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Molecular diagnosis of *Mycobacterium bovis* as the cause of tuberculosis in a camel

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Tuberculosis is a chronic contagious disease caused by mycobacterial species belonging to the *Mycobacterium tuberculosis* complex (Lachnik 2002). Tuberculosis in humans remains one of the major global reportable diseases, and a rise in its incidence has caused the World Health Organization (WHO) to declare the disease a global emergency (Nakajima 1993). Tuberculosis in camel has not been very extensively researched in comparison with those of other domesticated species. Camel tuberculosis is generally sporadic and has been diagnosed around the turn of the 19th century in Egypt (Littlewood 1988) and in India (Lingard 1905, Kinne *et al.* 2006). There are scanty reports on tuberculosis in camel. The present paper reports confirmed diagnosis of tuberculosis in camels based on conventional and molecular techniques.

Mycobacterial strains: The reference *Mycobacterial* strains used were *M. tuberculosis H37Rv* and *M. bovis AN5* maintained at Mycobacteria Laboratory, Indian Veterinary Research Institute, Izatnagar.

Sample: The clinical tissue samples the lung, spleen and liver from male Kachchhi camel of 10–11 years old from National Research Centre Camel, Bikaner (Rajasthan) were collected aseptically for bacterial isolation. Samples in 10% formalin were also collected for histopathology examination and in and buffered saline for isolation of mycobacteria.

Culture: The small pieces of cut specimens were decontaminated using 10 ml of 5% H_2SO_4. The small pieces of tissues were ground using a mortar and pestle, and the homogenate was transferred to a sterile universal container. A smear was made using a loop full of the homogenate, fixed and stained by Ziehl-Neelsen staining. The homogenate was centrifuged and the supernatant was discarded. The sediment was inoculated onto 2 Lowenstein-Jensen (LJ) slants and incubated at 37°C for 8 weeks to observe for the growth of *Mycobacterium* colonies.

Niacin production, nitrate reduction tests and sensitivity to Thiophen-2-corbonic acid hydrazide (T_2CH) were used for the conventional identification of the culture.

Molecular confirmation: DNA for PCR was isolated by suspending 2 loops full growth of mycobacteria from the LJ slopes in 200µl TE buffer [10 mM Tris, 1 mM EDTA pH 7.4] and incubated at 95°C for 10 min. The suspension was centrifuged at 12,000 rpm for 5 min and the supernatant with DNA was stored at -20°C till further use.

Total 5 primers were used including 2 for IS6110 and 3 for 12.7 kb fragment for the confirmation of *Mycobacterium* species.

IS6110 F: 5' gaccacgaccgaagaatccgctg 3'
IS6110 R: 5' cggacaggccgagtttggtcatc 3'
12.7 kb F: 5' caccccgatgatcttctgtt 3'
12.7 kb R1: 5' gccagtttgcattgctatt 3' and
12.7 kb R2: 5' gacccgctgatcaaaggtat 3'

The PCR was performed in a total volume of 25µl consisting 10µl of the sample template DNA, 5pM each of the 5 primers, 25µM of each dNTP's, 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin. The cycling conditions were standardized as initial denaturation at 95°C for 10 min, followed by 30 cycles of a denaturation step at 94 °C for1 min, primer annealing at 54°C for 1 min, extension at 72°C for 1 min, and the reaction continues for 30 cycles followed by the final extension at 72°C for 10 min. The amplicons were analysed by electrophoresis in a 1.5% agarose gel.

The smear prepared from homogenate of tissue (lung, spleen) showed acid-fast bacteria. Scanty growth was obtained on LJ slant containing 0.5% sodium pyruvate after 8 weeks of incubation indicating that bacteria grew very slowly during primary isolation. The cultured mycobacteria, negative for niacin production, were unable to reduce nitrate

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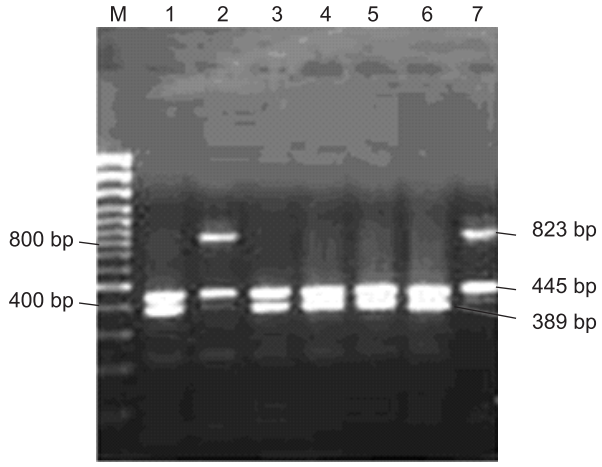

Fig. 1. PCR assay using IS 6110 and 12.7 Kb fragment. M-100 bp plus ladder; 1-$H_{37}R_v$; 2-*M bovis* AN5 3-6 Clinical samples (*M tuberculosis*); 7-*M.bovis* from a camel clinical sample.

and were found sensitive for T_2CH. The PCR assay performed for specific amplification of *IS6110* showed a 445 bp fragment. Second set of primers for 12.7 kb fragment consisting of a common forward primer and two reverse primers yielded amplification product of 389 and 823 bp in *M. tuberculosis* and *M. bovis* respectively (Fig. 1). The multiplex-PCR performed on mycobacteria isolated from camel showed the fragment indicative of *M. bovis*.

At necropsy, the male Kachchhi camel appeared severely emaciated and gross pathology revealed hard multiple nodules on the lung and spleen. Histopathology revealed granulomas with large, calcified central necrosis and some acid-fast bacteria surrounding epitheloid cells. Haematoxylin and eosin stained lung sections revealed tubercle granulomas characterized by presence of macrophages, lymphocytes, giant cells and fibroblasts (Fig.2).

Mason (1917 a, b) concluded that camel tuberculosis was caused by the same bacilli as that of the bovine type (*Mycobacterium bovis*). Besides high content of lysozyme, camel milk is reported to have bactericidal and viricidal properties and have been used in sanatorium to treat cases of tuberculosis (Yagil 2000). Camel milk is also claimed to destroy *M. tuberculosis* (Donchenko *et al*. 1975a). The disease is reported rare among camels kept under nomadic conditions. Out of 152 lymph nodes collected from slaughtered cattle, buffalo and camels, *M. bovis* was isolated from 13.25% cattle carcasses but none from camels (Bastawrows *et al*. 1995). Thus it becomes quite interesting to study and report camel tuberculosis. *M. tuberculosis*, *M. bovis* and atypical mycobacteria (*M. kansasii*, *M. aquae*, *M. fortuitum*, *M. smegmatis*) have been isolated from dromedaries (Elmossalami *et al*. 1971, Osman 1974). Grane and Higgins (1985) reported tuberculosis as an important disease of Bactrian camels where these were intensively farmed for milk production. Deen *et al*. (2004) have reported case of tuberculosis in camel at National Research Centre, Bikaner, based on lesions of lungs, lymph node and histopathological findings. Seven strains of *M. bovis* were isolated from 46 bulk milk samples taken from 712 lactating

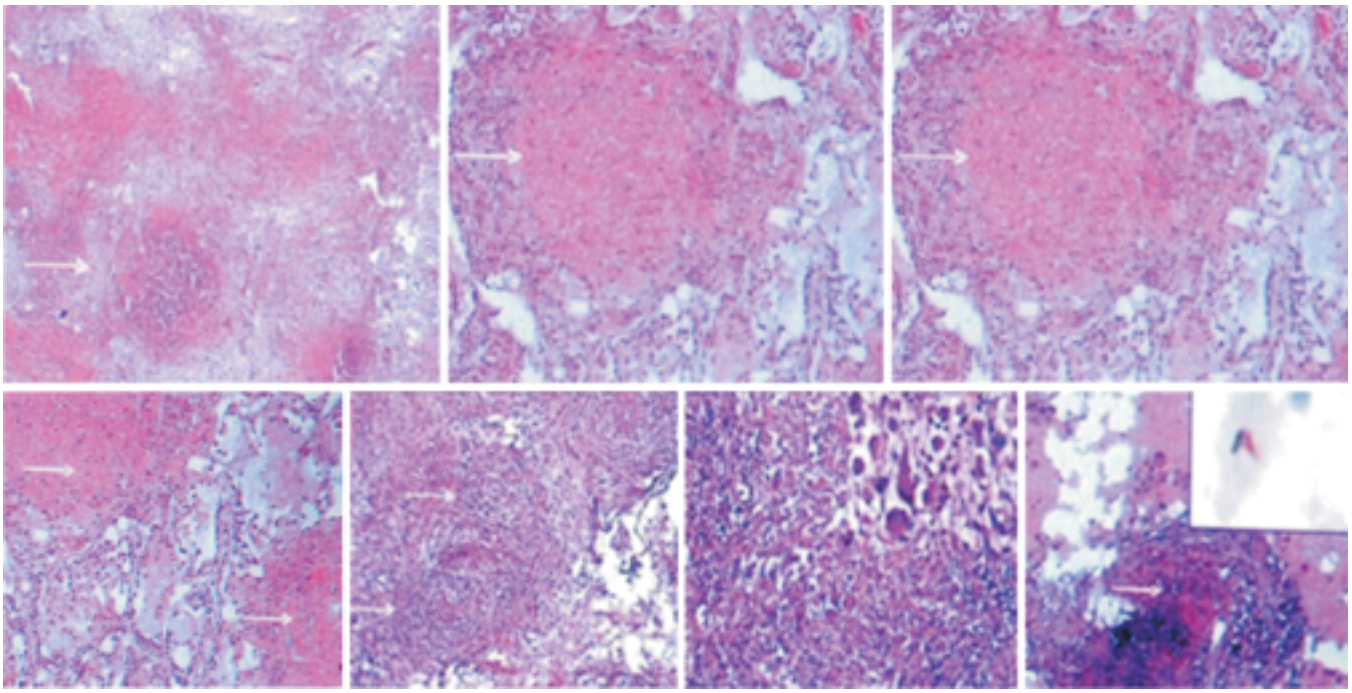

Fig. 2. H&E stained lung tissue section of camel showing tubercle granuloms (arrows) characterized by pressence of marophages, lymphocytes, giant cells (inset) and fibroblasts.

camels in 3 herds (Donchenko *et al*. 1975). Our report also strengthens the view that camel is susceptible to bovine type of tubercle bacillus. A guinea pig inoculated with the lung tissue from TB camel died after 3 weeks from typical TB. The spoligotype pattern of this isolated strain was designated SB1151 by www.M.bovis.org (Kinne *et al*. 2006). Our study conducted a multiplex PCR reported by our laboratory earlier, (Shah *et al*. 2002) which confirmed the strain to be *M. bovis*. The study concluded a confirmatory diagnosis of tuberculosis in camel and the strain so isolated from the camel may be a potential culture seed to produce homologous purified protein derivative (PPD) for screening of camels for tuberculosis.

SUMMARY

An adult male camel was diagnosed with tuberculosis (TB). It exhibited typical TB lesions in the lungs, liver and spleen. The histopathological examination of sections of tissues revealed presence of acid-fast bacilli. Mycobacteria were isolated from the camel's lung and were identified as the member of *Mycobacterium tuberculosis* complex (MTBC) subsequently confirmed as *M. bovis* by biochemical tests and multiplex PCR where 445 bp band indicative of MTBC and 823 bp band indicative of *M. bovis* was observed.

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