Isolation and identification of *Mycobacterium bovis* and *Mycobacterium tuberculosis* from animal tissues by conventional and molecular method

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ABSTRACT

Tuberculosis is a worldwide contagious and chronic disease of human as well as domestic animals with zoonotic potential. The *Mycobacterium bovis* and *Mycobacterium tuberculosis* are the main cause of tuberculosis. It has worldwide distribution with significant effect on animals and has public health importance. Therefore the present study was conducted to determine the prevalence of *Mycobacterium bovis* and *Mycobacterium tuberculosis* among the ruminant of district Kohat, Khyber PakhtunKhwa and also to evaluate the sensitivity and specificity of Microscopy and PCR. A total 200 tissue samples of lungs, lymph nodes and liver from cattle, buffaloes, sheep and goats were collected from Abattoir Kohat. All the tissue were first examined by direct smear microscopy by Ziehl Neelsen staining and then subjected to the PCR for the detection of *M. bovis* and *M. tuberculosis*. The overall prevalence of tuberculosis was 6.5% by PCR.Prevalence of tuberculosis was recorded in7.87% of lungs samples followed by 5.26% lymph node. Moreover the prevalence was found 5.2%, 4%, 10.6% and 6.5% in cattle, buffalos, Goats and sheep respectively. Furthermorethe sensitivity and specificity of PCR and microscopy in term of detection of TB was determined that PCR was found less sensitive then microscopy because of other species which was not amplified due to non availability of specific primer and were found positive in microscopy.In conclusion PCR is more reliable diagnostic tool for diagnosis of bovine tuberculosis. It is recommended that PCR based diagnostic reference laboratory may be established at district level and Tuberculosis awareness campaign must be arranged.

Key words: *M. bovis*, Microscopy, *M. tuberculosis*, PCR, Tissue.

INTRODUCTION

Tuberculosis (TB) is a chronic, contagious and infectious disease of domestic animals as well as humans, and wild animal species, caused by a group of interrelated acid fast bacteria, forming the *Mycobacterium tuberculosis* complex (Ghumman *et al.,* 2013). It includes *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, and *Mycobacterium caprae*. (O. Thoen *et al.,* 2009). *M. tuberculosis*is the principal microorganism related with human tuberculosis whereas *M. bovis* is most important in causing bovine tuberculosis (BTB) (Tenguria *et al.,* 2011). Moreover BTB also has a zoonotic potential which affects different groups of animals, farm animals and other domesticated animals (Arshad *et al.,* 2012).

Early infections are frequently asymptomatic. In late stages, animals via pulmonary association frequently have a moist cough (Tschopp *et al.,* 2010). Moreover in some cases, animal lymph nodes and retropharyngeal expands and some time might rupture. By postmortem examination BTB illustrated the tubercles formation where localization of bacteria occurs. Granulomas are most frequently found in the mediastinal and mesenteric lymph nodes. They are also common in the lungs and liver (Shirtaye *et al.,* 2006).The Office International des Epizooties classifies BTB as a List B disease (Javed *et al.,* 2006).

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The diagnoses are usually carried out through isolation of sputum, milk, faeces as well as other body fluids for microbiological analysis for the presence of *mycobacterium* (Ashford *et al.,* 2001). Postmortem lesions from various organs include lungs liver and lymph nodes BTB can be diagnosed by histopathology, microscopic demonstration of acid fast bacilli (AFB). Direct smear microscopy through fluorescent acid fast stain technique, the Ziehl Neelsen (ZN) staining obtained from tissues or other clinical samples also used (Ashford *et al.,* 2001). *M. bovis* growth and isolation after culturing on selective media, confirmed the diagnosis of *M. bovis. Mycobacterium* are slow growing bacteria, and their colony growth appears in 3 to 6 weeks after incubation of mycobacterium culture for 8 weeks. Culturing has specificity that approach 100% and permits susceptibility testing of the isolates but the main disadvantages is more time consuming (*Ramadan et al., 2012*). The identification of the *mycobacterium* can easily be confirmed through polymerase chain reaction (PCR) assays.Amplification of the *Mycobacterium* DNA by PCR is a more reliable method for rapid diagnosis and has sensitivity equal or greater than that of the culture method (Ramadan *et al.,* 2012).

Livestock is the most important sector of agriculture in Pakistan(Iqbal *et al.,* 2012).Also it plays an important role in national economy as well as in Khyber PakhtunKhwa, mainly in rural areas where livestock is the earnings source for the people of these rural areas. Regarding prevalence of BTB, no previous work has been done so far in District Kohat, Khyber PakhtunKhwa, therefore the present study was planned to evaluate theprevalence of bovine tuberculosis in dairy animals in district Kohat and compare the sensitivity and specificity of microscopy and PCR for diagnosis of *Bovine tuberculosis*.

MATERIALS AND METHODS

Study area: The study was carried out in main abattoir of district Kohat, Khyber PakhtunKhwa Pakistan, that lies between north latitude 32° 47' and 33° 53' and east longitude 70° 34' and 72° 17' with area of 2,545 km^2.

Collection of samples: Different type of biopsy / tissue samples of approximately 50 gram each of lungs, lymph nodes and livers were randomly collectedfrom cattle, buffaloes, sheep and goats at the abattoir. These samples were placed in sterile plastic bags and labeled with the name of animal species, age, sex, and collection date. These samples were then transported to the veterinary research institute (VRI) Peshawar through cold chain for further processing.

Ziehl-Neelsen's (ZN) acid fast staining: All the tissue samples were first examined by direct smear microscopy by Ziehl Neelsen stainingfor recording the presence of acid fast bacilli(Quinn *et al.*1994).

Isolation of Mycobacteria from tissue samples: Isolation of *mycobacteria* from tissues samples was carried out by differential and density gradient centrifugation method asdescribed by (Victor *et al.,* 1992) with slight modification.

DNA extraction: The pallet obtained through differential and density gradient centrifugation form tissue samples were further subjected to PCR for identification of *M. tuberculosis* and *M. bovis* with specific primers amplification.DNA was extracted from Pellets (tissue) by using tissue DNA extraction Kit (Bio Basic Canada Inc. EZ)with standard procedure of the manufacturer.

PCR Amplification: Purified genomic DNA isolated from pallet was subjected to PCR by targeting the pncA for *M. tuberculosis* and species specific 500bp fragment for *M. bovis.* The primer used for both organisms are listed in Table 1. The reaction mixture for PCR contained 5 µl of 10 × PCR buffer, 1 µl of the primer mix, 2 ìl of dNTP mix (5 mM each), 3 ìl of 25 mM MgCl$_2$, 1 unit of Taq DNA polymerase (Fermatas)**,**5µl of sample DNA in a volume of 50 µl. The reaction was performed in a thermal cycler (MµltigeneLabnet international Inc. USA). The cycling conditions were an initial denaturation at 95°C for 10 minutes followed by a 30 cycles of denaturation at 94°C for 1 minutes, annealing at 55°C for 30 seconds, and primer extension at 72°C for 1 minutes, with a final extension at 72°C for 10 min.The PCR products were resolved and analyzed by using 1.2% of agarose gel electrophoresis and photographed on UV photo-documentation system (Multi-gene Labnet international Inc.USA).

Prevalence rate: The prevalence of TB was calculated by the following formula:

Prevalence (%) = Number of animals positive through PCR / Total number of animals tested.

TABLE 1: List of primer used for *M. tuberculosis* and *M. bovis*

Species	Primers	Primer sequence	Product size	Target region
M. tuberculosis	Forward pncATB-1.2 Reverse pncATB-2	(5'ATGCGGGCGTTGATCATCGTC3') (5'CGGTGTGCCGGAGAAGCGG3')	185bp	pncA gene
M. bovis	Forward JB21 Reverse JB22	(TCGTCCGCTGATGCAAGTGC) (CGTCCGCTGACCTCAAGAAG)	500bp	*M. bovis* specific fragment

Sensitivity and specificity of PCR and microscopy techniques: The sensitivity and specificity of PCR and microscopy was determined among the 200 tissue sample in detection of TB by following formula

Sensitivity: No of true positives / No of true positives + No of false negative X 100

Specificity: No of true negatives / No of true negatives + No of false positives X 100

Statistical analysis: The data was analyzed by using SPSS 16.0 software, chi-square test and ANOVA for determination of p value.

RESULTS AND DISCUSSION

In the present study, total 200 tissues samples were collected from cattle, buffaloes, sheep, and goats from abattoir of district Kohat as shown in (Table 2) were examined by microscopy and PCR. Overall prevalence was 6.5 % (13/200) was determined by PCR and 7.5% (15/200) was determined by microscopy. Significant difference (P<0.05) was determined when analyzed the data statistically (Fig 1).

Prevalence of tuberculosis in tissue samples: In the present study a total of 13 (6.5%) tissue were found positive by PCR, amongst these 7.87% (10/127) of lungs samples followed by 3 (5.26%) lymph node. No positive results were found in liver samples during the experiment (Table 3).

Prevalence of tuberculosis in animal species: Overall Prevalence of TB 13 (6.5%) in the samples was recorded, amongst 5.2% in cattle, 4% in buffaloes, 10.6% in goat and 6.5% in sheep respectively. Significant association was found among the animals species is p<0.05 Table 4

Sex wise prevalence: In the present study it was shown that 2/66 (3%) were found positive in males and 11/134 (8.2%) were positive in females in the tissue samples collected from ruminants respectively (Fig 2).

Sensitivity and specificity of different techniques: The sensitivity and specificity of PCR and microscopy in term of detection of TB was determined that PCR was found less sensitive then microscopy because of other species which was not amplified due to non availability of specific primer and were found positive in microscopy. So the results show the contrast with world communities finding. While was coincided with others (Table 5, Fig 3, 4).

Tuberculosis is a chronic infectious and important disease of zoonotic potential. Detection of tuberculosis in animal has become essential because of milk, meat and dairy products which are the main source of nutrients and proteins but it might be the important source for the transmission of TB from animals to humans (Al-Saqur *et al.,* 2009).

Total of 200 samples of tissue were analyzed revealed the overall prevalence of TB in ruminants of district

TABLE 2: Number of Tissue Sample Collection from Each Animal Tissue

Animal tissue	Type of samples	Cattle	Buffaloes	Goats	Sheep	Total
	Lungs	41	26	27	33	127
	Lymph nodes	11	15	17	13	56
	Liver	5	5	4	3	17
Total		57	46	48	49	200

TABLE 3: Prevalence of Tuberculosis in Different Type of Animal Tissue Samples in District Kohat.

Samples	Total no. of sample	PCR Positive samples		Total
		M. tuberculosis	*M. bovis*	
Lungs	127	03	07	10 (7.87%)
Lymph nodes	57	01	02	03 (5.26%)
Liver	16	0	0	0
Total	200	04	09	13 (6.5%)

TABLE 4: Prevalence of Tuberculosis in animal species

Animal species	Total samples	PCR positive		Total Positive	P-Value
		M. Bovis	*M. Tuberculosis*		
Cattle	57	03	0	03 (5.2%)	
Buffaloes	50	02	0	02(4%)	P<0.002
Goats	47	01	04	05 (10.6%)	(Chi-Square
Sheep	46	03	0	03 (6.5%)	= 22.64)
Total	200	09	04	13 (6.5%)	

TABLE 5: Sensitivity and Specificity of PCR and Microscopy N=200

Samples	PCR			Microscopy		
	Sensitivity	Specificity	True positive	Sensitivity	Specificity	True negative
Tissue	6.9%	93.5%	13	7.5%	92.5%	185

Kohat, Khyber PakhtunKhwa was 6.5% (13/200).The prevalence was found higher than the prevalence reported by (Ifrahim 2001and Javed *et al* 2006)which was 1.7% and 5.48% respectively.Similar study was also conducted by (Khan and Khan 2007 and Khan *et al* 2008) reported relatively high prevalence 12.72% and 10.6% respectively compared to the present study. This shows that variation in prevalence rates occurfrom region to region and even from farm to farm in the same region.

Slaughtered animals with visible TB lesions could be identified by examination of the lungs and associated lymph nodes (Aliyu *et al* 2009). This can further be confirmed by diagnostic techniques in laboratory. In the present study 7.87% of lungs samples followed by 5.26% lymph node were found positive by PCR. The overall prevalence of TB from slaughterhouse was recorded 6.5% (13/200). (Ewnetu *et al* 2012) reported prevalence and distribution bovine TB lesions in cattle at Akaki abattoir which is in agreement with present study.In one of the study that the 2.7% suggestive lesion was of bovine TB in nature that was based on the detailed inspection procedure (Bekele and Belay 2011). Their finding was low, but was not as such lower than reported by (Shitaye *et al* 2006). Similarly study reported 3.5% and 3% in Addis Ababa municipal abattoir respectively (Teklue *et al* 2004*)*. It was revealed from their study that, the spread of bovine tuberculosis among cattle was linked to the type of production system (Shitaye *et al*., 2006) for this reason; the low prevalence of their study might be due to the fact that all animals were kept under extensive production system.

(Ewnetu *et al* 2012) reported in their study that gross lesions of TB in lungs (69.79%), lymph nodes of the head (18.75%) and mesenteric lymph node (9.38%) and hepatic lymph node were found (2.08%). The results of the present study are in agreement with the finding of (Ewnetu *et al* 2012) that lungs shows higher prevalence of TB compared to other tissues. (Corner 1994) has also reported that up to (95%) of cattle with visible TB lesions could be identified by examination of the lung and associated lymph nodes. Hence these finding indicated that inhalation might be the principal route of TB infection in animals. Therefore focus should be given on lungs and associated lymph nodes during postmortem examination.

In present study prevalence of TB in cattle, buffaloes, goat and sheep was recorded (5.2%), (4%), (10.6%) and (6.5%)respectively.(Ghumman *et al* 2013*)*in their study reported the prevalence of tuberculosis in cattle and buffaloes were reported (11.71%) and (11.96%) respectively. Results of the present study showed similarity with the results
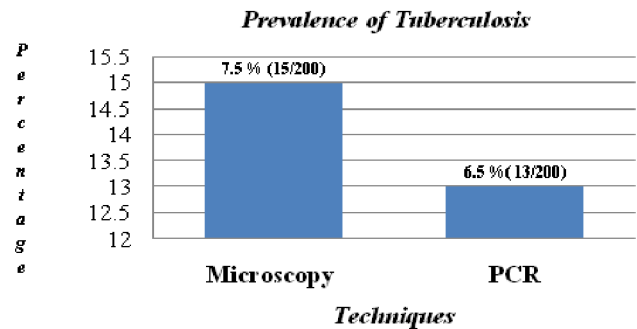
FIG 1: Prevalence of tuberculosis on the basis of microscopy and PCR
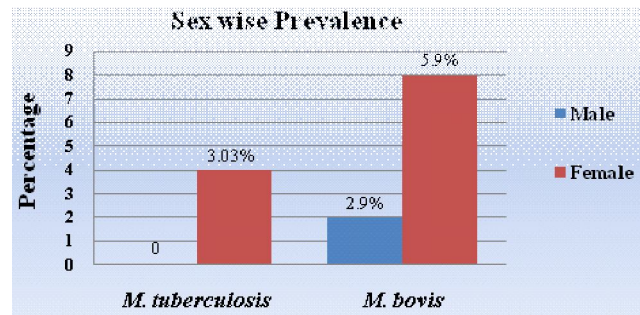
FIG 2: Sex wise prevalence of tuberculosis.
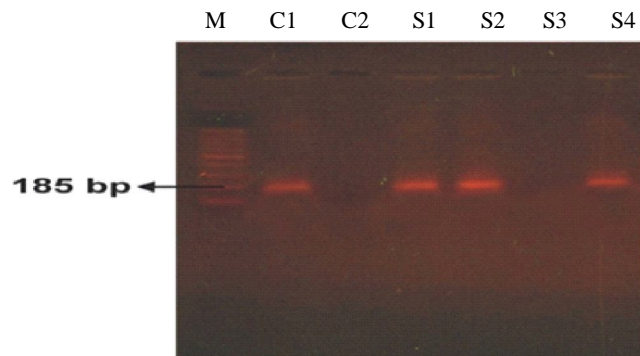
M: Marker (100bp), C1: Control Positive of M. tuberculosis, C2: Negative control of M. tuberculosis, S1-S4: Clinical samples.
FIG 3: PCR showing 185bp DNA of *M. tuberculosis*.

of(Ghumman *et al* 2013), showed the prevalence of TB was insignificantly different in both cattle and buffaloes.(Hena *et al* 2012) in their study reported the prevalence of tuberculosis in sheep and goats were (0.99%) and (1.34%) respectively. Their findings are much lower than the findings of the present study i.e. (10.6%) in goats and (6.5%) in sheep. These differences could be explained by many factors including differences in the disease status in the animal populations and various environmental influences. In many other studies it has been reported that bovine TB in small ruminant would become a problem only when they are in close contact with cattle with high disease prevalence (Tschopp *et al.,* 2011). It has been observed in the present study that majority of the farmers kept their sheep and goat inside their house at night this could be the reasons for high prevalence in small ruminants.

In the present study females showed high prevalence compared to male.(Kassa *et al.* 2012) in their study also reported high rate of tuberculosis prevalence in females compared to males.

Present study reveals the prevalence of TB by microscopy was7.5% (15/200) followed by the PCR 6.5% (13/200). Although PCR was a more sensitive technique than ZN staining, the results obtained in the present study showed a higher number of positive samples with ZN staining as compared to PCR. It might be due to the presence of other bacteria of tuberculosis because in our study only *M. bovis* and *M. tuberculosis* was differentiated by PCR. Similar results were also obtained by (Khan et al 2010) that ZN staining showed the AFB presence in 17.4% intestinal and 16.4% in lymph node tissue of buffalo, while PCR confirmed 12.8% in intestinal and 12.4% in lymph node sample positive for Paratuberculosis, which show higher number of positive results by microscopy than PCR. Moreover staining microscopy could not permit differentiation between species of MTB complex, only PCR methods can differentiate between them.

The use of direct smear microscopy as the only method for the diagnosis of suspected TB, although an essential requirement of any national TB program, could partly explain the relative low notification rate of disease caused by *mycobacterium* (*Grange et al.,* 1996). Moreover reduced number of positives results may also be obtained by using ZN staining, because it might b due to fact that *mycobacterium* often found low number in bovine samples and they could not be visualized by acid fast staining, only if a limited quantity (at least 5x10 Mycobacteria /ml) present (Bekele and Belay 2011).

Furthermore, the result of acid fast staining may also be affected by the sample taking technique during smear preparation as Mycobacteria are not evenly distributed in the tissue sample (Bekele and Belay, 2011). Although PCR have been widely evaluated in diagnosis of TB and useful for confirmed the result of the conventional methods such as staining and culturing. Moreover PCR give high sensitivity and specificity for the *Mycobacterium* and the method is more accurate and faster than conventional method for TB diagnosis (Al-Saqur *et al.,* 2009).The sensitivity and specificity of PCR was compared to ZN staining and culturing by (Ndugga *et al* 2004)and PCR was considered as an alternative to ZN staining for diagnosis of TB. In another study, comparative study of AFB staining, culturing, histopathology and PCR for *M. tuberculosis* revealed that PCR has high sensitivity and specificity and has a potentially important role in improving the diagnostic accuracy of extra pulmonary tuberculosis (Al-Saqur *et al.,* 2009).

It is concluded that tuberculosis in small ruminants were more prevalent as compared to large ruminants. PCR method was found more reliable diagnostic tool for diagnosis of bovine tuberculosis but having contrast result with rest of the world author conclusion. It is recommended thatPCR based diagnostic reference Laboratory may be established at district level.Tuberculosis awareness campaign and workshops may be arranged at each calendar year.Further work may be carried out on drug resistance and vaccine production etc.

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REFERENCES

Aliyu, M.,Adamu,M.J.Y., and Bilyaminu Y.A. (2009). Current Prevalence of Tuberculosis Lesions among Slaughtered Cattle in Northeastern States of Nigeria. *Revue d'elevage et de medecineveterinaire des pays tropicaux*, **62:** 13-16.

Al-SaqurM, Al-Thwani, A.N.and Al-Attar, I.M. (2009). Detection of Mycobacteria spp in cow's milk using conventional methods and PCR. *Iraqi Journal of Veterinary Sciences*, **23**: 259-262.

Ashford,D.A., Whitney,E., Raghunathan,P and Cosivi,O. (2001). Epidemiology of selected mycobacteria that infect humans and other animals. *Rev. sci. tech. Off. int. Epiz.*, **20**: 325-337.

Bekele, M and Belay, I. (2011). Evaluation of routine meat inspection procedure to detect bovine tuberculosis suggestive lesions in Jimma Municipal Abattoir, South West Ethiopia. *Global Veterinaria*, **6:** 172-179.

Collins, C.H and Grange, J.M. (1994). The bovine tubercule bacillus" *Journal of Applied Bacteriology*, **55**: 13-29.

Corner L.A. (1994) "Post mortem diagnosis of Mycobacterium bovis in cattle. *Veterinary Microbiology*, **40**: 53-63.

Ewnetu, L., Melaku, A. and Birhanu, A. (2012). Bovine Tuberculosis Prevalence in Slaughtered Cattle at Akaki Municipal Abattoir, Based on Meat Inspection Methods. *Global Veterinaria* **9**: 541-545.

Ghumman, M.A., ManzoorA.W., NazS., Ahmad R. Ahmad R. (2013). Prevalence of Tuberculosis in Cattle and Buffalo at Various Livestock Farms in Punjab. *International Journal of Veterinary Medicine*, Article ID 145084, DOI: 10.5171/2013.145084.

Hena,S.A., Tanimomo,B.K., Tarhyel,R., Ngbede,E.O., Oluwasina,O.O., Jagtap,G.P., Dhavale,M.C., and Dey, U. (2012). A tentative case of tuberculosis detected at postmortem examination of animal carcasses slaughtered at some local slaughter slabs in north-western Nigeria. *Scientific Journal of Agricultural*, **1:** 27-32.

Iqbal, M.J., Ali, Z.U. and Ali, S.S. (2012). Agroclimatic modeling for estimation of wheat production in the Punjab province, Pakistan. *Proceeding of Pakistan academy of Science*, **49**: 241- 249.

Ifrahim, M. (2001). Epidemiological studies on tuberculosis in cattle and buffalo population in villages around Faisalabad. (M.Sc. thesis), Department of Veterinary Microbiology, University of Agriculture, Faisalabad.

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

KassaG.M., AbebeF., WorkuY., LegesseM., MedhinG., BjuneG., and Ameni G. (2012). Tuberculosis in Goats and Sheep in Afar Pastoral Region of Ethiopia and Isolation of Mycobacterium tuberculosis from Goat. *Veterinary Medicine International*, 1-8.

Khan.F.A., Chaudhry, Z.I., Ali, M.I., Khan, S., Mumtaz, N and Ahmad, I. (2010). Detection of Mycobacterium avium subsp. Paratuberculosis in tissue samples of cattle and buffaloes. *Tropical Animal Health and Production*. **42**: 633-638.

Khan,I.A., and Khan A. (2007). Prevalence and risk factors of bovine tuberculosis in Nili-Ravi buffaloes in the Punjab, Pakistan. *Italian Journal of Animal Science*, **6**: 817-820.

Khan,I. A., KhanA., Mubarak A.and Ali S. (2008). Factors Affecting Prevalence Of Bovine Tuberculosis In Nili Ravi Buffaloes. Pakistan Veterinary Journal, **28**: 155-158.

Muhammad A., Ifrahim,M., AshrafM., Rehman S.U., and Khan H.A. (2012). Epidemiological studies on tuberculosis in buffalo population in villages around Faisalabad. *The Journal of Animal and Plant Sciences*, **22**: 246 – 249.

Ndugga, K.L., Cleeff, M.V., Juma, E., Kimwomi, J., Githuui,W., oskam, L., Schuittema, A., Soolingen, D.V., Nganga, L., Kibuga, D., Odhiambo, J.and Klatser, P. (2004), Comparison of PCR with routine procedure for diagnosis of tuberculosis in population with high prevalence of tuberculosis and human immunodeficiency virus. *Journal of Clinical Microbiology*, **42**, 1012-1015.

Quinn,P.J., Carter,M.E., Markey, B.K. and Carter, S.G.R. (1994). Clinical veterinary microbiology. (Wolfe, London), 144-155.

Ramadan, H. H., El-Gohary, A.H.N., Mohamed, A.A. and Nasr, E.A. (2012). Detection of Mycobacterium Bovis and Mycobacterium Tuberculosis from Clinical Samples by Conventional and Molecular Techniques in Egypt. *Global Veterinaria*, **9** : 648-654.

Shirtaye, J. E., Getahun, B., Alemayehu, T., (2006). A prevalence study of bovine tuberculosis by using abattoir meat inspection and tuberculin skin testing data, histopathological and IS1610 PCR examination of tissues with tuberculous lesions in cattle in Ethiopia. *Veterinary Medicine*, **51**: 512-522.

Shitaye, J.E., Getahun, B., Alemayehu, T., SkoricM., Treml, F., Fictum, P., Vrbas,V., Pavlik, I. (2006). A prevalence study of bovine tuberculosis by using abattoir meat inspection and tuberculin skin testing data, histopathological and IS 6110 PCR examination of tissues with tuberculosis lesions in cattle in Ethiopia. VeterinarniMedicina, **51**: 512–522.

Teklue, A., Asseged, B., Yimer, E., Gebeyehu, M.,Woldesenbet, Z. (2004). Tuberculosis lesions not detected by routine abattoir inspection: the experience of the Hossana municipal abattoir, southern Ethiopia. *Rev. Sci. Tech. Off. Int. Epiz.*, **23**: 957-964.

Tenguria, R.K., Khan, F.N., Pandey, S.Q.A. (2011). Epidemiological study of zoonotic Tuberculosis complex (ZTBC). *World Journal of Science and Technology*, **1**: 31-56.

Thoen, C.O., Lobue, P.A,.Enarson, D.A., Kaneene, J.B., and de Kantor, I.N. (2009). A re-emerging disease in animals and humans. *Veterinariaitaliana*, **45**: 135-181.

Tschopp, R., Aseffa, A., Schelling, E., Berg, S., Hailu, E., Gadisa, E., Habtamu, M., Argaw, K. and Zinsstag, J. (2010). Bovine Tuberculosis at the wildlife livestock-Human interface in Hamer Woreda, South Omo, Southern Ethiopia. *PLoS ONE*, **5**: 1-7.

Tschopp, R., Bobosha, K., Aseffa, A., Schelling, E., Habtamu, M., Iwnetu, R., Hailu, E., Firdessa, R., Hussein, J., Young, D., and Zinsstag, J. (2011). Bovine tuberculosis at a cattle-small ruminant-human interface in Meskan, Gurage region,Central Ethiopia. *BMC Infectious Diseases*. **11:**318.

Victor, T., Dutoit, R., and Vanhelden, P.D. (1992). Purification of Sputum Samples through Sucrose Improves Detection of Mycobacterium tuberculosis by Polymerase Chain Reaction. *Journal Of Clinical Microbiology*. 1514-1517.