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

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Received: 01-01-2015 Accepted: 26-06-2015 DOI: 10.18805/ijar.5583

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 in 7.87% 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. Furthermore 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. 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 canetti, 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).
The diagnoses are usually carried out through isolation of sputum, milk, feces 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 the prevalence 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 km².

**Collection of samples:** Different type of biopsy / tissue samples of approximately 50 gram each of lungs, lymph nodes and livers were randomly collected from 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 staining for 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 as described 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 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 µl of dNTP mix (5 mM each), 3 µl of 25 mM MgCl₂, 1 unit of Taq DNA polymerase (Fermatas), 5 µl of sample DNA in a volume of 50 µl. The reaction was performed in a thermal cycler (Multi-gene Labnet 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 minute, annealing at 55°C for 30 seconds, and primer extension at 72°C for 1 minute, 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 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. tuberculosi and M. bovis

<table>
<thead>
<tr>
<th>Species</th>
<th>Primers</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Target region</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>Forward pncATB-1.2</td>
<td>(5’ ATGCCGGCGCTTGATCATCCTGC3’)</td>
<td>185 bp</td>
<td>pncA gene</td>
</tr>
<tr>
<td></td>
<td>Reverse pncATB-2</td>
<td>(5’ CGGTGTTGGCAGGAAGCGG3’)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. bovis</td>
<td>Forward JB21</td>
<td>(TCGTCGCTGATCGAAATGTC)</td>
<td>500 bp</td>
<td>M. bovis specific fragment</td>
</tr>
<tr>
<td></td>
<td>Reverse JB22</td>
<td>(CGTCCGCTACCTCAAGAG)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**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) × 100
- **Specificity:** No of true negatives / (No of true negatives + No of false positives) × 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

<table>
<thead>
<tr>
<th>Animal tissue</th>
<th>Type of samples</th>
<th>Cattle</th>
<th>Buffaloes</th>
<th>Goats</th>
<th>Sheep</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lungs</td>
<td>41</td>
<td>26</td>
<td>27</td>
<td>33</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>11</td>
<td>15</td>
<td>17</td>
<td>13</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>46</td>
<td>48</td>
<td>49</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total no. of sample</th>
<th>PCR Positive samples</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M. tuberculosis</td>
<td>M. bovis</td>
</tr>
<tr>
<td>Lungs</td>
<td>127</td>
<td>03</td>
<td>07</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>57</td>
<td>01</td>
<td>02</td>
</tr>
<tr>
<td>Liver</td>
<td>16</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>04</td>
<td>09</td>
</tr>
</tbody>
</table>

### TABLE 4: Prevalence of Tuberculosis in animal species

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Total samples</th>
<th>PCR positive</th>
<th>Total Positive</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. Bovis</td>
<td>M. Tuberculosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>57</td>
<td>03</td>
<td>03 (5.2%)</td>
<td>P&lt;0.002</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>50</td>
<td>02</td>
<td>02(4%)</td>
<td></td>
</tr>
<tr>
<td>Goats</td>
<td>47</td>
<td>04</td>
<td>05 (10.6%)</td>
<td>(Chi-Square = 22.64)</td>
</tr>
<tr>
<td>Sheep</td>
<td>46</td>
<td>03</td>
<td>03 (6.5%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>09</td>
<td>04</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 5: Sensitivity and Specificity of PCR and Microscopy N=200

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>True positive</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>True negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>6.9%</td>
<td>93.5%</td>
<td>13</td>
<td>7.5%</td>
<td>92.5%</td>
<td>185</td>
</tr>
</tbody>
</table>

Kohat, Khyber Pakhtunkhwa was 6.5% (13/200). The prevalence was found higher than the prevalence reported by (Ifrahim 2001 and 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 occur from 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.
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 was 7.5% (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 be 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-Saqr 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-Saqr 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 that PCR 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.

ACKNOWLEDGEMENT

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding the work through Research group no. (RG 1435-014).

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