* and other mycobacterial species (Cardoso et al., 2004). Three to four weeks old culture was added in 0.5 ml distilled water, 2 drops of extract of the culture were dropped into a porcelain depression spot test plate, 2 drops of 4% aniline bromide solution and 2 drops of 10% cyanogen bromide solution were added to the extract, plate was gently rotated to mix the liquid and the final reading was made at the end of 10 minutes.

For nitrate reduction test, a 0.2 ml of distilled water was added to screw-cap tube, 2 loop full of colonies were taken from positive culture and emulsified in water, a 2.0 ml of $NaNO_3$ substrate was added to the test tube and mixed well, and tubes were placed in the water-bath for 2 hours at 37 °C. One drop of hydrochloric acid solution, 2 drops of sulfanilamide solution, and 2 drops N- naphthylene diamine solution were added in the test tubes. Tubes were examined and observed for development of pink to red colour which showed positive.

Statistical Analysis

Collected data was compiled and analyzed using statistical software (SPSS version 11 statistical package program). The chi-square tests where applied and P value of ≤ 0.5 was considered statistically significant.

RESULTS

Incidence of *M. bovis* in Milk and Nasal Secretions

As shown in Table 1, incidence of *M. bovis* was found higher (p<0.05) than double in nasal secretions (1.875%) compared to that of milk samples (0.833%). Overall prevalence of 1.42% was recorded in both types of samples/districts. Individually, a three times higher (p<0.05) number of *M. bovis* isolates was recovered from Hyderabad (2.143%) compared to that of Tando Allahyar district (0.714%).

Table 1: Incidence of *Mycobacterium bovis* in milk and nasal discharge samples of cattle

Specimen	Hyderabad	Tando Allahyar	Overall incidence No. (%)
	Incidence No.(%)	Incidence No.(%)	
Nasal Discharge*	3 (3.75)	0 (0.0)	3 (1.87) ***
Milk**	0 (0.0)	1 (1.25)	1 (0.83)
Overall	3 (2.14)***	1 (0.71)	4 (1.42)

*: total number of samples analyzed for each district were n=80;
**: total number of samples analyzed for each district were n=60;
***: $p < 0.05$

Prevalence of *M. bovis* in Cattle Related to Various Risk Factors

As shown in Table 2, nasal secretion samples of male cattle originated from Hyderabad and 1 female milk sample of Tando Allahyar district was positive for the *M. bovis* pathogen. The relationship of associated risk factors including age, breed, sex, type of farming, pregnancy status, parity, milk production and stage of lactation was found statistically non-significant (p >0.05).

Table 2: Prevalence of *Mycobacterium bovis* in cattle in Hyderabad and Tando Allahyar districts in relation to epidemiological risk factors

Factors	Hyderabad	Tando Allahyar
	Prevalence No. (%)	Prevalence No. (%)
Sex		
Male*	3 (15)	0 (0)
Female **	0 (0)	1 (1.67)
Age ***		
5-8 Years	3 (7.5)	0 (0)
>8 Years	0 (0)	1 (2.5)
Breed ***		
Local Breed	0 (0)	0 (0)
Exotic Breed	3 (7.5)	1 (2.5)
Farming ***		
Rural	0 (0)	1 (2.5)
Peri Urban	3 (7.5)	0 (0)
Pregnancy Status**		
Non-pregnant	0 (0)	1 (3.33)
Pregnant	0 (0)	0 (0)
Parity *		
1 Parity	0 (0)	0 (0)
2-4 Parity	0 (0)	0 (0)
>4 Parity	0 (0)	1 (5)
Milk Production *		
2-4 Liters	0 (0)	0 (0)
4-8 Liters	0 (0)	1 (5)
>8 Liters	0 (0)	0 (0)
Stage of lactation *		
Early	0 (0)	0 (0)
Mid	0 (0)	0 (0)
Late	0 (0)	1 (5)

*: total number of samples analyzed for each district were n=20;
**: total number of samples analyzed for each district were n=60;
***: total number of samples analyzed for each district were n=40

DISCUSSION

Mycobacterium is the causative agent of bovine tuberculosis, which is a chronic bacterial disease of animals and humans. Bovine tuberculosis is considered as a major infectious disease of cattle and other mammals and wild life in many parts of the world (OIE, 2009). It is mostly seen as latent infection, whereas the clinical signs are not specifically differential, these includes anorexia, weakness, dyspnea, enlargement of lymph nodes and emaciation. In dead animals, disease may diagnose through histopathological techniques, necropsy and culture, while in live animals delayed hypersensitivity is widely using tool to diagnose the infection however, this approach is not considered reliable due to some fraction of false positive results (Gumi et al., 2012). Bovine tuberculosis is an endemic disease of cattle and buffaloes in Pakistan (Jalil et al., 2003). Animal are at risk to get infection through ingestion of feces, urine, wound, lymph discharge and infected milk. Milk is the main source to spread the infection from adult to young stock and also its transmission to human beings (Kazoora et al., 2016).

Culture technique is considered as a gold standard method for the diagnosis of bacterial diseases *Mycobacterial* infections (Khan et al., 2016; OIE, 2008). In present study, we used this technique to record the prevalence of *M. bovis* in nasal secretions and milk samples collected from apparently healthy cattle. Our results regarding incidence of *M. bovis* in these specimens are in consistent with previous literature. Sulieman and Hamid (2002) stated that infected animal may shed the pathogen in its nasal secretions along with milk. Disease has the significance importance because of its transmission from animal to human and may consider as milk born zoonotic disease. The chances of transmission of *M. bovis* will increase with the use of unpasteurized milk.

Our present results and previous studies (Hassanain et al., 2009; Javed et al., 2012) have demonstrated that prevalence of bovine tuberculosis could be diversified from region to region and even from farm to farm in the same region due to difference in the herd management, hygienic conditions, animals density, nutrition *etc*. (Hussin et al., 2016; Peter et al., 2015). Moreover, it has been revealed that different species/breeds have different susceptibility/resistant levels for bacterial pathogens (Mangi et al., 2015). A higher prevalence in Hyderabad district could be due to that it is a most populous city of Sindh province after Karachi and also a border city where animals brought from several neighboring animal markets for sell (Yousaf et al., 2016).

Literature shows that there are many roots of excretion of the *M. bovis* pathogen such as milk, blood, nasal secretions, lymph glands, rectal pinch and feces (Srivastava et al., 2006; Sgarioni et al., 2014; Jalil et al., 2003). Therefore, in present study, milk and nasal secretion were collected to isolate *M. bovis*. Three nasal samples from male in Hyderabad and 1 milk sample of Tando Allahyar district showed growth on Lowenstein Jensen medium and those isolates were further confirmed through biochemical tests as *M. bovis.* Our study showed that nasal secretion and milk were the most appropriate specimens for isolation of *M. bovis* from infected cattle (however nasal secretion is comparatively superior to milk specimen). There have been other studies showing relatively higher isolation of *M. bovis* from lymph glands and pus as compared to other specimens (Niaz et and Siddiqi, 1979; Sulieman and Hamid, 2002; Leite et al., 2003). While some other workers recommended the milk and faces as the preferred specimens for the recovery of *M. bovis* pathogen (Leite et al., 2003). Kleeberg (1984) indicated that one cow with tuberculosis can excrete enough viable tubercle bacilli to contaminate the milk of up to 100 cows when milk pooling and bulk transportation is used. The same author noted tubercle bacilli in milk products such as yogurt and cheese made from non-pasteurized milk 14 days after processing and in butter as long as 100 days after processing. Because most of the farmers either sell their milk to local people or pool milk in units for selling milk products without treating it with heat, hence risk of milk contamination with *M. bovis* is a potential major health hazard to consumers.

Our study could also be compared with the report of Jalil et al. (2003) who reported that nasal secretion and milk are the main routes of excretion of acid fast bacteria. They also reported that *Mycobacteria* can survive and retain infectivity in moist conditions up to 18 days. Our results regarding associated risk factors are in agreements with the studies of Arshad et al. (2012) and Javed et al. (2012).

CONCLUSION

From the current study it could be concluded that bovine tuberculosis is prevailing in the cattle of both districts (Hyderabad and Tando Allahyar) of Sindh province. Moreover, *Mycobacterium bovis* can transmit through animal to animal and animal to human as it is shaded by nasal secretions and milk samples. The contamination level of nasal secretions is slightly higher than milk. It is strongly recommended that milk must be heat treated before its human consumption.

ACKNOWLEDGMENT

The authors highly acknowledged the CVDL (Central Veterinary Diagnostic Laboratory) Tando Jam for providing research facilities to carry out this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTION

This work is a part of M. Phil project of first author Ambreen Leghari. Asghar Ali Kamboh, Parkash Dewani and Aslam Parvez Umrani were the mentors of her project. Kanwar Kumar Malhi, Zahid Iqbal Rajput and Shakeel Ahmed Lakho helped in writing and Iqra Bano and Jan Mohammad shah helped in revision of this manuscript.

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