Research Article

Bovine Tuberculosis (bTB): Detection of *Mycobacterium bovis* Infection with Specie-specific Primers in Sputum Samples in Pakistan

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Abstract | Tuberculosis (TB) is a communicable and chronic infection triggered by *Mycobacterium tuberculosis* (MTB). Bovine tuberculosis (bTB) is one amongst neglected zoonotic disease (NZD) caused by *Mycobacterium bovis*; has an economic importance with public health significance and life-threatening infection at the human-animal-ecosystem interface in low-resource settings and developing countries globally. Our study aimed to investigate the incidence of pulmonic tuberculosis caused by *M. bovis* in specific occupational groups; to study the socio-demographic conditions, awareness, level of knowledge and practices about tuberculosis; to investigate the molecular prevalence of bTB. Overall, 390 no of sputum samples collected as of 800-participants comprised of TB patients (100), livestock(L/S) farm employees(200), abattoir employees(174), butchers(294), veterinarian(10) and veterinary assistants(22). Two out of 100 TB patients (2/100), 3 out of 200 livestock farm workers (3/200) and 3 out of 23 abattoir workers (3/23) were found positive for the presence of *M bovis* through PCR technique. A significant association ($p<0.05$) was found between livestock farm workers and abattoir workers with the prevalence of zoonotic tuberculosis. The level of knowledge and awareness about the zoonotic bTB were very low and considered high-risk occupational groups of gaining zoonotic TB. Bovine tuberculosis is a main public health issue in different job-related groups in district Peshawar, Khyber Pakhtunkhwa, Pakistan. The surveillance, prevention and control programs of this NZD in the KP province, Pakistan is necessary because of the continual unrestricted movement of the animals which might result in an increased spread of bTB to humans.

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Introduction

Tuberculosis (TB) remains the principal cause of high death ratio across the world with severe public health concern (Adesokan *et al*., 2012). More than 95% of TB death occur in the developing world (Lolch *et al*., 2003). *Mycobacterium tuberculosis* is known to account for most cases of human TB; however, the proportion due to *M. bovis* is unknown. Bovine tuberculosis (bTB) is mainly caused by *M. bovis* ssp. *bovis* and to a lesser extent by *M. bovis* ssp. *caprae* (Ayele *et al*., 2004; Cosivi *et al*., 1998). *Myco-*

bacterium tuberculosis (MTB), *M. africanum, M. pinnipedii, M. canetti*, *M. microti* along with vaccination strain *Bacillus* Calmette-Guerin form the *Mycobacterium tuberculosis complex* (MTC). *Mycobacterium* spp. are Actinomycetales like Corynbacteriaceae, Tsukumurellaceae and Nocardiaceae (Tortoli, 2003; Tortoli *et al*., 2006a; 2006b). Cattle signify the chief reservoir of bTB however there is a rare human-human transmission (Evans *et al*., 2007). The incidences which are reported under WHO are 0.002 % (2/100,000) in African region, 0.007 % (7/100,000) in American region,0.03 % (30/100,000) in European region (Muller *et al*., 2013). Bovine TB is one of the neglected zoonotic diseases (NZD) at the human-animal-ecosystem interface and has been reported to cause disease in humans in widespread regions across the world (Thoen *et al*., 2010). A survey was conducted by the World Health Organization in 2017, wherein approximately 10.4 million people become ill with TB infection including 90% adults, 65% male, 10% people living with HIV infected individuals (74% in Africa) and 56% were found infected in five countries listed in descending order due to number of incident cases *i.e*; India, Indonesia, China, the Philippines and Pakistan (WHO, 2017). Globally, Pakistan is estimated to have the 4th highest prevalence of multidrug-resistance (MDR) TB (WHO, 2017). An increase in AIDS and drug resistance has been result in high incidence of human tuberculosis (TB) (Zumla *et al*., 1999). The tuberculosis issue has been intensified by increase in incidence of human immunodeficiency virus (HIV) and emergence of multidrug resistance (MDR) (Zumla *et al*., 1999). Human immunodeficiency virus (HIV) plays a significant role in development and succession of active TB in individual's septic with TB and HIV (Narain *et al*., 2004). *Mycobacterium tuberculosis* is the most common cause of TB in humans but humans are also infected by *Mycobacterium bovis* (*M. bovis*) that causes bovine tuberculosis (bTB) (Hlavsa *et al*., 2008). The diseases that are transmitted from animals (vertebrates) to humans are called zoonoses. In human, majority of newly emerging infections are of zoonotic origin (Quammen, 2012). Most zoonoses occure during preparation of infected meat and ingestion or close contact to animals duing hunting, herding or slaughtering animals (Karesh *et al*., 2012). Transmission of *Mycobacterium bovis* occurs either inhalation or oral route (Winthropet *al*., 2005). All the developed countries have been succeeded in reducing the transmission of TB in humans with the help of proper pasteurization of dairy products and proper control of bTB infection in bovines (Michel *et al*., 2010; Torgerson *et al*., 2008). Although TB in humans is important zoonotic disease reported globally where milk and other dairy products are utilized with improper pasteurization (Ullah *et al*., 2020; Moda *et al*., 1996). All the developed countries are poorly documented regarding data of human TB caused by *M. bovis* (zoonotic TB) due to poor diagnostic facilities such as sputum cytology only. Pakistan has been ranked fifth (5th) country amongst high burden TB countries globally where 193 million total populations has been reported (WHO, 2017).

The World Health Organization (WHO) has suggested proper gathering of information regarding zoonotic diseases in all unindustrialized states (WHO, 1994). Here in Pakistan, we have no regular system for screening of animals for presence of bTB using tuberculin dermal test. Different researchers reported dissimilar prevalence in different areas of research institutions such 6%, 8% and 2.5% &10% in Peshawar, Lahore and different livestock farms in Punjab area respectively (Irfan *et al*., 2016; Jalil *et al*., 2003; Javed *et al*.,2006; Khan *et al*., 2008). In Peshawar, a study was conducted at a slaughterhouse to record bTB in bovines and as result 13% and 14% prevalence was detected in cattle and buffaloes respectively (Khan *et al*., 2014).

In the present study prevalence of bTB in bovines and the zoonotic aspect was studied because limited data was available about livestock (L/S) farm employees, abattoir employees, veterinarians and butcher's population. Unluckily, here in Pakistan we have no known data to operate bTB control program successfully and aware our public against prevention strategies (Adesokan *et al*., 2012). Therefore, this study was designed and conducted to find out the prevalence and associated risk factors responsible for an outbreak of pulmonary TB (*M. bovis*) in livestock farm workers, slaughter house workers, animal handlers, butchers, veterinary assistants and veterinarians. Furthermore, the awareness and different practices of these professionals regarding TB was also studied.

Materials and Methods

Ethical approval

This research study was carried out in accordance with the code of ethics and was duly approved by the Ethical Review Committee (ERC), The University of Agriculture Peshawar, Khyber Pakhtunkhwa (KPK), Pakistan.

Figure 1: *Sample collection site, district Peshawar Khyber Pakhtunkhwa (KP) province. Pakistan.*

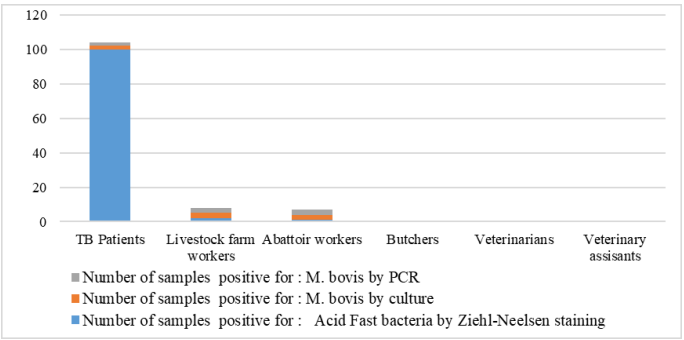

Figure 2: *Graph showing the overall results of Ziehl-Neelsen staining, Culture and PCR of the occupational groups in the study area.*

Sampling and methodology

The present research study was performed in district Peshawar, Khyber Pakhtunkhwa (KPK) province, Pakistan. A total of 390 no of sputum samples were collected from 800-participants comprised of TB patients (100) who were admitted in different hospitals in the study area, livestock(L/S) farm employees (200) who were exhibited clinical signs of cough up sputum, abattoir employees (174), butchers (294), veterinarian (10) and veterinary assistants (22). A total of 100 meat retail shops were selected randomly in the study area. Out of 294 butchers working in these meat retail shops, 35 sputum samples were collected from those butchers who were doubted of having TB. All the collected sputum samples were processed and analyzed for the detection of *M. bovis* with Ziehl-Neelsen (ZN) staining, bacterial culture growth and polymerase chain reaction (PCR). Random sampling technique was followed. Different dairy farms and farmer's community associations were visited and history, physical examination and family information were recorded. Similarly, history of chronic cough and sputum samples were collected from veterinarians (V.O's) and veterinary assistants(VA's) employed in research area.

The author(s) used a predesigned feedback form and carried out the exchange of ideas in Pushto (local dialectal). The interview was focused on tuberculosis and the knowledge, awareness, practices and socio-demographic conditions of the participants with respect to tuberculosis. Distinct feedback form was designed for abattoir workers, slaughterers, veterinarians, veterinary assistants and livestock farm workers.

Ziehl-Neelsen (ZN) acid fast staining

The lipoid-capsule of acid fast-bacterium takes-up carbol-fuchsin & repels-decolorization by weak acid-rinse. The lipoid-capsule (waxy on room-temperature) of mycobacteria having high-molecular weight & penetrates effectively through aqueous-based staining-solutions. Glass slides, filtered water, carbol fuchsin, acid-alcohol, methylene-blue, burner & microscope were use. The slide was first deparaffinizeed and hydrated to distilled H_2O. A thin smear was formed and then heat-fixed the smear on slide. The slide was poured with carbol fuchsin and steamed

for 5-10 minutes. Then the slides were washed and few drop of acid alcohol (20% H_2SO_4) was drove on the smear for decolorization for 01 minute. Then it was wash away with consecutively tap-water for 5 minutes. Then rinsed in distilled-water. The working methylene blue was poured for30 second followed by rinsed in water. Finally, glass-slide dehydrated, cleared & covered with cover slip. The slide was first examined with 10x power and then with 100x (Quinn *et al*., 1994).

Isolation of bacterial culture

For bacterial isolation, selective culture media was used which is Stone brinks for *M. bovis* and Lowenstein and Jensen medium for *Mycobacterium tuberculosis*. After the processing of sputum samples, the settled pellet was spread onto the slants of culture media in the glass tubes and incubated at 37 ^{0}C for 8-weeks (Payeur *et al*., 1993). The growth appeared on culture media was morphologically characterized and then confirmed by using PCR technique.

DNA extraction and PCR

The processing of sputum samples and DNA isolation was performed with Geno Lyse ® DNA kit by Hain Lifesciences GmbH, in biosafety level III Lab at Provincial Tuberculosis Reference Laboratory (PTRL), Peshawar. Procedure for PCR (Irfan *et al*., 2016) was followed with some modification for molecular detection of *Mycobacterium bovis* in sputum samples. The detection of *M. bovis* was carried out by amplification of 500bp PCR product with specie-specific primers as JB primers (JB21 as Forward:5'-TCGTCCGCTGATGCAAGTGC-3'andJB22 as Reverse: 5'-CGTCCGCTGACCTCAAGAAG-3'). The optimized PCR was performed in BIORAD T100™, Biorad® thermal cycler per 25µl reaction volume for each study sample with 1.75µl of forward (F) and reverse (R) primer each, 10µl mastermix, 2.5µl of DNA sample and 9µl DNA as-free deionized water. The PCR amplification protocol was involved 3 steps *i.e*; denaturation (95^{0}C for 30sec), annealing (55^{0}C for 30sec) and extension (72^{0}C for 1minute). The very sample were processed to 35cycles before final extension (72^{0}C for 5 minutes) and 500bp amplified PCR product was obtained. The PCR amplified products were visualized with UV illumination (Figure 3).

Gel electrophoresis

A 10X Tris-acetate-EDTA(TAE) buffer of 100ml was taken in 100ml flask with 1.5gram gel agarose and was dissolved using the microwave oven (1minute and 30sec). Soft gel stain of 4µl SYBR® (Thromo-Fisher Scientific) was added to flask and then the final flask solution was poured into gel tray already fitted with comb. The soft gel stain helps in visualization of amplified PCR product. Gel tray was placed in electrophoresis tray already filled with TAE buffer (10X). The PCR amplified products along with 6µl DNA-ladder (1000bp) was transferred to gel wells by means of micropipettes and finally power supply with 120 volte Voltage, 500 mAmp current for 35minutes was applied. Lastly, the gel was seen in gel doc system and was photographed (Figure 3).

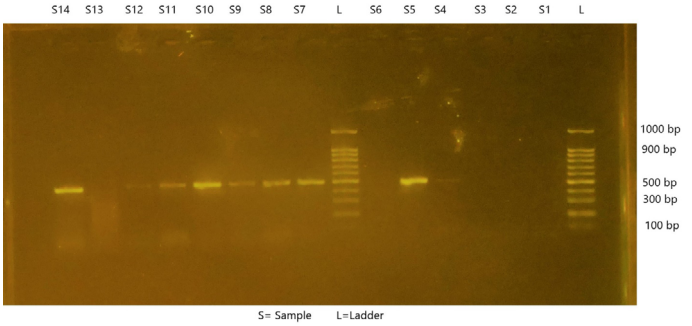

Figure 3: *PCR amplicon showing specie-specific 500bp DNA of Mycobacterium bovis (S5, 7-12 and S14). S1-4, 6 & 13 are negative samples.*

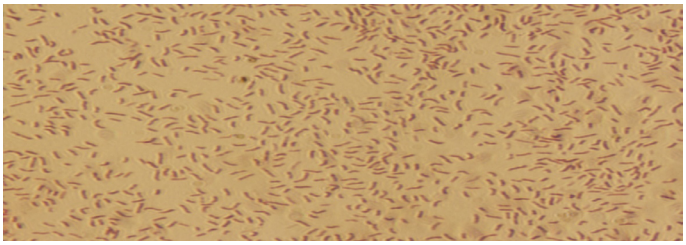

Figure 4: *Ziehl-Neelsen staining of sputum samples of TB patients showing acid-fast bacilli.*

Statistical analysis

The bivariate frequency analysis of various parameters were calculated by applying Chi squared test after the data was processed and analyzed with SPSS (Version 23.0). The percent prevalence of the disease was presented in the form percentages.

Results and Discussion

Amongst the 100 sputum samples collected from TB patients, all samples (100%) were found positive for acid fast bacteria through Ziehl-Neelsen staining (Figure 4) while two samples (2%) were found positive for *M. bovis* through culture and PCR each (Table 1, Figure 2). Similarly, a total of 200 livestock farm workers were interviewed and sputum samples

Table 1: *Sample distribution and PCR results in the study area.*

Group	Total number in study area	Number interviewed	Number of sputum samples collected	Number of samples positive by Ziehl-Neelsen for Acid Fast bacteria (%)	Number of samples positive for M. bovis by culture	Number of samples positive for M. bovis by PCR
TB Patients	Data not available	100	100	100 (100%)	2 (2%)	2 (2%)
Livestock farm workers	Data not available	200	200	2 (1%)	3 (1.5%)	3 (1.5%)
Abattoir workers	Data not available	174	23	1 (4.35%)	3 (13.04%)	3 (13.04%)
Butchers	Data not available	294	35	0 (0.0%)	0 (0.0%)	0 (0.0%)
Veterinarians	33	10	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Veterinary assistants	76	22	22	0 (0.0%)	0 (0.0%)	0 (0.0%)

Table 2: *Bivariate frequency analysis of various parameters of the livestock farm workers.*

Parameter	Negative	Positive N(%)	Chi-square
Type of work at livestock farm			
Watering & Feeding	9	1(10.00)	p= 0.014
Milking	11	1(8.33)	
Cleaning	92	0(0.0)	
All tasks	21	1 (4.55)	
Others	64	0(0.0)	
Duration of work (years) at livestock farm			
< 6	137	0(0.0)	p=0.036
7–15	21	1(4.55)	
>15	39	2(4.88)	
Age of farm worker (years)			
upto 18	40	0(0.0)	p= 0.015
19–25	54	0(0.0)	
26–35	66	0(0.0)	
36–45	26	2(7.14)	
> 45	11	1(8.33)	
Education of farm worker			
Illiterate	21	2(8.70)	p=0.005
Primary	10	1(9.09)	
Secondary	78	0(0.0)	
Higher Secondary	39	0(0.0)	
Graduate	49	0(0.0)	

were collected and analyzed for the presence of *Mycobacterium bovis*. Two samples (1%) were found positive through Ziehl-Neelsen staining (Figure 5) while three samples (1.5%) were found positive through culture and PCR each for the presence of *M. bovis* (Table 1, Figure 2). Type of work, duration of work (years), age (years) and education of the L/S farm employees was associated significantly ($p<0.05$) through occurrence of zoonotic tuberculosis (Table 2). Among the farm workers, 10 (5%) were involved in watering and feeding while 12 (6%) farm workers were involved in milking, 92 (46%) in cleaning the overall farm, 22 (11%) performed all tasks and 64 (32%) in other farm activities *i.e*; procuring and carriage. Nobody of the farm employees had undertaken job related formal education at livestock farm. Similarly, the farm workers working for >15 years at the farm were found more positive for TB as compared to the workers working for < 15 years at the livestock farm. During the research, the disease found significantly associated with old age of the farm workers. The livestock farm workers with age >45 years were found more positive for TB as compared to young workers. Likewise, the disease was not detected in the educated farm workers and was found in the illiterate group or those who got primary education only (Table 2).

During the investigation, all the livestock farm workers included in this study were male and interviewed. 39 % (78) were found with age above 45 years & 41% (82) with education at secondary level. No single farmers was found with any proper work related training. Similarly, 57% (114) of the livestock farm workers consumed boiled milk regularly, 12% (24) consumed boiled milk occasionally whereas 7% (14) used whole milk rarely. Simply 9% (18) of livestock farm workers used gumboots and no one used gloves in the course of animal management and cleaning of the livestock shed. Only 59% (118) of the farm workers used soap for hand and body washing. 100% of the farm workers had heard about TB. 84% (168) of the livestock farm workers assumed that human TB can be treated completely by following the treatment. None of the farm worker was aware of the amalgamation of 3 key symptoms of TB. Nobody of the L/S farm employee had tested their animal farm for bTB

infection. Only 3% (6) properly indicated that bTB is not curable in animals. As the farmers was not aware that globally the bTB treatment is not recommended so when they were enquired about their strategy to adopt if an animal was found affected by bTB infection. Only 68% (136) of the respondents specified that they would do medication for the same. 22% (44) identified that TB infected animals would be sold in the market. Nobody of the farmer indicated that TB infected animal would be culled and buried. Almost 81% (162) of the farm workers revealed ignorance in education and training about infectious and zoonotic animal diseases.

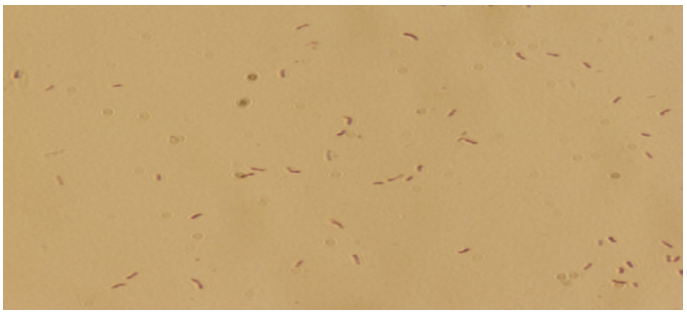

Figure 5: *Ziehl-Neelsen staining of sputum samples of livestock farm workers showing acid-fast bacilli.*

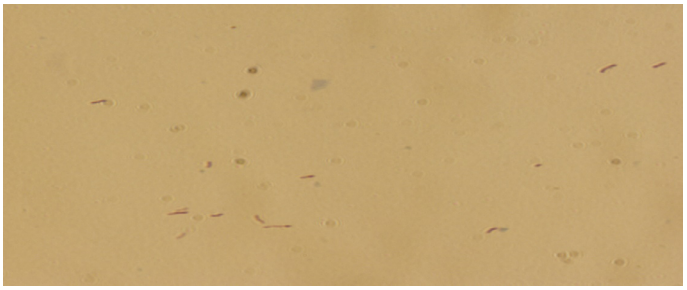

Figure 6: *Ziehl-Neelsen staining of sputum samples of abattoir workers showing acid-fast bacilli.*

A total 174 abattoir workers were interviewed where 23 had been suffering from chronic cough. Twenty-three (23) Sputum samples were collected for the presence of biological agent *i.e*; *M. bovis*. Of these, one samples (4.35%) was found positive through Ziehl-Neelsen staining (Figure 6) while three samples (13.04%) were found positive for the presence of *M. bovis* through culture and PCR each (Table 1, Figure 2). All the abattoir workers included in this study were male and the reply rate of the slaughter house labor was around 93% (174/187). Type of work, duration of work (years), age (years) and education of the abattoir worker was significantly associated ($p<0.05$) with incidence of zoonotic TB. Those who had worked for more than 10 years in the abattoir were more affected by zoonotic TB as compared to those who worked for a short time. All the selected slaughter house workers were interviewed and it was documented that 77% (134) were having direct interaction with animal blood and dead bodies. Personal protective equipment (PPE) like gloves, masks and hair nets were used by only 2 % (6) of the abattoir workers while gumboots and aprons were used by 35% (61) and 19% (33) respectively. Similarly, before going to start work, 88% (153) of abattoir workers took baths. In winter 59% (103) took steady bath before going to start work and 73% (127) took bath when finished their job while during summer, all workers at slaughter house took baths when finished their job. Other facilities like warm water, antiseptics and disinfectants were not offered at slaughter house to workers for their washing and for the equipment they used during work at abattoir. It was stated by the abattoir workers that on job veterinarian do anti-mortem inspections normally. As animals were not screened for bTB hence, no animal had been rejected due to the presence of bTB infection, during the inspection. Further, after butchery, due to the presence of tuberculous lesions, no animal had been put away neither partly nor as a whole so far. Similarly, knowledge about bTB and its transmission to human was also poor. It was documented that 23% (40) of abattoir workers had no knowledge of TB and its zoonotic transmission. Only 86% (150) of slaughter house workers indicated that they had heard about TB while 39% (68) workers indicated the transmission of TB occur from animals to human via the consumption of raw milk. Likewise, 54% (94) declared that the transmission occurs via the consumption of raw meat and 46% (80) declared aerosols route for the transmission of TB from animals to humans. It was found that the majority of abattoir workers have no knowledge about bTB and its zoonosis.

Only 7% (14) livestock farm workers and 3% (5) abattoir workers showed health-certificate stating them zoonotic sicknesses free. The 14 L/S farm workers and 5 abattoir workers considered themselves at risk of getting diseases while working with animals at livestock farm and in abattoir respectively. These occupationally exposed workers conducted complete body checkup once per annum in a tertiary care hospital and obtained health certificate for their own satisfaction. Although, proper annual body check-up and getting health certificate is not compulsory for the occupationally exposed workers in the country. However, the attitude of the livestock farm and abattoir workers weather or not they consider themselves

Table 3: *Distribution of signs and symptoms of bovine tuberculosis known to the livestock workers in the study area.*

Signs and symptoms	Group	Yes, n (%)	No, n (%)
Coughing up blood or sputum	TB patients	100 (100)	0 (0)
	Livestock workers	48 (24)	152 (76)
	Abattoir workers	35 (20)	139 (80)
	Butchers	76 (26)	218 (74)
Chronic cough lasting for >3 weeks	TB patients	100 (100)	0 (0)
	Livestock workers	40 (20)	160 (80)
	Abattoir workers	33 (19)	141 (81)
	Butchers	74 (25)	220 (75)
Pain in the chest	TB patients	99 (99)	1 (1)
	Livestock workers	44 (22)	156 (78)
	Abattoir workers	26 (15)	148 (85)
	Butchers	71 (24)	223 (78)
Weight loss	TB patients	100 (100)	0 (0)
	Livestock workers	132 (66)	68(34)
	Abattoir workers	120 (69)	54 (31)
	Butchers	197 (67)	97 (33)
Weakness or fatigue	TB patients	100 (100)	0 (0)
	Livestock workers	100 (50)	100 (50)
	Abattoir workers	90 (52)	84 (48)
	Butchers	173 (59)	121 (41)
Annorexia	TB patients	99 (99)	1 (1)
	Livestock workers	160 (80)	40 (20)
	Abattoir workers	146 (84)	28 (16)
	Butchers	229 (78)	65 (22)
Fever	TB patients	99 (99)	1 (1)
	Livestock workers	156 (78)	44 (22)
	Abattoir workers	136 (89)	19 (11)
	Butchers	235 (80)	59 (20)
Chills	TB patients	99 (99)	1 (1)
	Livestock workers	80 (40)	120 (60)
	Abattoir workers	42 (24)	132 (76)
	Butchers	82 (28)	212 (72)
Sweating at night	TB patients	99 (99)	1 (1)
	Livestock workers	32 (16)	168 (84)
	Abattoir workers	30 (17)	144 (83)
	Butchers	47 (16)	247 (84)
Diarrhoea	TB patients	36 (36)	64 (64)
	Livestock workers	140 (70)	60 (30)
	Abattoir workers	92 (53)	82 (47)
	Butchers	153 (52)	141 (48)
Vomiting	TB patients	41 (41)	59 (59)
	Livestock workers	160 (80)	40 (20)
	Abattoir workers	66 (38)	108 (62)
	Butchers	126 (43)	168 (57)

at risk of infection of zoonotic diseases are reflected by acquiring a proper health certificate from the office of the district TB control officer (DTCO). The level of knowledge of the workers about signs-symptoms related to TB were very pitiable (**Table 3**). National Tuberculosis Control Program (NTCP), Pakistan have declared the amalgamation of three signs *i.e*; "chronic cough lasting for more than 3 weeks, weight loss and fever" as the main symptoms of TB. Hence, in this study, only 3 (2%) of the abattoir workers knew the combination of these three signs. Similarly, 83% (144) of the slaughter house workers were demanding training on zoonotic diseases.

All the sputum samples (35 no) collected from butchers were found negative for the presence of *M. bovis*. All the butchers answered that they washed fingers afore starting and afterward finishing the labor regularly. Only 29% (85) butchers replied that they acquired bath after finishing the work during winter while 86% (253) did so during summer. 33% (97) butchers identified that they prepared carcasses free of observable lesions whereas 67% (197) indicated that customer's desire is an important factor to prepare carcasses free of observable lesions. All the butchers quantified that they would remove only the bTB infected area along with some normal tissues and would not discard the whole carcass, when they were asked if carcass confirmed bTB infected. Furthermore, 89% (262) butchers indicated their direct communication with blood and dead bodies of animals. 19% (56) of the butchers used aprons while gloves, facemasks or hair nets were used by only 2% (6). No butcher had a health-certificate and no one got formal work related training. During the investigation, all the butchers specified that buffalo and cattle can be infected with TB. Similarly, 58% (171) identified that transmission of bTB from animal to human arise through ingesting of raw-meat, 43% (126) butchers stated zoonotic transmission of bTB occur via aerosol route while 39% (115) stated that the zoonosis of bTB occur through faeces and urine. So far the awareness about three standard signs of TB according to NRCP of Pakistan, no single butcher identified the amalgamation of three (3) major signs which are "chronic cough lasting for more than 3 weeks, weight loss and fever" as the main symptoms of TB.

Similarly, the sputum samples were collected from 10-veterinarian and 22-veterinary assistant employed in current research area. All the collected samples

were processed and analyzed through Ziehl-Neelsen staining, culture and final confirmation was made through PCR. All the samples were negative for the presence of *M. bovis*. The veterinarians and the veterinary assistants were also interrogated about the TB, the etiological agent, the signs & symptoms of TB and the zoonotic transmission of bTB. The knowledge of veterinarians was outstanding but veterinary assistants had poor understanding of the same.

Our study found that as compared to other professions studied in this study, the livestock farm workers and slaughter house manual labors were affected as a result of zoonotic tuberculosis more frequently. As bovine tuberculosis is prevalent in study area and the livestock farm workers during farm management were found unprotected from live farm animals. Similarly, abattoir labors were also found uncover equally from live animals (in the course of pre-slaughtering management) and carcasses (during and after slaughtering). Moreover, livestock farm workers and the abattoir workers, daily manage variety of large number of animals and personal protective equipment's (PPE's) were accessible to none of them. Several research studies were conducted in many endemic countries of bTB and have reported that abattoir workers were at increased risk of getting *M. bovis* infection (Robinson *et al*., 1988; Georghiou *et al*.,1989; Cousins *et al*., 1999; Barrera *et al*., 1987). As compared to livestock farm workers, the slaughter house workers were found at a much greater risk of contracting zoonotic TB due to the aerosol dispersion of bacilli as they were involved in carrying out necropsies and slaughtering the animals having direct contact with blood and carcasses of infected animals (Winthrop *et al*., 2005).

Majority of the slaughter house workers were unacquainted about bTB, its way of transmission, its zoonosis and public health significance. No single slaughter house worker had got any formal training with respect to bTB. Globally in unindustrialized republics, maximum cases of zoonotic TB have been reported in livestock farm labors, abattoir staffs, zoo workers, predators and veterinarians (Winthrop *et al*., 2005; Moda *et al*., 1996; Robinson *et al*., 1988; Cousins *et al*., 1999; Fanning *et al*., 1991). The level of knowledge of livestock farm workers, abattoir workers and butchers about signs and indications of TB and the ways of its spreading was negligible which on priority basis signifying the prerequisite for proper and specific job related education and training of high risk groups (Moda *et al*., 1996; Anaelom *et al*., 2010). Amongst high risk crowds, the awareness and approaches were evaluated and were found in line with the results of investigation carried out in some African states. Similarly, a study was conducted in the republic of Tanzania at country side regions which revealed 40% respondent's animal husbandry practices are to be high risk on behalf of contracting bTB and 75% of the respondents were reported with underprivileged awareness of TB (Mfinanga *et al*., 2003). A similar study was conducted in Tanzania on awareness and usual farm worker's practices and livestock healthcare workers. The study determined an increased risk to expose the respondents of contracting zoonotic diseases are due to certain factor like; lake of specific and job related trainings, lake of PPE's, un-hygiene food intake habits, poor livestock rearing practices and lake of awareness about zoonosis of certain chronic diseases (Swai *et al*., 2010). Similarly, Lack of awareness on spreading of zoonotic infections and occurrence of high risk behavior, for example consumption of raw animal products and unsafe slaughtering practices, have also been recognized in Ethiopia (Amenu *et al*., 2010). In Pakistan, the studies among abattoir workers, livestock farm workers and butchers the awareness and usual practices about the zoonotic bTB are very rare. In this study, the veterinarians and veterinary assistants had proper schooling amongst the occupationally unprotected groups related to their occupation. The standard of veterinarian's awareness about bTB is associated to their proper education in veterinary medicine while veterinary assistants had poor understanding and awareness about bTB demonstrating a reduced standard of education related to their job and demanding to improve their knowledge by giving them job related different types of trainings with more importance on zoonotic transmission of different infections, personal protective measures and management of sick animals. In our study, the results are grounded on sputum samples analysis, the extra-pulmonary TB and the latent TB could be the cases in the high risk occupationally exposed groups which were not detected. We have merely considered the occurrence of active pulmonary TB cases in these high risk professions. Although, all segments of the public are affected by bTB and human TB but poor community are most susceptible (Awah *et al*., 2010). In Peshawar, livestock farming is growing fast in and around the city. Majority of the livestock farmers have countable animals at their farm. However, maximum livestock farmers, livestock farm workers and profes-

sionals are poor and lacking basic knowledge related to their job. In this connection, it is serious to improve the capacity building by giving proper livestock farming training, supporting the education and perpetual re-evaluation of the livestock workers and allied professionals for the welfare and good health of both animal and human hence improving animal productivity in Peshawar.

Conclusions and Recommendations

Bovine tuberculosis (bTB) is a main community health concern in specific job-related clusters in district Peshawar, Khyber Pakhtunkhwa, Pakistan. In medical and veterinary laboratories, PCR could be applied to the clinical samples obtained from both human and animals for the detection of *M. bovis*. Though, a bit expensive, the PCR can be used as a routine diagnostic test to check the prevalence of bovine tuberculosis in cattle/buffaloe in Pakistan. On the basis of our findings, the surveillance, prevention and control programs of this NZD in the province Khyber Pakhtunkhwa, Pakistan is necessary because of the continual unrestricted movement of the animals which might result in an increased spread of bTB to humans.

1. The occupational groups neither used personal protective equipment's (PPE's) nor adopted suitable safe working procedures, putting them to hazard of getting zoonotic diseases like bTB in Peshawar city, Khyber Pakhtunkhwa, Pakistan.
2. The type/duration of work at L/S farm and age/education of the L/S farm employees were linked with the prevalence of zoonotic TB in this occupationally exposed group.
3. The livestock farm workers, abattoir workers, butchers and veterinary assistants who were interrogated in this study had less awareness and negligible knowledge about TB.

The findings of this research work stated that better risk/hazard evaluation is mandatory to pinpoint various prospects to end work-associated communicable infections. The results also specify the intense need of political/government commitment to mitigate the impact of bTB on the citizen's health by implementing control programs to address this neglected zoonotic disease (NZD) at the human-animal-ecosystem interface. The investigation emphasized the want for better awareness of zoonotic TB amongst specific occupational group's *i.e*; livestock farm workers, slaughter house labors and butchers along-with formal education and job specific trainings. The results of this research study recommend the necessity for further and thorough research of this specific work-related infectious and zoonotic disease in and around the capital city- Peshawar of Khyber Pakhtunkhwa, Pakistan.

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Novelty Statement

The research and experimental work on the subject title is original and new in the field of Veterinary Pathology in Khyber Pakhtunkhwa, Pakistan.

Author's Contribution

Asad Ullah: Conceptualized, Investigated, Wrote original draft.
Umar Sadique: Supervised, Resources.
Ibadullah Jan: Wrote Methodology.
Imad Khan: Software.
Raheela Taj: Visualized, Validation.
Mumtaz Ali Khan: Formal analyzed.
Salahud-din: Data Curation, Project administration, Visualization.
Naimat Ullah Khan: Wrote review, edited and Validation.

Conflict of interest
The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication with the work submitted.

References

Adesokan, H.K., A.O. and Jinkens, D. van Soolingen, S.I. Cadmus. 2012. *Mycobacterium bovis* infection in livestock workers in Ibadan, Nigeria: evidence of occupational exopure. Int. J. Tuberc.

Lung Dis., 16: 1388-1392.

Amenu, K., E. Thys, A, Regassa and T. Marcotty. 2010. Brucellosis and Tuberculosis in Arsi-Negele District, Ethiopia: Prevalence in Ruminants and People's Behaviour towards Zoonoses Tropicultura. 28: 205-210.

Anaelom, N.J., O.J. Ikechukwu, W.W. Sunday and U.C. Nnaemeka. 2010. Zoonotic tuberculosis: A review of epidemiology, clinical presentation, prevention and control. J. Pub. Health Epidemiol., 2: 118-124.

Awah N.J., A.C. Kudi, G. Bradley, I.N.A.Anyangwe, S.F.Tebug and J. Tchoumboue. 2010. Prevalence of bovine tuberculosis in abattoirs of the littoral and Western Highland Regions of Cameroon: A Cause for Public Health Concern Veterinary Medicine International. 2010:

Ayele, W.Y., S.D. Neill, J. Zinsstag, M.G. Weiss and I. Pavlik. 2004. Bovine tuberculosis: an old disease but a new threat to Africa. Int. J. Tuberc. Lung Dis., 8(8): 924-937.

Barrera, L. and I.N. De Kantor. 1987. Nontuberculous mycobacteria and *Mycobacterium bovis* as a cause of human disease in Argentina. Trop. Geogr. Med., 39:222-227. PMID: 3124310

Cosivi, O., J.M. Grange, C.J. Daborn, M.C. Raviglione, T. Fujikura, D. Cousins, R.A. Robinson, H.F. Huchzermeyer, I. de Kantor and F.X. Meslin. 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg. Infect. Dis., 4(1): 59-70.

Cousins, D.V. and D.J. Dawson. 1999. Tuberculosis due to *Mycobacterium bovis* in the Australian population: cases recorded during 1970-1994. Int. J. Tuberc. Lung. Dis., 3:715-721.

Evans, J.T., E.G. Smith, A. Banerjee, R.M. Smith, J. Dale, J.A. Innes, D. Hunt, A. Tweddell, A. Wood, C. Anderson, R.G. Hewinson, N.H. Smith, P.M. Hawkey and P. Sonnenberg. 2007. Cluster of human tuberculosis caused by Mycobacteruim bovis: evidence of person-to-person transmission in the UK. Lancet, 369: 1270-1276.

Fanning, A., S. Edwards and G. Hauer. 1991. *Mycobacterium bovis* infection in humans exposed to elk in Alberta. Can. Dis. Wkly Rep., 17:239-243. PMID: 1841004.

Georghiou, P., A.M. Patel and A. Konstantinos. 1989. *Mycobacterium bovis* as an occupational hazard in abattoir workers. Aust. N.Z. J. Med., 19:409-410. https://doi.org/10.1111/j.1445-5994.1989.tb00289.x

Hlavsa, M.C., P.K. Moonan and L.S. Cowan. 2008. Human tuberculosis due to *Mycobacterium bovis* in the United States, 1995-2005. Clin. Infect. Dis., 47:168-175. https://doi.org/10.1086/589240

Irfan, K., M.H. Mushtaq, M.U.D. Ahmad, M.S. Khan and J. Haider. 2016. Zoonotic tuberculosis in occupationally exposed groups in Pakistan. Occup. Med., 66:371-376. https://doi.org/10.1093/occmed/kqw039

Jalil, H., P. Das and A. Suleman. 2003. Bovine tuberculosis in dairy animals at Lahore, threat to the public health. Metropolitan Corporation Lahore, Pakistan.

Javed, M.T., M. Usman, M. Irfan and M. Cagiola. 2006. A study on tuberculosis in buffaloes: some epidemiological aspects, along with haematological and serum protein changes. Vet. Arh., 76:193-206.

Karesh, W.B., A. Dobson, J.O. Lloyd-Smith, J. Lubroth, M.A. Dixon, M. Bennett, S. Aldrich, T. Harrington, P. Formenty, E.H. Loh, C.C. Machalaba, M.J. Thomas and D.I. Heymann. 2012. Ecology of zoonoses: natural and unnatural hostories. Lancet, 380:1936-1945. https://doi.org/10.1016/S0140-6736(12)61678-X

Khan, I.A., A. Khan, A. Mubarak and S. Ali. 2008. Factors Affecting Prevalence Of Bovine Tuberculosis In Nili Ravi Buffaloes. Pak. Vet. J., 28:155-158.

Khan, J., S. Ayaz, N.M. AbdElsalam, R. Riaz Ullah and T. Shah. 2014. Prevalence of Tuberculosis in Buffalo and Cattle. J. Pure Appl. Microbiol., 8:721-726.

Khattak, I., M.H. Mushtaq, M.U.D. Ahmad, M.S. Khan, M. Chaudhry and U. Sadique. 2016. Risk factors associated with *Mycobacterium bovis* skin positivity in cattle and buffalo in Peshawar, Pakistan. Trop. Anim. Health Prod., 1-7.

Lolch, I., I. Hernandez, M. Barragen and C. Trance-Paredes. 2003. Infectious diseases, non-zero sum thinking and the developing world. Am. J. Med. Sci., 376:66-77. https://doi.org/10.1097/00000441-200308000-00003

Mfinanga, S.G., O. Morkve and R.R. Kazwala. 2003. Tribal differences in perception of tuberculosis: a possible role in tuberculosis control in Arusha, Tanzania. Int. J. Tuberc. Lung Dis., 7:933-941.

Michel, A.L., B. Muller and P.D. van Helden.

2010. *Mycobacterium bovis* at the animal-human interface: a problem, or not? Vet. Microbiol., 140:371-381. https://doi.org/10.1016/j.vetmic.2009.08.029

Moda, G., C.J. Daborn, J.M. Grange and O. Cosivi. 1996. The zoonotic importance of *Mycobacterium bovis*. Tuberk. Lung Dis., 77: 103-108.

Muller, B., S. Durr, S. Alonso, J. Hattendorf, C.J. Laisse, S.D. Parsons, P.D. van Helden and J. Zinsstag. 2013. Zoonotic *Mycobacterium bovis*-induced tuberculosis in humans. Emerg. Infect. Dis., 19(6): 899-908.

Narain, J.P. and Y.R. Lo. 2004. Epidemiology of HIV-TB in Asia. Indian J. Med. Res., 120:277-289.

Payeur, J.B., J.G. Jarnagin, L.A. Marquardt and B.M. Martin. 1993. Laboratory Methods in Veterinary Microbiology for the Isolation and Identification of Mycobacterium National Veterinary Service Laboratories Veterinary Services/Animal Plant Health Inspection Service/United State Department of Agriculture, Ames, IA.

Quammen, D. 2012. Spillover. Animal Infections and the Next Human Pandemic. W.W.Norton & Company, New York, USA. Yale J. Biol. Med., 86(1):107–112. PMCID: PMC3584488

Quinn P.J., M.E. Carter, B. Markey and G.P. Carter. 1994. Mycobacterium species: In: Clinical Veterinary Microbiology, Wolfe Publishing, London. 156–170.

Robinson, P., D. Morris and R. Antic. 1988. *Mycobacterium bovis* as an occupational hazard in abattoir workers. Aust. N.Z. J. Med., 18:701-703. https://doi.org/10.1111/j.1445-5994.1988.tb00156.x

Swai, E.S., L. Schoonman and C.J. Daborn. 2010. Knowledge and attitude towards zoonoses among animal health workers and livestock keepers in Arusha and Tanga, Tanzania. Tanzan J, Health Res., 12: 280-286.

Thoen, C.O., P.A. Lobue and I.N. de Kantor. 2010. Why has zoonotic tuberculosis not received much attention? Int. J. Tuberc. Lung Dis., 14: 1073–1074.

Tipu, M.Y., Z.A. Chaudhary, M. Younus and M. Rabbani. 2012. A cross sectional study of *Mycobacterium bovis* in dairy cattle in and arround Lahore city, Pakistan. Pak. J. Zool. 44:393-398.

Torgerson, P. and D. Torgerson. 2008. Does risk to humans justify high cost of fighting bovine TB? Nature, 455:1029-1029.

Tortoli, E. 2003. Impact of genotypic studies on mycobacterial taxanomy: the new mycobateria of the 1990. Clin. Microbiol. Rev. 16:319-354. https://doi.org/10.1128/CMR.16.2.319-354.2003

Tortoli, E., A, Mantella, A. Mariottini, G. Mazzarelli, P. Pecile, P.G. Rogasi, G. Sterrantino, E. Fantoni and F. Leoncini. 2006a. Succesfully treated spondylodiscitis due to a previously unreported mycobacterium. J. Med. Microbiol. 55:119-121.

Tortoli, E., R. Mattei, C. Russo and C. Scarparo. 2006b. Mycobacterium lentiflavum, an emerging pathogen? J. Infect., 52: e185-e187.

Ullah, A., U. Sadique, S. Ayaz, M.S. Qureshi and F.A. Khan. 2020. Bovine tuberculosis (bTB)-isolation and species-specific identification of Mycobacterium bovis from bovine raw milk in Pakistan. Sarhad J. Agric., 36(2): 489-498.

Winthrop, K.L., J. Scott and D. Brown. 2005. Investigation of human contacts: a *Mycobacterium bovis* outbreak among cattle at a California dairy. Int. J. Tuberc. Lung Dis., 9:809-813.

World Health Organization (WHO). Global Tuberculosis Report (GTBR). 2017. http://www.who.int/tb/publications/global_report/gtbr2017_executive_summary.pdf.

World Health Organization (WHO). http://www.emro.who.int/pak/programmes/stop-tuberculosis.html.

World Health Organization (WHO). 1994. Zoonotic tuberculosis (*Mycobacterium bovis*). 1994. Memorandum from a WHO meeting (with the participation of FAO) Bull World Health Organ Annon., 72:851-857.

Zumla, A., S.B. Squire, C. Chintu and J.G. Grange. 1999. The tuberculosis pandemic: implications for health in the tropics. Trans. Royal Soc. Trop. Med. Hyg., 93:113-117. https://doi.org/10.1016/S0035-9203(99)90099-8