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CASE REPORT

Use of Molecular Probes for Presumptive Diagnosis of Tuberculosis Associated with Mycobacterium Tuberculosis and Mycobacterium Bovis Infection in Antelopes in Pakistan

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ABSTRACT

The etio-prevalence of tuberculosis complex and diagnostic significance of cytokines in antelopes of Lahore, Pakistan was determined by multiplex polymerase chain reaction and cytokine ELISA. One hundred blood samples of five different types of captive zoo antelopes including Mouflon sheep, black buck, gorial, hog deer and urial were tested. The percent prevalence of *Mycobacterium bovis* and *M. tuberculosis* was 30% and 20% respectively. All five categories of antelopes were infected with *M. bovis* and *M. tuberculosis* and none of the animals was positive for *M. avium.* Mouflon sheep, black buck and hog deer were significantly more affected by *M. bovis* as compared to *M.tuberculosis.* While Gorial had non-significantly more *M.tuberculosis* as compared to *M. bovis.* Urial had lowest tuberculosis incidence and was equally infected by both *Mycobacterium* species. The levels of IFN-γ and TNF-α were significantly higher in TB infected animals as compared to negative controls ($P<0.05$). From PCR positive animals two black bucks died later and the histopathological analysis of their lungs revealed pathognomic granuloma lesions.

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INTRODUCTION

Bovine tuberculosis is an infectious chronic disease of domestic and wild animals with serious zoonotic implications in humans. Previous studies have shown that there are at least nine members of the MTBC infecting animals other than humans; these have also been referred to as ecotypes (Brites *et al*., 2018). In antelopes tuberculosis (TB) is caused by tuberculosis complex including *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium caprae* (Angela *et al*., 2017). *Mycobacterium bovis* and *Mycobacterium caprae* on the other hand are mainly found in domesticated cattle and goats but are also frequently isolated from several wild animal species which can act as reservoirs. Socio-economic and public health concern of tuberculosis enlists it in world top priority disease to be eliminated by World Health Organization. Pakistan ranks 6th among TB burden countries (Munir *et al.,* 2018). In livestock, bovine tuberculosis (bTB) has been confirmed in the majority of countries from all parts of the continent but wildlife infection is confirmed in only seven countries from southern and eastern Africa (Garine-Wichatitsky *et al.,* 2013). In most Asian countries, little data is available about tuberculosis in wildlife, especially antelope. Although it can be a potential source of infection for bovines and humans. There has been a single study on wildlife tuberculosis in Islamabad, Pakistan with overall prevalence of 3.3% in zoo animals and 3.2% in cervidaes (Shahid *et al.*, 2012) but no information is available about antelopes TB in other big cities such as Lahore that has an antelopes population of around 300. Lahore zoo, Safari zoo and Jallo zoo are the main hubs of wildlife in Lahore. Keeping in view the wildlife population of Lahore it is imperative to study the disease prevalence here. Another novel aspect of this study was to compare that which *Mycobacterium* specie is mainly responsible for disease occurrence in different antelopes. An additional aim of this study was to compare two cytokines of TB in infected and non infected antelopes for future use of cytokines as diagnostic markers.

MATERIALS AND METHODS

An improval from ethical committee of University of Veterinary and Animal Sciences Lahore, Pakistan was obtained before sampling from suspected animals. A total of 100 blood samples were collected from tuberculosis suspected antelopes from wildlife parks and zoo in and around Lahore, Pakistan. The inclusion criteria for these animals were emaciation, chronic weight loss, nasal discharge, dyspnea, coughing, rough body coat and enlarged lymph nodes. These animals were equally divided into five groups including Mouflon sheep, black buck, gorial, hog deer and urial. Multiplex PCR was conducted for confirmation of *M. bovis, M. tuberculosis* and *M. avium*. The cytokine ELISA was performed to evaluate the difference of IFN-γ and TNF-α in infected and non infected animals.

DNA was extracted from blood samples using DNA extraction kit (Exgene TM blood SV Cat #105-152). DNA quantification was performed by using Nanodrop spectrophotometer (Thermo Scientific Spectrophotometer ND-2000). In order to conduct multiplex PCR three sets of primers were designed for *M. avium, M. tuberculosis* and *M. bovis* using Primer 3. We designed the primers for *M. avium* k10 gene (Accession: NC-002944.2), *M. tuberculosis* rpoB gene (Accession: AL123456.3) and *M. bovis* mpb70 gene (Accession: NC-002945.3). The sequences of the primers are given in Table 1.

Table 1: Primers designed for Mycobacterium complex

Sr. No.	Primer Name	5'-3' Sequence	Tm (°C)	Product Size(bp)
1.	JSM170-F	GCTGATCCAAAACCAGATCC	51.8	400
	JSM170-R	GTTCATCGAAACGCCGTAC	53.8	
2.	Avium-F	GGCGTGTCTTCTTGACACC	53.2	470
	Avium-R	CTGGACGTCTGCCACAAG	52.6	
3.	Bovis-F	ACGATCGACGAGCTCAAGAC	53.8	240
	Bovis-R	GATTGACAGCGTGCTAATGC	51.8	

PCR was performed in total volume of 25 µL containing 1µL DNA (50 ng/µL), 2.5 µL PCR buffer (2 mM), 2.5 µL dNTPs (25 mM), 2 µL $MgCl_2$ (20 mM), 0.5µL of each primer (10 pmol/mL), 0.3 µL taq polymerase (5 U/µL) and 15.7µL distilled water.

The PCR conditions were initial denaturing at 95°C for 5 minutes followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing of primers at 53°C for 30 seconds and extension for one minute at 72°C followed by final extension for 10 minutes at 72°C. PCR product was visualized on 1.5% agarose gel. *Mycobacterium* culture obtained from Veterinary Research Institute (VRI) Lahore was used as positive control while distilled water was used as negative control.

Sandwich ELISA was designed to determine the levels of TNF-α, IFN-γ were assessed with the commercially available ELISA kits including Bovine TNF-α (R&D System Europe Ltd., UK) and Bovine IFN-γ (Mabtech AB, Sweden). The results were calculated from sample OD values in relevance to standard curve using SigmaPlot®11 Systat Software, Inc (San Jose, CA, USA). The final concentration was measured in pg/mL. All the samples were run in duplicate (Liu *et al.,* 2018). Statistical analysis was conducted with Chi square-test.

RESULTS AND DISCUSSION

The present study was conducted to determine the molecular prevalence of tuberculosis complex in antelopes of Lahore, Pakistan as well as to determine the role of cytokines (IFN-γ TNF-α) in TB diagnosis. The results indicated that all five types of antelopes screened in present study were positive for tuberculosis by PCR (Fig. 1). This shows that all kinds of antelopes suffer from TB. Particularly, it is important to note and report the detection of *M. bovis* and *M. tuberculosis* in antelope as these were all captive zoo animals. However most of the positive samples were obtained from Mouflon sheep and black buck. This may be due to more susceptibility of these antelopes towards TB as compared to other antelopes.

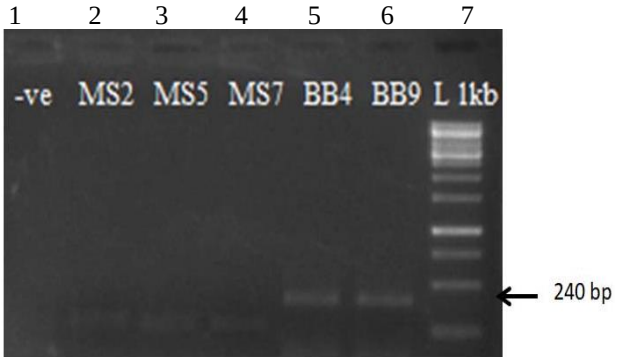

Fig. 1: Lane 1: Negative control, Lane 2-4: Negative samples, Lane 5 &6: Positive samples with amplification of 240bp product of *Mycobacterium bovis* in infected antelopes, Lane 7: DNA ladder.

Overall there was an increased incidence of *M. bovis* (30%) as compared to *M. tuberculosis* (20%). Mouflon sheep, black buck and hog deer showed significantly more incidence of *M. bovis* as compare to *M. tuberculosis* ($P<0.05$). Gorial had non-significantly more *M. tuberculosis* than *M. bovis* while urial had least TB incidence with equal infection from *M. bovis* and *M. tuberculosis* (Fig. 2a). This high incidence of TB in antelopes may be possible reason for non eradication of this disease from cattle. This added in previously available information that wild antelopes may be the maintenance hosts or reservoirs for both of *Mycobacterium* species (Meunier *et al.,* 2017) however the present study for the first time reported the increased affinity of antelopes for *M. bovis*. This may be due to host specificity of *Mycobacterium* as antelopes belong to bovidae family and *M. tuberculosis* is host specific for humans (Cambier *et al.,* 2014). Moreover, when *M. tuberculosis* and *M. bovis* have been contrasted directly in a neutral host, such as mice, guinea pigs and rabbits, it has been *M. bovis* that is the more virulent of the two (Medina *et al.,* 2006). However, the sequence analysis of these will be helpful for further investigational studies as previous studies report the intermediate forms of the *M. tuberculosis* complex (*Mycobacterium orygis*) that separate *M. tuberculosis* from *M. bovis* are successfully spreading from antelope to antelope (Behr and Jordon, 2015).

As there is not a single report about the presence of Mycobacterium caprae in Pakistan that is mostly prevalent in wildlife of European countries such as Spain, Poland, Italy, Austria and Germany (Krajewska-Wędzina *et al.,* 2018).

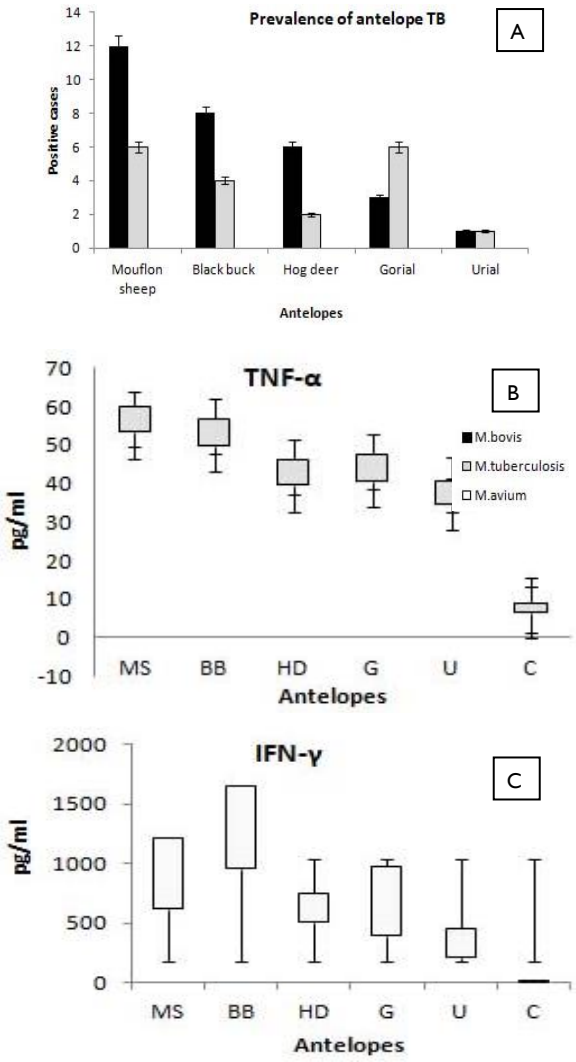

Fig. 2: Comparative prevalence of tuberculosis complex in various antelopes (a) and their cytokine profile (b & c).

The results of present study indicated a significantly increased level of both IFN-γ and TNF-α in TB infected antelopes as compared to non infected antelopes (Fig. 2b & 2c). This highlights the significance of these two cytokines that can be potentially used as diagnostic or prognostic markers for TB detection in antelopes. All the infected animals had greater values of IFN-γ as compare to TNF-α. This may be explained by the major role of IFN-γ in pathogenesis of bovine tuberculosis in contrast to humans that normally have increased TNF-α (Joshi *et al.*, 2015). Another reason for increased IFN-γ as compare to TNF-α may be initial priming of macrophages by IFN-γ to secrete more TNF-α post-infection.

Two of the black bucks with severe TB infection died and were brought to department of Pathology, University of Veterinary and Animal Sciences, Lahore for necropsy examination. The gross lesions indicated presence of caseous nodules on lungs (Fig. 3a) and trachea fill with frothy discharge mixed with blood. Histopathological analysis showed encapsulated granuloma formation and accumulation of inflammatory cells in lungs (Fig. 3b & 3c). As there is no previous data available about the histopathological lesions of TB in antelopes to the best of our knowledge this is the first study explaining the histopathological lesions in antelopes infected with TB.

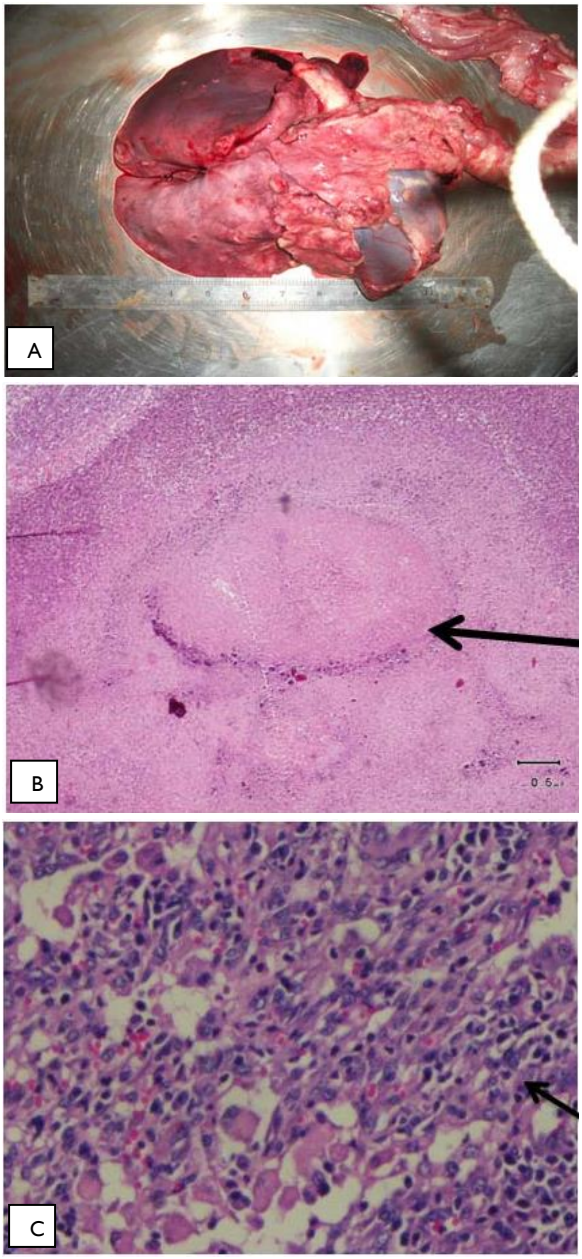

Fig. 3: Caseous nodule formation (a) encapsulated granuloma formation (b) with accumulation of macrophages and acid fast bacteria (c) in lungs of black buck.

Conclusions: The information obtained from the present study describes that TB exits in antelopes of Lahore, Pakistan. The affecting species include *Mycobacterium bovis* and *Mycobacterium tuberculosis*. These results can be implemented to start TB eradication program in wildlife and ultimately in domestic bovines. Moreover, the cytokines levels of TNF-α and IFN-γ were high in infected as compare to non-infected antelopes. Therefore, these cytokines levels could be use as diagnostic biomarkers for TB diagnosis in antelopes.

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Authors contribution: RA and MYT designed the study. MS and MRK collected the samples. RA, MS and GM run

the samples in lab. MI and BZ statistically analyzed the data. RA drafted the manuscript. All authors gave approval for final version.

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