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BACTERIAL AND PCR BASED DIAGNOSIS OF NATURALLY OCCURRING BOVINE TUBERCULOSIS IN CATTLE AND BUFFALOES

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Bovine tuberculosis causes huge economic losses to the dairy industry in terms of poor growth, reduce milk production potential of infected animals, emaciation and poor quality of carcass. Bovine tuberculosis due to Mycobacterium bovis is a major threat to public health. Therefore, this study was aimed at to know the frequency of Mycobacterium bovis infection in cattle and buffaloes. A total of 265 animals, including adult cattle (132) and buffaloes (133) of different age and sex were screened for tuberculosis infection with tuberculin test. Equal numbers of milk and nasal swab samples were collected from tuberculin reactor and non-reactor cattle and buffaloes for the isolation and identification of the causative organism. The infectious agent was identified and confirmed through procedures such as culture characteristics, ZN (Ziehl-Neelsen) staining, and PCR (Polymerase Chain Reaction) was also performed. The study revealed an overall 10.56% (95% C.I. 7.28 to 14.71) prevalence of bovine tuberculosis, the frequency of infectious agent was higher in buffaloes (11.04%) than cattle (9.76%). TB (Tuberculosis) was present in 100% herds. Multivariate logistic regression analysis with the backward elimination procedure revealed that, age (OR=1.564), body weight (OR=1.008) and status (OR=5.72**)** showed a significant association with the occurrence of tuberculosis. Nasal samples yielded more positive PCR results than milk samples. By considering PCR as a gold standard, ZN was more sensitive and PPD was a more specific test. Among all tests, PCR proved most accurate and fast test for the confirmation of bovine tuberculosis.

Keywords: Stonebrink's, DNA, skin, Lactation, bacteria, tuberculin, PCR, disease.

INTRODUCTION

Tuberculosis is an oldest disease known to man. As the disease has a long incubation period, it is very difficult to judge clinical signs and symptoms in the early phase. Bovine tuberculosis is caused by *Mycobacterium bovis* (*M. bovis*) (OIE, 2015). The natural host of *M. bovis, is* cattle, but it can affect nearly all warm-blooded mammals, including humans (Perry *et al.,* 2002). In the animals, the major transmission route is an aerosol in adult animals and the pulmonary tuberculosis is the most common form than extra-pulmonary tuberculosis (Radostits *et al.,* 2007). In less number of cases spread of disease may occur through milk, colostrum (Daborn *et al*., 1996). In animals, mainly the disease remains subclinical, if the clinical signs appear it may include mild degree pyrexia, weakness, anorexia, emaciation, dyspnoea and varied degree of coughing (Cassidy, 2006). The disease can easily transfer from one animal to others through aerosol droplets, saliva and nasal secretions so, the animal living in high densities, confined housing and using common water drinkers are at higher risk of contracting the disease within the herd (Raghvendra *et al.,* 2010; Aziz-ur-Rehman, *et al*., 2017; Hussain *et al*., 2018). The disease has been reported across the globe. Bovine tuberculosis is consistently present in developing countries, especially countries those can't bear the cost of test and slaughtering program (Muller, 2010). Different studies in Pakistan on bovine tuberculosis in cattle, buffalo, sheep and goat have shown its consistent presence in the animals, which in general vary from 5-10% (Javed *et al*., 2006). In 2013, the disease was estimated to vary from, 6.62 to 11.96% in buffalo while 5.53 to 11.71% in cattle (Ghumman *et al*., 2013).

For the diagnosis of tuberculosis in the animals, main emphasis is based on tuberculin testing, observing clinical signs, necropsy findings, isolation on selected culture medium and Zhiel-Nelsen (ZN) staining (OIE, 2009). Tuberculin testing is a standard technique being used all over the world in the control and eradication program against tuberculosis (Cousins, 2001). Tuberculin testing can identify the disease usually after 30-50 days of infection (Paylor, 2014). Tuberculin testing is a cheaper test, but it has certain issues of false positive results. Culture isolation is needed for the confirm of the presence of bacterial agent, however, it requires a long time to obtain the viable growth on culture medium (Collins *et al.,* 1994). PCR is the advanced diagnostic technique with high specificity and sensitivity and it can produce results in a very short period and can easily be performed on direct clinical samples (Schiller *et al.,* 2010). The combined use of all these methods can increase the detection rate and improve the diagnosis of tuberculosis

(Ramosa *et al.,* 2015). Keeping in view all the factors the present study was planned to find out the prevalence of tuberculosis and associated risk factors in cattle and buffaloes of two colonies surrounding Faisalabad. To compare the usefulness of diagnostic tests in field conditions was another important objective of the study.

MATERIALS AND METHODS

***Study animals and sample size*:** Two cattle/buffalo colonies of Faisalabad, one on Aminpur road and second on Satiana road were included in the study and a total of 133 and 132 adult cattle and buffaloes, respectively above two years of age were screened for Tuberculosis on the basis of expected prevalence. For the calculation of the sample size the relevant formula for a 95% confidence limit was used with 10% expected prevalence and 5% desired absolute precision (Thrusfield, 2007):

$$n = \frac{1.96^2 \, Pexp \, (1-Pexp)}{d^2}$$

***Intradermal tuberculin testing*:** The intradermal tuberculin testing (IDTT) was performed by injecting 0.1 ml bovine purified protein derivative (PPD; produced by Instituto Zooprofilattico, Perugia, Italy) at cervical region. Skin thickness at administration sites was measured by using Vernier caliper by the same operator for all the animals. The animals were classified (positive and negative) on the basis of skin thickness as criteria described by Aagard *et al.* (2003). Certain epidemiologically important risk factors were also recorded on a specialized proforma, including specie, age, sex, status of animal and milk production.

***Sample collection*:** The study population (both cattle and buffaloes) was divided into different age groups, weight groups, status of milking and milk yield groups. From all reactors animals and equal number of non-reactor, but suspected animals, the sampling was done by observing all precautionary measures for collection of sample to maintain sterile conditions. Two ml milk sample from each animal was taken into a sterile screw capped container. The nasal secretions were also collected with sterile nasal swabs. All the samples were placed in ice buckets and transported to the lab for further processing.

The decontamination of all the milk samples was done with 4% NaOH solution. Processed samples were incubated for 30 minutes at room temperature. After neutralization with HCl solution, the samples were centrifuged at 1000 rpm/ 15 minutes. The supernatant was removed carefully, and sediment was used for slide preparation, inoculation on culture medium and DNA extraction for PCR (Akhtar *et al.,* 2015). Nasal swabs were dipped into a normal saline solution for 30 minutes. The swabs were well squeezed against the wall of the tubes, decontamination was done with 4% NaOH solution, after centrifugation at 1000 rpm for 15 minutes, the supernatant was discarded, and the pallet was used for slide preparation, inoculation on culture medium and DNA extraction for PCR (Akhtar *et al.,* 2015).

***Polymerase chain reaction technique*:** A set of primers specific for *Mycobacterium bovis* specie gene was used with the sequence (JB21 F-5′-TCGTCCGCTGATGCAAGTGC-3′; JB22 R-5′-CGTCCGCTGACCTCAAGAAG-3′) previously described by Rodriguez *et al.* (1995). The PCR was performed in a total volume of 25 µl containing 1x reaction buffer, 1.5mM Magnesium Chloride (Fermentas, USA), 2.5 U of *Taq* Polymerase (Fermentas, USA), 0.2 mM of each deoxynucleoside triphosphate (Fermentas, USA), 2µg of template DNA and 100 pmole of each primer (forward and reverse).PCR was performed in a thermal cycler (Qantarus, UK) with the following thermal cycling conditions were initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 1 min and extension at 72°C for 1 min with a final extension of 72°C for 10 min (Shah *et al.*,2002). The amplified product was subjected to gel electrophoresis for band separation. The DNA was stained with ethidium bromide and visualized under ultraviolet trans-illuminator (Syngene, USA).

***Statistical analysis*:** The data were analyzed by Mental Haenszel Chi-Square. The 95% confidence interval was also worked-out for various parameters (SAS, 2007). The describe section in WinPepi software and screening and diagnostic test validity of measure component were used. The comparison of two test tool was used to elaborate results on sensitivity and specificity also add logistic regression analysis.

RESULTS

The study revealed an overall prevalence of 10.56% (95% C.I. 7.28 to 14.71) at two cattle and buffalo colonies based on tuberculin testing (Fig. 1).

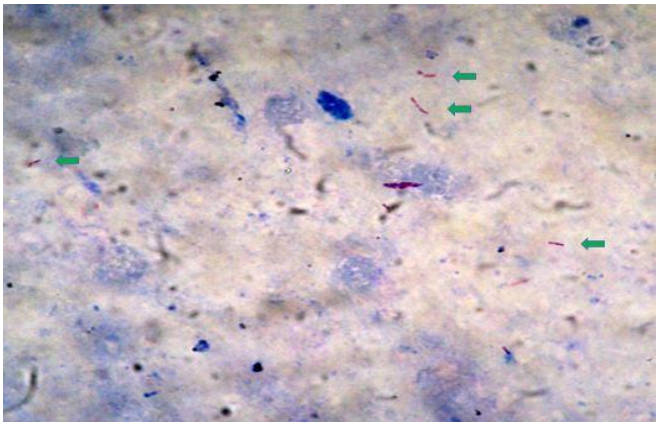

Figure 1. ZN stained positive results (pinkish rod shape organism is visible in the field).

Table 1. Different colonies and species based prevalence percentage at two cattle/buffalo colonies.

Parameters	Tuberculin		95% CI	Mentel Haenszel Chi.
Parameter	Positive/Negative	Positive %age		Square Test
Colony				
Satiana Road	12/132	9.09	5.02 to 14.94	P=0.436
Aminpur Road	16/133	12.03	7.29 to 18.41	
Species				
Buffalo	19/172	11.04	6.99 to 16.41	P= 0.729
Cattle	9/93	9.67	4.82 to 17.01	

Table 2. Different parameter wise prevalence percentage at two cattle/buffalo colonies.

Parameter	Tuberculin		95% C.I.	Mantel-Haenszel chi-sq
	Positive/ Negative	Positive (%)		
Herd				
1	3/19	15.7	4.18 to 37.21	P= 0.561
2	2/21	9.5	1.63 to 28.05	
3	2/25	8	1.36 to 24.00	
4	1/19	5.2	0.26 to 23.33	
5	1/26	3.8	0.19 to 17.54	
6	3/23	13	3.43 to 31.53	
7	2/20	10	1.71 to 29.29	
8	4/30	13.3	4.38 to 29.10	
9	3/30	10	2.61 to 24.85	
10	2/20	10	1.71 to 29.29	
11	5/32	15.6	5.96 to 31.29	
Age (years)				
3-7	24/222	10.81	7.21 to 15.42	P=0.768
8 or more	4/43	9.30	3.03 to 20.93	
Weight groups (Kg)				
<500	2/41	4.8	0.83 to 15.19	P= 0.198
>500	26/224	11.6	7.89 to 16.31	
Milking Status				
Dry	2/46	4.34	0.74 to 13.63	P= 0.131
Lactating	26/219	11.87	8.07 to 16.67	
Milk Yield (Liters)				
1-4.9	1/9	11.1	0.56 to 43.86	P=<0.120
5-9.9	23/154	14.93	9.95 to 21.23	
10-15	3/56	5.36	1.38 to 13.89	

In the individual colonies 9.09% (12/132) prevalence was observed in Satiana road colony, while 12.03% (16/133) prevalence rate was observed in the cattle/buffalos of Aminpur road. The herd-prevalence rate was 100%. Among species the prevalence was 11.04% in buffalos (95% CI 6.99 to 16.41) and 9.76% in cattle (95% CI 4.82 to 17.01). The statistical analysis revealed non-significant differences between different age groups, weight groups, status of lactation and milk yield groups (Table 2).

The bivariate logistic regression analysis in cattle and buffaloes revealed the significant association of age of the animals with positive skin test (Table 3). The results showed that with the increase in one-year age, there will be 56.4% more chance of a positive skin test.

Table 3. Parameters showed significant association with Tuberculosis in cattle and buffaloes in logistic regression analysis procedure.

Parameter	Odd Ratio	P-Value
Bivariate Logistic regression		
Age	1.564	0.006
Multivariate logistic regression		
Age	1.564	0.006
Body Weight	1.008	0.050
Status	5.720	0.050
After controlling age as constant bivariate logistic regression		
Specie	2.32	0.050
Status	9.19	0.035

Multivariate logistic regression analysis with the backward elimination procedure revealed that, age (OR=1.564), body weight (OR=1.008) and status of the animals (OR=5.72**)** showed a significant association with a positive skin test (Table 3). With a one-year increase in the age, there will be 56.4% more chance of a positive skin test and with the increase in one kg body weight there will be 0.008% more chance of a positive skin test. The results also revealed that the chances of a positive skin test for tuberculosis in buffalo were 5.72 times higher than in cattle. The data revealed that by adding other methods with tuberculin testing the detection rate of TB was increased from 10.56% (tuberculin testing) to 12.45% with ZN-staining (Fig. 2), and 13.58% with direct PCR (Table 4).

Table 4. Comparison of different diagnostic tests to detect TB rate.

Test	Positive %age	Negative	95% CI	P-Value
PPD	28 (10.56)	237	7.28-14.71	
ZN	33(12.45)	232	8.87-16.85	0.826
Culture	22(8.30)	243	5.41-12.10	
Direct PCR	36(13.58)	229	9.85-18.42	

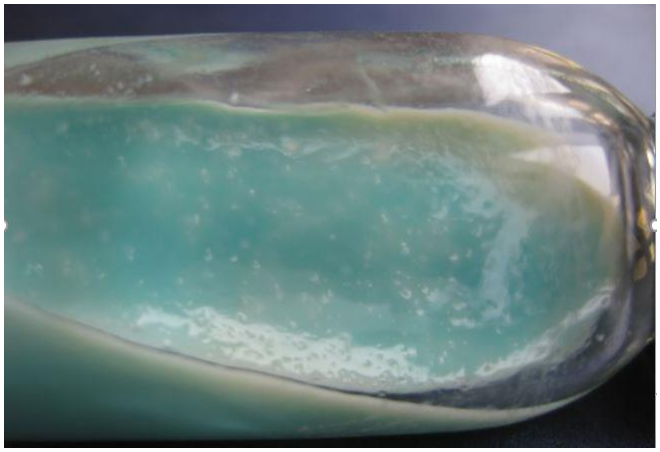

Figure 2. A positive growth on Stonebrink's medium (white color pin shape colonies are visible).

From clinical samples (PPD +ve plus suspects n=58) PCR was positive in 17.8% milk samples and 46.4% nasal samples. Individually, it was positive in 7.1% milk samples alone and 35.71% of nasal samples alone. In 10.71% cases, PCR was positive in both nasal and milk samples together (Table 5). A comparison was made in terms of specificity and sensitivity by considering PCR as a gold standard (Fig. 4), the PPD sensitivity and specificity were 77.8 and 100%, while ZN sensitivity and specificity were 86.1 and 99.1%, respectively. It shows that ZN is a more sensitive and tuberculin is a more specific technique.

Table 5. The results of PCR from clinical samples (n=28+28=56).

Type of clinical samples	Positive %age	95% CI
Milk	10 (17.8)	9.44-29.52
Nasal	26 (46.4)	33.71-59.51
Milk alone	4(7.1)	2.31-16.33
Nasal alone	20(35.7)	24.02-48.85
Milk+Nasal	6(10.7)	4.46-20.96

Table 6. The relationship between PPD and ZN at two cattle/buffalo colonies.

Parameters	Direct PCR			
	Positive		Negative	
ZN	PPD Positive	PPD Negative	PPD Positive	PPD Negative
Positive	28	3	0	2
Negative	0	5	0	227

PPD Sensitivity = 77.8%
PPD Specificity = 100%
False +Ve = 0%
Both tests Sensitivity = 77.8%
ZN is more sensitive, PPD is more specific.
ZN Sensitivity = 86.1%
ZN Specificity =99.1%
False +Ve = 0.9%
Both tests Specificity = 100%

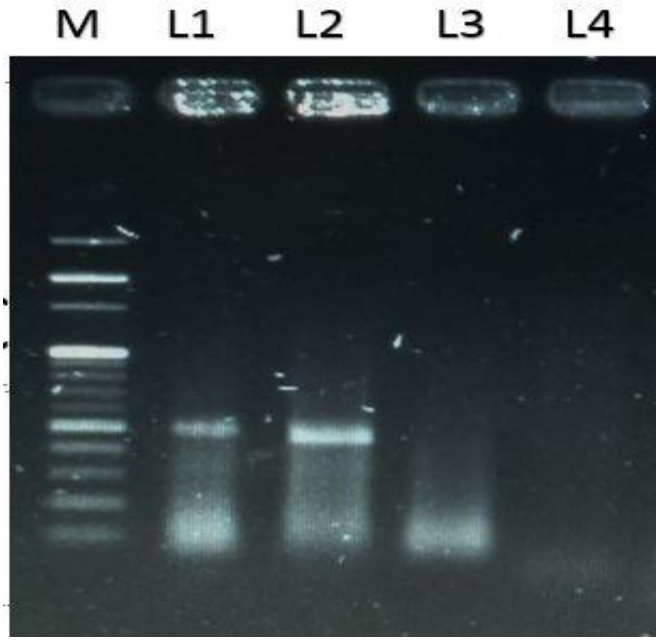

Figure 3. PCR positive results for *M. bovis* (500bp). From L to R: M=DNA ladder, L1-L2= 500 bp band positive results, L3= control, L4= Blank).

DSICUSSION

Different bacterial diseases with known and unidentified etiology are the major and a serious threat for both animal and public health in poor and developing regions of the world. These diseases not only influence the production potential of target animals, but also act as barrier in trade of different dairy animals. The dairy animals in Pakistan produce about 43.56 million tons of milk annually, which ranks the country at 4th position among different milk producing countries in the world (Ali *et al.,* 2016; Qayyum *et al.,* 2016). The present study revealed an overall prevalence of 10.56% at two livestock colonies. Individually, the prevalence was 9.09% at Satiana road colony and 12.03% at Aminpur road colony. The prevalence was high in buffaloes (11.04 %), while low in cattle (9.67%). The study conducted by (Ghumman *et al.,* 2013) revealed that the buffaloes were found to be more positive for the disease than cattle. Different prevalence based studies in Pakistan has been carried out on cattle and buffaloes and indicated a varied prevalence. A study showed the prevalence of bovine tuberculosis in buffaloes to be10.6 % (Khan *et al.,* 2008); whereas, Javed *et al.* (2011) reported 8% disease prevalence in cattle. The disease was present in all herds with varying frequency ranging from 3.8% as minimum to 15.7% as highest number of cases at two colonies. Maximum disease cases were observed in the older animals having age 3-7 years, followed by age more than 7 years. Similar findings were reported in another study conducted in India by Thakur *et al.* (2010), where most of the cases of bovine TB were observed in the age groups 6-9 years and 9-12 years. The high percentage of disease in older animals could be due to the prolonged exposure of organism in close confined areas. The other reason may be the decrease immunity with increase age in a very hot and humid environment.

The cattle and buffaloes were divided into two groups; prevalence was more in heavy animals (>500 kg) than lighter weight. A prevalence based study conducted by Javed *et al.* (2012) also showed that the occurrence of bovine tuberculosis was more in heavy animals (>500 kg body weight). Based on milking status, prevalence was more in lactating animals. Based on milk yield, animals were divided into 3 groups, i.e., 1-4.9, 5- 9.9 and 10-15 liters, maximum prevalence was observed in group having 5-9.9-liter milk production in a day. Similar type of findings were also reported by Khan *et al.* (2008) as in the study, higher number of positive reactors had a milk production of more than 7 liters. The probable reason of more the number of cases in high producing animals could be due to the reason that high milk yield, enhance the disease load and animals can become immunocompromised due to constant production stress.

Multivariate logistic regression analysis with the backward elimination procedure at two buffalo/cattle colonies revealed that, age, body weight and status showed a significant association ($P<0.005$) with the occurrence of tuberculosis, while the bivariate logistic regression analysis in buffalo and cattle revealed that after controlling the age as a constant factor, the specie and status of animals showed significant association with the occurrence of tuberculosis. From Pakistan an earlier study conducted by Javed *et al.* (2013), reported the association of disease with age and body weight of animals, additionally total number of animals were also associated with occurrence of disease.

The data revealed that by adding other methods with tuberculin testing the detection rate of TB was increased from 10.56% (tuberculin testing) to 12.45% ZN-staining and 13.58% with direct PCR. The major reason why the least number of tuberculin positive animals were obtained as compared to PCR positive results is that the tuberculin testing has a problem as in immunocompromised state the PPD results could be negative, but PCR can confirm the results at the species level (Wagari, 2016). A combined use can increase the detection rate. Clinical samples raw milk and nasal swabs were collected from a reactive and equal number of non-reacting animals (n=58). PCR was positive in 17.8% milk and 46.4% nasal samples. While, it was positive in 7.1% milk samples alone and 35.71% of nasal samples alone, in 10.71% cases, PCR was positive in both nasal and milk samples together (Table 5). These figures are lower than previous reports as, Mumtaz *et al*. (2008) showed 29% milk samples positive by PCR. Even higher detection rate (52.8%) from milk and (64.2%) from nasal samples was also observed in a study conducted by Akhtar *et al*. (2015) at Government livestock farms. PCR was proved most sensitive and specific method as it requires only 5 fg of DNA from clinical samples to produce positive results (Figueiredo *et al*., 2010)

In addition, with tuberculin testing, ZN staining, culture isolation and PCR was also performed. Less number of cultures isolate were observed due to the harsh environment and probably due to the lesser number of viable bacteria in the clinical samples. Considering PCR as a gold standard, the PPD sensitivity and specificity were 77.8% and 100%, while ZN sensitivity and specificity were 86.1 and 99.1%, respectively.

***Conclusions*:** The study revealed an overall 10.56% prevalence of bovine tuberculosis, the frequency of infectious agent was higher in buffaloes (11.04%) than cattle (9.76%). TB was present in 100% herds. Multivariate logistic regression analysis with the backward elimination procedure revealed that, age, body weight and status showed a significant association with the occurrence of tuberculosis. Nasal samples yielded more positive PCR results than milk samples. By considering PCR as a gold standard, ZN was more sensitive and PPD was a more specific test. Among all tests, PCR proved most accurate and fast test for the confirmation of bovine tuberculosis.

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