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A Cross Sectional Study of *Mycobacterium bovis* in Dairy Cattle in and Around Lahore City, Pakistan

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Abstract.- The objective of this study was to estimate the prevalence of *Mycobacterium bovis* (*M. bovis*) in dairy cattle in and around Lahore. During 10 months (May 2007 to February 2008) period, milk samples analyzed through conventional (culture) and modern (PCR) methods. Out of 1000 samples, 454 (45.4%) were positive with PCR. Out of 454, 69 samples (15%) were positive for direct acid fast staining and 31 samples (7%) were positive with isolation and identification of *M. bovis*. The presence of *M. bovis* in raw milk samples is an enormous health risk factor for milk handlers and end consumers. There is dire need to improve the health of milking animals and livestock owners that will mitigate the prevalence of bovine tuberculosis in bovines.

Key words: Prevalence, bovine TB, *Mycobacterium* species, PCR.

INTRODUCTION

Bovine tuberculosis (BTB) is not only a threat to animal but is also a zoonotic problem. *M. bovis*, the cause of BTB and *M. tuberculosis*, the cause of classical human tuberculosis, are genetically and antigenically very similar and cause identical clinical disease in humans (Danker *et al*., 1993). Apart from mortality, it is estimated that animal affected with tuberculosis lose 10-25% of their productive efficiency (Radostits *et al*., 2000). Key factors in the control of tuberculosis are rapid detection, adequate therapy and contact tracing to arrest further transmission (Broekmans, 1994).

The BTB infection in cattle is usually diagnosed in the live animal on the basis of delayed hypersensitivity reactions. After death, it is diagnosed by post-mortem examination and histopathological and bacteriological techniques. The presence of *M. bovis* in the milk of apparently healthy milking animals is detected either on the basis of cultural examination or more modern molecular diagnostic techniques. Bacteriological examinations may comprise the demonstration of acid-fast bacilli by microscopic examination (provides presumptive confirmation), isolation of *mycobacteria* on selective culture media and their subsequent identification by cultural and biochemical tests (OIE, 2004). Culture is gold standard, but a lot of time is required for this slow-growing organism. Serodiagnosis of tuberculosis also has many limitations (Daniel, 1990). As an alternative to these conventional methods, nucleic acid based techniques are more rapid, sensitive and specific means of identification of mycobacteria with some limitations (Bhattacharya *et al*., 2003).

Pakistan has been reported to be one of the twenty two countries accounting for 80% of total T.B burden worldwide and is one of the five countries responsible for half of the T.B worldwide (Metzger *et al.*, 2010). The TB cure rate in Pakistan is some 78% still a lot of efforts need to be made to improve the detection rate (27%), which is still very low (WHO, 2004). In Pakistan, bovine tuberculosis threatens cattle and buffaloes in both public and private sectors (Ali *et al.*, 2005). The diagnosis of TB at molecular level is not being used in routine and a few people have done research on this aspect. The tuberculin sensitivity test is a diagnostic tool for tuberculosis (Khan *et al.*, 2008). False positive test result may be caused by non-tuberculous *mycobacteria* or previous BCG vaccine. Prior BCG may result in a false-positive result for many years

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afterwards (Chaturvedi and Cockroft, 1992).

The prevalence of tuberculosis in large ruminants in Lahore varied from time to time and place to place. On the basis of tuberculin testing, the reported prevalence in Lahore was 6.9% (Jalil *et al.*, 2003) and 7.3% (Amin *et al.*, 1992). Mumtaz *et al*. (2008) reported 9.6% incidence of bovine TB after single intradermal tuberculin test, where as MTB complex and *M. bovis* was found to be 35% and 29% respectively by PCR in milk samples out of total 31 samples, collected from same buffaloes.

Keeping in view these facts, an attempt was made to estimate the prevalence of *M. bovis* in dairy cattle in and around Lahore City, Pakistan using modern (PCR) and conventional (Tuberculin testing and culture) laboratory methods.

MATERIALS AND METHODS

Study population and sampling

Out of total nine towns in the Lahore city, five peri urban towns, namely Ravi, Shalamar, Aziz Bhatti, Wagha and Nishtar Towns were selected for the study. Five union councils (out of 10-15) were selected from each peri urban town, and 40 lactating animals were selected out of each union council. Thus a total of 1000 milking animals were randomly selected from the five periurban towns. All selected animals were screened with single cervical inter-dermal tuberculin test (SCIDTT) and milk samples were collected from each animal for further analysis. About 50–100 ml of milk was aseptically collected from each animal in sterile plastic containers and transported in ice-packed cooler to the laboratory where they were stored at 4-8 ˚C until analysis.

Tuberculin testing

All the selected animals were screened with single cervical inter-dermal tuberculin test after preparing for the test through the procedure adopted from Sulieman and Hamid (2002). Briefly, the sites was shaved with a razor and disinfected. The initial reading of the thickness of the skin was measured using vernier calipers and noted. A measured quantity of 0.14 ml of PPD mammalian tuberculin was injected intradermally. Finally, the thickness of skin at the injected sites was measured after 72 hours (± 6 hours).

DNA extraction and PCR

DNA extraction

The DNA was isolated from milk with the help of QIAMP DNA mini kit, USA®. In brief, from each milk sample, 50 µl of milk was transferred to sterile 1.5 ml labeled microfuge tubes. 300 µl cell lysis solution was added to the sample and mixed well with the help of a vortex for a few seconds and heated for 65°C in a water bath for 60 minutes. Then 200 µl protein precipitation solution was added to the cell lysis solution and vortexed for 20 seconds to mix the solution. The solution was centrifuged at 13000 rpm for 15 minutes. Supernatant was transferred to new labeled 1.5 ml microfuge tubes leaving behind the protein pellet at the bottom. Then 300 µl 100% Iso propanol was added and centrifuged at 14000 rpm for 15 minutes. The supernatant was discarded and 300µl 70% ethanol was added to the pellet at the bottom. It was centrifuged at 14000 rpm for 1 minute for proper concentration of DNA. At the end, the ethanol was poured off carefully 20 µl of DNA hydration solution was mixed to pellet. It was incubated at 65^0C for 5 minutes. The extracted DNA from milk samples was analyzed by agarose gel electrophoresis to confirm the presence of DNA (Mumtaz *et al.*, 2008).

Gel electrophoresis

In the central well, 5 µl of 10 Kb DNA ladder was loaded while in remaining wells, 6µl of sample DNA with 2 µl DNA loading dye (50% glycerol, 6xTAE, 1%bromoethanol blue) were loaded by using micropipettes. The gel was stained with ethidium bromide solution having concentration of 10 mg / ml for 20 minutes. Then the gel was washed in water and observed under UV trans–illuminator, and it was photographed using a Polaroid camera.

PCR

The PCR protocol described by Bhattacharya *et al*. (2003) for detection of *M. bovis* in milk was followed with some modifications. Detection of *M. bovis* was carried out by amplification of 500 bp PCR product with JB primers (Forward: JB21 TCGTCCGCTGATGCAAGTGC; Reverse: JB22: CGTCCGCTGACCTCAAGAAAG)

For PCR analysis, a 50 µl volume reaction for each sample was prepared which contained 5 µl of 10X PCR buffer, 5 µl of 2.5 mM dNTPs, 0.5 µl of AmpliTaq DNA polymerase, 2 µl of forward primer, 2 µl of reverse primer, 5 µl of $MgCl_2$, 5 µl of DNA sample and 25.5 µl of distilled water. PCR reaction mixture tubes were transferred to a thermal cycler and 500 bp amplified products over 30 cycles were obtained by using the conditions: 94°C for 4 min., 93°C for 30 sec., 50°C for 30 sec., 72°C for 30 sec. and completed with a final extension step of 7 min. at 72°C. PCR product was identified by agarose gel electrophoresis (Fig. 1).

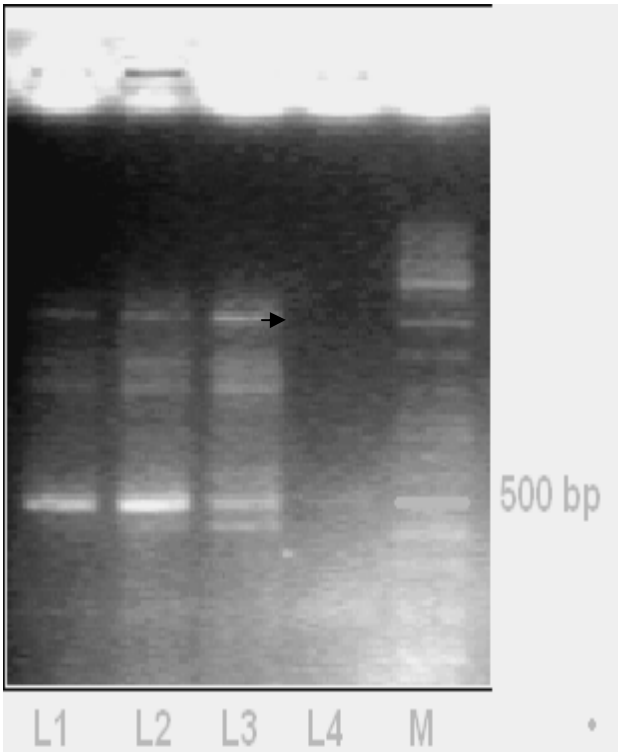

Fig. 1. Gel electrophoresis of milk samples after multiplex PCR
L1, Positive control of *M. bovis* (from culture of *M. bovis*)*;* L2 and L3, 500 bp products positive for *M. bovis*; L4, Negative sample; M, 10 Kb DNA Marker.

Conventional methods

All the PCR positive samples were subjected to screening with conventional methods (Quinn *et al*., 1994). These milk samples were smeared for acid fast staining and also inoculated on Stone brink's media and incubated at 37°C for six to eight weeks. Colony characteristics and growth were recorded. Smears were made from cultures and stained by Ziehl Neelsen staining technique.

The growth obtained after culture was subjected to biochemical tests for confirmation of *M. bovis* and *M. tuberculosis e.g.,* nitrate reduction test, niacin test, growth inhibition and tolerance test in presence of thiophene-2-carboxylic acid hydrazide (TCH) and pyrazinamidaze test for the identification of drug resistance using different laboratory techniques (Quinn *et al*., 1994).

Statistical analysis

Data was analyzed statistically by applying Chi-square test using SPSS 13.0 to compare prevalence of *M. bovis* in bovine milk from five different Towns of Lahore.

Table I.- Prevalence of bovine tuberculin positive animals in peri urban areas of Lahore.

Town	Tuberculin positive	Prevalence (%)
Ravi	35	17.5
Shalamar	22	11
Wahga	7	3.5
Aziz Bhatti	27	13.5
Nishtar	43	21.5
Total	134	13.4

RESULTS

Out of 1000 randomly selected animals, 134 animals were found reactive to tuberculin test. Town wise detail about tuberculin positive animals is given in Table I. Similarly, 454 animals were found positive to *M. bovis* by PCR in milk. Out of these 454 PCR positive samples, 69 samples were positive to acid fast staining and 31 samples could be cultured on the medium. Growth of primary culture took 6–8 weeks to show visible colonies. Colonies were buff colored and rough, having the appearance of bread curbs or cauliflower and not easily emulsified but gave a granular suspension. Direct smear staining with Ziehl Neelsen, showed red rod bacilli (acid fast bacilli) against a blue background. Town wise detail of positive samples with PCR, acid fast staining and culture is given in

Table II, while prevalence is given in Table III.

Table II.- Prevalence of Mycobacteria in dairy cattle from peri urban areas of Lahore based on testing of milk.

Town	Milk		
	PCR +ve	Acid fast + ve	Culture +ve
Ravi	97	11	9
Shalamar	72	06	7
Wahga	61	10	2
Aziz Bhatti	76	19	3
Nishter	148	23	11

Table III.- Comparative prevalence on the basis of different tests.

Town	Test wise prevalence (%)		
	PCR	Acid fast	Culture
Ravi	48	11	9
Shalamar	36	8	10
Wahga	30	3	3
Aziz Bhatti	38	25	4
Nishter	74	15	7

Table IV.- Statistical comparison of different towns for prevalence of bovine tuberculosis (P<0.05).

Test parameter	Pearson Chi-Square	
	Value	Asymp. Sig (2 sided)
Tuberculin	32.09	0.000*
PCR	96.23	0.000*
Acid Fast	9.50	0.50*
Culture	3.994	0.407

* = There was found a significant difference among towns for prevalence of bovine tuberculosis when checked with different test parameters

DISCUSSION

The animals infected with *M. bovis* secrete the causative organism in milk and nasal secretions. The disease has significant importance on account of its transmission to humans. Humans acquire infection mostly through consumption of un-pasteurized milk contaminated with *M. bovis*. In countries where bovine milk is not pasteurized before use, bovine tuberculosis has emerged as the single major cause of extra-pulmonary human tuberculosis (Sulieman and Hamid, 2002). In this study, overall prevalence on the basis of tuberculin test was 13.4% and 45.4% on the basis of PCR. There was a significant difference ($P<0.05$) among the five towns under study on the basis of prevalence of bovine tuberculosis. There was also a significant difference among different tests employed for diagnosis of tuberculosis in animals. On the basis of tuberculin testing, our findings (Ravi town 17.5%, Aziz Bhatti town 13.5%, Nishtar town 21.5%) are some what different from those of Amin *et al.* (1992) and Jalil *et al.* (2003) who reported 6.9% and 7.3% prevalence in Lahore respectively. This might be due to the difference in sample collection locations as these are some what close to the findings from Shalamar town (11%) and Wagha Town (3%). On the other hand, these findings are close to the findings of Mumtaz *et al.* (2008) who reported 9.6% incidence of bovine TB after single intradermal tuberculin test, where as MTB complex and *M. bovis* was found to be 35% and 29% respectively by PCR in milk samples out of total 31 samples, collected from same buffaloes.

Out of total 1000 animals, 454 (45.4%) showed amplified products at 500 bp specific for *M. bovis*. The application of PCR clearly distinguished the bovine TB caused by *M. bovis* when compared with routine laboratory tests. The 148 tuberculin positive animals' milk samples were also positive for *M. bovis* with PCR. These findings are in line with those of Figueiredo *et al.* (2009) who reported PCR using primers JB21/JB22 as a highly reliable method in identifying *M. bovis* isolates, presenting 100% concordance with the conventional microbiological method. The PCR method was fast, reproducible and useful for the study of slow-growing *mycobacteria*, particularly in cultures where the small number of bacilli hindered identification by classical methods. It also could be a valuable tool for the rapid identification of acid-fast bacilli isolated from suggestive bovine TB lesions.

Results of the present study indicated that there was a significant difference between the five towns on the basis of PCR based detection of *M. bovis*, and also when the milk samples were studied with acid fast staining but not when the samples were analyzed through culture examination (Table

IV). Although almost all of the animals under study were being reared in open farming systems, the hygienic conditions differ among towns. In Wagha Town, these were much better than others. This might be a reason for relatively lower prevalence. In this study PCR was considered as an alternative to ZN staining for diagnosis of TB. These findings are in line with Ndugga *et al*. (2004) and Grange and Yates (1994) who also reported that comparative study of AFB staining, culture, and PCR for *M. bovis* revealed that PCR had high sensitivity and had a potentially important role in improving the diagnostic accuracy of tuberculosis. Direct smear microscopy does not permit differentiation between species of the *M. tuberculosis complex*. It was clear from the results that *M. bovis* was present in considerable number of milk samples and PCR was more sensitive than the conventional methods for its detection. These results were in agreement with the findings of Cegielski *et al*. (1997) and Romero *et al*. (1999) who also reported that PCR has been found more specific and sensitive as compared to tuberculin or skin test.

The present study indicated that there is a high prevalence (45%) of bovine tuberculosis in periurban areas of Lahore. The prevalence of tuberculosis on the basis of PCR positivity in this study was different from Mumtaz *et al*. (2008) who reported 29% prevalence (on the basis of PCR) in local milk samples of Lahore. The difference in the findings may be due to sample population (as shown by the differences we detected between our populations) which was more random and organized in this study as compared to only 31 samples in the study of Mumtaz *et al.* (2008).

CONCLUSIONS

The bovine tuberculosis is prevalent in Lahore with a high rate in some periurban areas. PCR could be applied directly to clinical samples in medical and veterinary laboratories, and can be used to detect *M. bovis* in bovines. Although some what expensive, PCR for diagnosis of bovine tuberculosis in buffaloes/cattle can be used as a routine diagnostic test to check the prevalence of bovine tuberculosis in Pakistan. On the basis of findings, the regular monitoring of the milking animals, milk men and milk handlers is suggested for presence of *M. bovis* in areas with high burden of the disease.

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