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A PRELIMINARY STUDY ON PREVALENCE OF BOVINE TUBERCULOSIS IN CATTLE AND BUFFALO IN OUTSKIRTS OF LAHORE, PAKISTAN

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This study was conducted to identify mycobacteria in cattle and buffalo by using conventional method of Tuberculin and then by ELISA using serum samples in randomly selected animals. Positive sample of ELISA were further run on PCR for confirmation. Furthermore, along with the checking of its comparative efficacy, the disease prevalence in cattle and buffalo on the basis of associated risk factors including the species, age, sex and management, in vicinity of Lahore, Pakistan were investigated. One hundred and ninety-two clinically suspected animals of different breeds, ages (2-8 years) and weights for detection of tuberculosis, were selected randomly from four organized farm of Lahore district. The animals were divided into two main experimental groups designated as group B (buffalos) and group C (cows). Each main group was further sub-divided into 3 sub-groups B1, B2, B3 and C1, C2, C3 with age group of below 4y, 4-6y and over 6y, respectively. On the basis of tuberculin test number of positive cases of bovine tuberculosis in buffalo were 7.29 % while in cattle were 11.46% out of total of 96 animals of each.

Tuberculin positive cases were further tested with PCR and ELISA. Number of positive cases of buffalo and cattle were 85.71% and 90.91%, respectively when tested with PCR. The expected size of the amplified DNA fragment was 317 bp. On the basis of diagnosis with ELISA-IFN-γ assay, number of positive cases in buffalo and cattle were 71.43%% and 72.73%, respectively.

Keywords: Bovine Tuberculosis, Tuberculin Test, PCR, ELISA, Cattle, Buffalo, Lahore

Tuberculosis is an infectious bacterial zoonotic globally important disease, that affects the lungs but can also affect the other systems of body, commonly known to be pulmonary and extra-pulmonary TB, respectively(Bannalikar and Verma 2007). Firstly in 1882, Robert Koch discovered *M. tuberculosis*, the cause of TB, an aerobic non-motile, bacillus (Mandell et al. 2005). TB is a highly contagious disease and through coughing, sneezing or talking, it can be transferred from one person to other, as organism is shed into the air by an infected person (Beresford and Sadoff 2010). *M bovis* is a major problem, for both wildlife and in particular for livestock, for which the economic losses to farmers are very severe (Andersen et al. 2000).

The most frequent causative agent of TB in humans is M. tuberculosis, whilst M. bovisis mainly responsible for bovine TB but can also cause TB in humans. In developed countries, zoonotic TB is not a public health problem because of milk pasteurization process (Thoen et al. 2006). Approximately 90% of the people infected with TB carry the bacilli for their whole life and may remain unaware of the fact that they are infected with TB (Beresford and Sadoff 2010). M. bovis mainly infects cattle but it is an important zoonosis in several countries of the world, which is a serious public health problem and also results in economic losses (De Kantor and Ritacco 1994). The control of bovine TB is difficult because the causative agent, M. bovis infects both animals of agricultural importance and wild mammals act as a reservoir (O'Reilly and Daborn 1995).

None of the currently available diagnostic tests can be considered as having both 100% sensitivity and 100% specificity, however Food and Rural Affairs (DEFRA) claims a

specificity of the single intradermal comparative cervical tuberculin test (SICCT) of 99.9%(Hartnack and Torgerson 2013). The tuberculin skin test is the primary surveillance test for the diagnosis of bovine TB. Single intradermal comparative cervical test (SICCT) is used, where avian PPD, prepared from a culture of Mycobacterium avium, and bovine PPD are injected into adjacent sites in the mid-cervical region and increases in skin swelling between the two sites are compared at 72 h post-injection (De la Rua-Domenech et al. 2006). Still, in the case of disease with a low prevalence and a test specificity of less than 100%, the number of false-positive individuals can be higher than the number of infected animals (Hartnack and Torgerson 2013).

The aim of the present study was to identify mycobacteria in cattle and buffalo by using conventional method of Tuberculin and then by ELISA using serum samples in randomly selected animals. Positive sample of ELISA were further run on PCR for confirmation. Furthermore, along with the checking of its comparative efficacy, the disease prevalence in cattle and buffalo on the basis of associated risk factors including the species, age, sex and management, in vicinity of Lahore, Pakistan was evaluated.

MATERIALS AND METHODS

Study population and study design

The samples were collected from the four organized cattle and buffalo farms in vicinity of eastern Lahore, Pakistan. One hundred and ninety-two clinically suspected animals of different breeds, ages (2-8 years) and weights for detection of tuberculosis, were selected randomly from four organized farm of Lahore district. The animals were divided into two main experimental groups designated as group B (buffalos) and group C (cows). Each main group was further sub-divided into 3 sub-groups B1, B2, B3 and C1, C2, C3 with age group of below 4y, 4-6y and over 6y respectively as described in table 3.1.

Diagnostic Tests

Single Intradermal Tuberculin Test

Animal testing was performed through skin testing using the single intradermal tuberculin test(SITT) as described by (Marsot et al. 2016). The following procedure was used for single intradermal tuberculin test. The injection site was clipped and cleaned aseptically. A fold of skin within each clipped area was measured with calipers and the site marked prior to injection. A short needle, bevel edge outwards and graduated syringe charged with tuberculin attached, was inserted obliquely into the deeper layers of the skin. The dose of tuberculin (less than 2000 International Units (IU) of bovine tuberculin) was then injected in the center of the middle third of the neck. A correct injection was confirmed by palpating a small pea-like swelling at each site of injection. The skin-fold thickness of each injection site was re-measured 72 hours after injection.

Molecular Identification using RT-PCR

The expected size of the amplified DNA fragment was 317 bp, and the bands were visualized after electrophoresis of 1/10 of the reaction mixture in an agarose gel stained with ethidium bromide (0.5 mg/ml). The amplification parameters included an

Table 1 Experimental Design

Group B (Buffalos)						**Group C (Cows)**					
B1 (below 4 years)		B2 (4-6 years)		B3 (over 6 years)		C1(below 4 years)		C2 (4-6 years)		C3 (over 6 years)	
M	F	M	F	M	F	M	F	M	F	M	F
16	16	16	16	16	16	16	16	16	16	16	16

M: Male, F: Female, B: Buffalo, C: Cows

Table 2 Primer sequences for DNA amplification

Primer	**Sequence (5'---------3')**	**Product size**
Forward	AGAGTTTGATCCTGGCTCAG	317bp
Reverse	TGCACACAGGCCACAAGGGA	

initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 68°C for 1.5 min, and extension at 72°C for 2 min. The extension step in the 35th cycle was held for 10 min before the samples were shifted to 4°C for storage.

Enzyme Linked Immunosorbent Assay-Gamma-Interferon

The whole blood culture system and Enzyme Immunosorbent Assay (EIA) for bovine IFN-γ was used for cattle samples as described by (Rothel et al. 1990). The usefulness of this assay had previously been evaluated in a preliminary trial (GutieÂrrez et al., 1993). Heparinized blood was processed within 8 hours post-collection. Aliquots (100 ml) of either bovine PPD, avian PPD (both at 300 mg ml ÿ1, CZ Veterinaria, Spain), or PBS were added to 1.5 ml blood, incubated for 20-24 hours at 37 °C in a humidified atmosphere before plasma was harvested. The plasma samples were assayed for IFN-γ in a sandwich EIA utilizing two monoclonal antibodies to bovine IFN-γ, accordingly to the manufacturer's indications (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). For the purposes of this trial, an animal was considered to react to M. bovis if the OD of the bovine PPD stimulated sample was 1.5 times greater than or equal to the OD of the no-antigen sample and greater than or equal to the OD of the avian PPD-stimulated sample. The interpretation of the avian PPD-stimulated sample was similar except that samples reacting to both PPDs were considered positive only where the OD of the avian PPD was greater than the bovine PPD.

Associated Risk Factors

We also checked the associated risk factors of tuberculosis including species, age, sex, body condition, clinical signs and vaccination status**.**

Statistical Analysis

Data was analyzed by using Chi-square analysis test.

RESULTS

In this study a total of one hundred and ninety-two animals were tested to find out incidence of tuberculosis in cattle and buffalo by using different diagnostic techniques such as tuberculin test, ELISA and PCR and to compare their reliability on the basis of their sensitivity and specificity and to study associated risk factors of the disease.

Diagnosis of Tuberculosis in Bovine using Different Molecular Techniques

On the basis of tuberculin test number of positive cases of bovine tuberculosis in buffalo were 7.29% while in cattle were 11.46% out of total of 96 animals of each. Tuberculin positive cases were further tested with PCR and ELISA. Number of positive cases of buffalo and cattle were 85.71% and 90.91% respectively when tested with PCR. The expected size of the amplified DNA fragment was 317 bp. On the basis of diagnosis with ELISA-IFN-γ assay, number of positive cases in buffalo and cattle were 71.43%% and 72.73% respectively (Table 4).

Associated Risk Factors

Age

Table 4 indicated the association of age and incidence of bovine tuberculosis in buffalo and cattle. Number of positive cases in buffalo at age below 4 years, 4-6 years more than 6 years were observed as 2,4 and 6 respectively. While in cows, number. of positive cases of Bovine Tuberculosis at ages below 4 years, 4-6 years more than 6 years were found as 5, 11 and 21. There was no significant difference ($p>0.05$) between buffalo and cows at age below 4 years regarding the bovine tuberculosis incidence. Moreover, a clear significant difference ($p<0.05$) was observed between buffalo and cows at ages 4-6 years and more than 6 years on the basis of bovine tuberculosis prevalence.

Gender

In case of Buffalo, number of positive cases of bovine tuberculosis in male and female 02 and 05 respectively, while negative cases observed in male and female were 46 and 43 respectively. In cows, 04 males and 07 females were declared as positive while remaining 44 males and 41 females were declared as negative cases of bovine tubercu -losis. No significant difference ($p>0.05$)

Table 3 Associated Risk Factors regarding the Prevalence of Bovine Tuberculosis in Buffalo

Parameters	No. of Positive Cases	No. of Negative Cases	P-Value (p<0.05)	Is Discrepancy Significant (p< 0.05)?
Age				
Below 4 years	1	31		
4-6 years	2	30	0.340	No
Above 6 years	4	28		
Gender				
Male	2	46	0.2176	No
Female	5	43		
Body Condition				
Good Condition	1	28	0.3594	No
Fair Condition	2	33		
Poor Condition	4	28		
Breed				
Nili Ravi (pure breed)	2	46	0.2176	No
Nondescript	5	43		

Table 4 Associated Risk Factors regarding the Prevalence of Bovine Tuberculosis in Cows

Parameters	No. of Positive Cases	No. of Negative Cases	P-Value (p<0.05)	Is Discrepancy Significant (p< 0.05)?
Age				
Below 4 years	2	30	0.263	No
4-6 years	3	29		
Above 6 years	6	26		
Gender				
Male	4	44	0.218	No
Female	7	41		
Body Condition				
Good Condition	1	27	0.128	No
Fair Condition	3	29		
Poor Condition	7	29		
Breed				
Indigenous	3	45	0.109	No
Exotic	8	40		

was observed in buffalo and cows on the basis of gender with respect to incidence of bovine tuberculosis (Table 4).

Breed

In case of buffalo, number of positive cases of bovine tuberculosis in Nili Ravi (purebreed) and non-descript breeds were 2 and 5 respectively. While, number of negative cases of bovine tuberculosis in Nili Ravi (pure breed) and non-descript breeds were 46 and 43 respectively. A significant difference ($p<0.05$) was observed between the pure and non-descript breeds of buffalo (Table 4.3). Number of positive cases of bovine tuberculosis in indigenous and exotic breeds of cows were 3 and 8 respectively. Moreover, number of negative cases of bovine tuberculosis in indigenous and exotic breeds of cows were 45 and 40 respectively. A significant difference ($p<0.05$) was observed between the indigenous and exotic breeds of cows (Table 4).

Body Condition

In buffalo, only one animal with good body condition was found positive against bovine tuberculosis, while two animals with fair body condition was declared as positive, four animals with poor body condition were observed as positive cases. Number of negative cases in buffalo with good, fair and poor body conditions were twenty-eight, thirty-three and twenty- eight respectively. Regarding the prevalence of bovine tuberculosis, no significant difference ($p>0.05$) was found in buffalo on the basis of body condition. In case of cows, one animal with good body condition was found positive against bovine tuberculosis, while three animals with fair body condition was declared as positive, seven animals with poor body condition were observed as positive cases. Number of negative cases in cows with good, fair and poor body conditions were twenty-seven, twenty-nine and twenty-nine respectively. Regarding the prevalence of bovine tuberculosis, no significant difference ($p>0.05$) was found in cows on the basis of body condition.

DISCUSSION

In Australia, low specificity and sensitivity of the single intradermal test with both no visible lesion (NVL) reactors and infected anergic non-reactors has been a problem in some circumstances. In New Zealand, lack of sensitivity is not a major contributor to the TB problem in cattle, but has caused problems in deer herds. On a population basis specificity is good (> 99.5%). However, it does cause problems for individual cattle and deer herds, producing NVL reactors (particularly in deer herds where 30% of reactors had visable TB lesions compared to 42% in cattle) or requiring ancillary testing. The comparative cervical intradermal test was widely used to clarify the status of reactors in herds with no apparent history of *Mycobacterium bovis* and in herds with specificity problems (Rushford 1964; Rushford 1966). A standard autopsy procedure (Corner and Presidente 1980) for reactors and culture of lymph nodes from NVL reactors enabled the true status of these animals to be established.

The gamma interferon test (Wood and Rothel 1994), the result of research aimed to overcome these sensitivity problems, may also have a role in herds with non-specificity problems. However, stressed cattle, believed to be a significant contributor in northern Australia to the low sensitivity of the tuberculin test, respond very poorly to both the gamma interferon and single caudal fold tuberculin test (L.A. Corner, personal communication, 1991). In New Zealand, the LTT has been introduced as an ancillary test for deer to improve test sensitivity and specificity. Complete musters, destruction of unmusterable animals, age group segregation and age group culling are also important factors in an effective testing program.

In our study, it was proved that the older animals are mostly infected and become carrier of infection in spite of the younger ones. Animals of less than of 4 years of age are less infected than animals of age 4-6 years or more than of 6 years. One of the main individual risk factors identified by numerous studies in both developed and developing countries is the age of animals. The duration of exposure increases with age; older animals are more likely to have been exposed than younger ones, as shown by several cross-sectional studies carried out in Tanzania, Zambia and Chad (Cook et al. 1996; Kazwala et al. 2001; Delafosse et al. 2002; Cleaveland et al. 2007; Inangolet et al. 2008; Munyeme et al. 2009). In a 1996 cross-sectional study, which included more than 2000 individuals issued from 200 herds, Irish authors observed that calves were less likely to be positive reactors than older animals. Animals might get infected at a young age, but only express the disease clinically when they are adults (Griffin et al. 1996). Mycobacteria have the ability to remain in a latent state for a long period before reactivation at an older age (Pollock and Neill 2002). Nevertheless, scientists have not proved that a true dormant state exists in cattle (Van Rhijn et al. 2008). A lot still remains to be done in terms of scientific research to obtain an experimental latency model in cattle and to assess if there are any consequences, such as under diagnosis of the

disease, particularly in developed countries, in relation to it.

In our study, female cattle and buffalo were more prone to be effected by the bovine tuberculosis rather than the male cattle and buffalo. Gender has only been mentioned as a risk factor in studies carried out in Africa. Opinions diverge regarding its influence on the susceptibility to a M. bovis infection. A cross-sectional study conducted in Tanzania from 1994 to 1997, which included 5692 indigenous and 244 exotic cattle, revealed that male cattle were significantly more affected by bovine tuberculosis than female animals (Kazwala et al. 2001). Male cattle are mostly used as oxen, which are kept longer in the herd than females. Due to this particular longevity, it is more probable that they get in contact with infected cattle from other affected herds and in turn get infected; this would imply that between-herd contact is a major source of bovine tuberculosis transmission (Kazwala et al. 2001). From 2006 to 2007, a cross-sectional study on 1470 animals in Uganda revealed significantly more females positive to the skin test than males (Inangolet et al. 2008). Gender-linked factors are probably related to management practices or behavioral habits; males and females are managed differently, both in developed and developing countries. In developed countries, dairy cows usually reach an older age than males because of their role in calving and milk production.

In our study it can be concluded that exotic breeds are more susceptible to *Mycobacterium tuberculosis* infection rather than indigenous breeds. Studies performed in Africa also identified the animals' breed as a risk factor for a positive skin test. In 1998, a cross-sectional study carried out on 1813 animals (494 intensive dairy farms) of Eritrea suggested that imported breeds, used to improve the dairy industry in tropical areas, may be less resistant to bovine tuberculosis compared to indigenous breeds, e.g. zebu (Omer et al. 2001). Elias et al. observed a (not significantly) higher bovine tuberculosis infection rate in imported cattle during their 2005–2006 survey including 1572 dairy cattle in Ethiopia (Elias et al. 2008). The difference of susceptibility between breeds is likely to be related to differences in management: imported dairy animals are generally kept under intensive conditions. This suggested that a risk factor for bovine tuberculosis needs confirmation through additional studies (Cleaveland et al. 2007). Another question is whether a variability of the reaction to the skin test exists depending on the cattle breed. If established, this variability would imply that diagnostic tests should be suitably designed and applied according to the animal breed to be tested.

We concluded that animals with good body conditions were less susceptible to infection than the animals with fair and poor body conditions. The body condition score (BCS) relies on palpation of the sharpness, muscle and fat covering the backbones and lumbar processes and is determined on a 1 (emaciated) to 5 (obese) scale (Edmonson et al. 1989). In 1996, Cook et al. linked a low BCS to an increased risk of a positive skin test result in their cross-sectional study including 2226 animals in Zambia (Cook et al. 1996). During a matched case-control study including 80 chronic bovine tuberculosis herds carried out in 1990 in Ireland, Griffin et al. demonstrated that an animal's resistance to tuberculosis was reduced by a shortage of feed and/or an unbalanced diet (Griffin et al. 1996). This follow-up was carried out during three months, thus it is not sure a possible link between diet shortage and bovine tuberculosis could have been observed. A more recent study carried out in the same country over a one-year period dealing with 20 young steers positive to the Single Intradermal Comparative Cervical Tuberculin Test (SICCT), fed a restricted diet and housed in contact with positive reactor steers showed no evidence that dietary restriction had any effect on bovine tuberculosis transmission (Costello et al. 1998). Skin test reactors might have a poor BCS as a consequence of an advanced stage of bovine tuberculosis, as suggested by a cross-sectional study including 5692 indigenous cattle carried out in Tanzania between 1994 and 1997; animals suffering from a clinically advanced bovine

tuberculosis often present a low BCS as a result of the long-lasting pathological process (Kazwala et al. 2001). These cross-sectional studies were carried out during a definite period of time, so that this parameter should be considered carefully. In cross-sectional studies, scientists do not know the initial status of animals. It is not possible to distinguish a low BCS as a risk factor or as a consequence of clinically advanced bovine tuberculosis. The real impact of BCS should be the subject of directed studies dealing with diet restriction.

Conflict of interest

The authors declare that they are not in a situation of conflicting interests.

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