Tuberculosis in buffalo: the first report on the phenotypic and genetic characteristics of the isolated organism in Western Azarbaijan, Iran

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ABSTRACT

In most countries, tuberculosis caused by *Mycobacterium bovis* is mainly a disease of cattle but can infect buffalos too. The disease can be controlled successfully by mean of a test-and-slaughter program. In Iran test-and-slaughter program has started since 1971 and prevalence of bovine tuberculosis reduces from 5% to less than 0.12% in recent years. In Western Azarbaijan, North West of Iran, the prevalence of bovine tuberculosis is 0.06%. Tracing the source of infection and finding the animal reservoirs is one of the important parts of an eradication program against bovine tuberculosis and that can be achieved by differentiation of *M. bovis* isolates. Molecular typing techniques are powerful tools for epidemiological investigations and have been extensively used for this purpose. To understand the molecular epidemiology of bovine tuberculosis in the region and possible role of buffalos, 140 specimens collected from buffaloes at abattoirs bacteriologically were cultured for Mycobacteria. Only one specimen was positive in culture. The isolate was identified as *M. tuberculosis* complex by conventional biochemical tests and PCR targeting the insertion sequence *IS*6110. Restriction fragment length polymorphism (RFLP) analysis with polymorphic GC rich repeat (PGRS) and direct repeat (DR) probes showed that this isolate is distinct from *M. bovis* BCG and *M. bovis* isolates (n=10) isolated from cattle at different provinces. This is the first case of infection with *M. Tuberculosis* complex in buffalos in Iran. The role of buffalos in transmission of infection to humans and animals needs to be investigated.

Keywords: Bovine tuberculosis, *Mycobacterium bovis*, Buffalo, Iran, RFLP

INTRODUCTION*

Mycobacterium tuberculosis complex group comprises of *M. tuberculosis, M. bovis, M. africanum, M. microti* (Runion *et al* 1980) and a

newly described species *M. canetti.* (Van Soolingen *et al* 1997). *M. tuberculosis* is primarily the causative agent of human tuberculosis, but may also infect animals in contact with infected human (Michalak *et al* 1998). *M. bovis* is pathogenic for many domesticated and wild animals in particular

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bovidae, cervidae and occasionally carnivores. Human infection with *M. bovis* is well described and historically has been a common cause of tuberculosis (TB) transmitted through contaminated dairy products. It is interesting to note that out of total Asian cattle and buffalo populations, only 6% and less than 1%, respectively, are found in countries where bovine TB is notifiable and a test-and-slaughter policy is used while 94% of the cattle and more than 99% of the buffalo population in Asia are either only partly controlled for bovine TB or not controlled at all (Cosivi *et al* 1998). Thus, 94% of the human population lives in countries where cattle and buffaloes undergo no control or only limited control for bovine TB (Singh *et al* 2004). Tuberculosis in wild and domesticated animals is mainly caused by *Mycobacterium bovis*. Using intradermal tuberculin test in a national program for eradication of bovine tuberculosis, all reactors have been culled and slaughtered since 1971 in Iran. Consequently, the prevalence of bovine tuberculosis has been reduced from 5% to 0.12% in the whole country (Annual Reports of Iranian Veterinary Organization 2006). However, the prevalence of bovine tuberculosis in Western Azarbaijan is close to 0.06%. Buffalos (*Syncerus caffer*) can be an important maintenance host for *M. bovis* (Michel 2002). These animals are bred in close contact with cattle in Western Azarbaijan areas, Iran, thus increasing the possibility of contact transmission between both animals. No information was available on the situation of tuberculosis in the population of buffalos in Iran. Nor, the organism involved in tuberculosis in buffalos has been isolates and characterized either phenotipically or genetically in Iran. The identification of sources of infection and routes of transmission are important parts of an eradication scheme and new molecular techniques for the strain differentiation of *M. bovis* isolates provide a powerful set of tools for epidemiological investigations (Liebana *et al* 1997). Until recently, the only methods available to type *M. tuberculosis*

complex strains was phage typing, however this method does not provide sufficient strain differentiation for use in epidemiology of *M. bovis* (Crawford & Bates 1984). Different genetic markers have been used to study the epidemiology of bovine tuberculosis in different countries (Van Soolingen *et al* 1994a, Gutierrez *et al* 1995, Szewzyk *et al* 1995, Romano *et al* 1996). A DNA probe prepared from IS*6110* has low discriminatory power in typing of *M. bovis* isolates since strains of *M. bovis* contain low copy number of this insertion sequences (Romano *et al* 1996, Romano *et al* 1998). Polymorphic GC rich repeat (PGRS) sequences in *M. tuberculosis* complex have been used as probe in RFLP analysis of *M. tuberculosis* complex (Ross *et al* 1992). This probe can produced more DNA banding patterns than IS*6110* in RFLP analysis of *M. bovis* and has proved itself as a useful for differentiation of strains (Cousins *et al* 1993, Cousin *et al* 1998 (a), Aranaz *et al* 1998, Cousins *et al* 1998 (b), Guitierrez *et al* 1995, Romano *et al* 1996, Skuce *et al* 1996, Van Soolingen *et al* 1994b). There are approximately 30 copies of PGRS in the *M. bovis* genome (Costello *et al* 1998). Generally, only the larger PGRS fragments are analyzed, as these show the greatest degree of polymorphism and resolution of the low-molecular-weight fragments is difficult (Aranaz *et al* 1998; Skuce *et al* 1996). The direct repeat (DR) region consists of a series of virtually identical DR sequences, each 36 bp long, interspersed with variable spacer sequences from 35 to 41 bp long (Groenen *et al* 1993, Hermans *et al* 1991). Each DR sequence and the adjacent variable spacer sequence are termed a direct variable repeat (DVR) (Groenen *et al* 1993).

This study was designed to isolate *M. tuberculosis* complex from slaughtered buffalos. Restriction fragment length polymorphism analysis (RFLP) was then used to genetically characterize the isolated organism and to compare it with 10 isolates of *M. bovis* cultured from cattle and one *M. bovis* BCG 1173P2 strain.

MATERIALS AND METHODS

Collection of specimens. Lymph nodes (mediastinal mesenteric and retropharyngeal) were collected from 140 carcasses of buffalos without background of tuberculin test at abattoirs in Western Azarbaijan. To compare the phenotypic patterns and RFLP profiles of *M. bovis* isolates, 10 isolates recovered from cattle and *M. bovis* BCG1173P2 were also included in this study.

Mycobacterium isolation and identification. All specimens were digested and decontaminated by 5 gr/l *N*-acetyl-L-cysteine in 2% NaOH (0.5 M) and 0.05 Na citrare (Goyal *et al* 1999). The digested specimens were centrifuged and neutralized with HCL (0.1N). The sediments were inoculated on two slopes of Lowenstein-Jensen (L-J) medium with both pyruvate and glycerin. All mycobacterial isolates were identified as *M. bovis* on the basis of colonial morphology, growth in L-J with pyruvate, retardation of growth in L-J medium with glycerol, accumulation of niacin and, susceptibility to 10 mg/ml thiophene -2- carboxylic acid hydrazide (TCH). Production of niacin, growth in the presence of glycerol and resistance to TCH was used to speciate the isolates as *M. tuberculosis* (Sreevatsan *et al* 1996).The genomic DNA for PCR and RFLP was extracted from the visible colonies according to the method described by Van Embden *et al* (1992) as below. At least one loopfull of *Mycobacteria* were harvest from Lowenstein-Jensen slopes, placed into 400 μl of 1X TE buffer (10mM Tris/Hcl, 1 mM EDTA, pH= 8.0), and incubated at 80 ˚C for 20 min to kill the cells. 50 μl of lysozyme, (10 mg/ml) were added to the heat killed cells and the suspension were vortexed shortly and incubated overnight. It followed by addition of 75 μl of proteinase K (10mg/ml) in 10% SDS. The suspension was vortexed and incubated for 10 min. at 65 ˚C followed by treatment with 100 μl of 5 M NaCl and 100 μl of CTAB/NaCl (4.1 gr NaCl in 80 ml distilled water with 10 gr CTAB) solution. Subsequently, 750 μl of chloroform-isoamyl alchol (24:1) was added and mixed gently and centrifuged at 6500 rpm for 15 min. The supernatant was transferred to microfuge and cold isopropanolol was added at equal volume. The solution was cooled at -20 ˚C for two hours and DNA was precipitated by centrifugation at 6500 rpm for 15 min. The isopropanol was discarded and sedimented DNA was dissolved in 1X TE buffer. The concentration of extracted DNA was determined by NanoDrop (Nano Drop, USA). To identify the isolates by PCR, the primers INS-1: 5'CGTGAGGGCATCGAGGTGGC and INS-2: 5'GCGTAGGCGTCGGTGACAAA were used to amplify *IS*6110 which is specific to *M. tuberculosis* complex (Van Soolingen *et al* 1996). PCR reactions consisted of 5 μl of 10x PCR buffer, 4 μl dNTP mix (each dNTP; 2.5 mM), 5 μl of each primer (50ng/μl), 0.25 μl Taq polymerase (1.25 U) and approximately 100-150 ng of template DNA. The conditions used for PCR were 3 min at 94˚C, followed by 25 cycles of 1 min at 94 ˚C, 1 min at 65 ˚C, and 2 min at 72 ˚C and final extension at 72 ˚C for 4 min (Van Soolingen *et al* 1996). Then PCR products examined by electrophoresis and staining with ethidium bromide .

Digestion of chromosomal DNA, electrophoresis of DNA fragments and southern blotting. Approximately 6 μg of DNA from each isolate was digested with 30 unit of *Pvu*II at 37 ˚C overnight in a final volume of 50 μl. DNA fragments were separated in 1% agarose gel by electrophoresis for 10 min. at 3.2 V/cm (100 V) and then decreased the voltage to 0.8 V/cm (30 V) and run overnight. DNA was transferred to positively charged nylon membrane by southern blotting. Prior to the blotting, the gel was exposed to UV transluminator for 5 minutes and treated with HCL (0.25 M) to depurinate DNA. The gel was then rinsed shortly with distilled water and placed in 500 ml 0.4 M NaOH for 20 minutes following rewashing with distilled water. After blotting, DNA was fixed on by

heating it at 120 ºC for 30 minutes (Van Soolingen *et al* 1996).

Hybridization with oligoprobes (oligo-nucleotides) and detection. Oligonucleotide probes PGRS (5' CGG CCG TTG CCG CCG TTG CCG CCG TTG CCG CCG) and DR (5' CCG AGA GGG GAC GGA AAC) were labeled by digoxigenin at 3' end by tailing method (MWG Germany). The membrane containing DNA were initially prehybridized with hybridization buffer (5×solid sodium citrate [SSC], 0.1% N-laurylsarkosine, 0.02% SDS, 1% blocking reagrent in MQ water) for 4 hours at 65 ºC. The probe was then added to fresh hybridization at concentration of 1 pmol/ml. Hybridization was continued at 65 ˚C in rolling bottle at 6 rpm overnight. The membrane was processed for washing as recommended by the suppliers (Roche Germany). Anti-digoxigenin antibody conjugated with alkaline phosphatase diluted in 1/5000 in detection buffer (Tris/HCl 0.1 M, NaCl 0.1 M) was added to the membrane and shaked gently for 30 minutes. The membrane washed twice with washing solution (100 m1 of 20 SSC+10 ml 0f SDS 10% and then 5 ml of 20 SSC+ 10 ml of SDS 10%) to remove unbounded antibody. The signals were detected on the membrane by adding of the substrate BCIP/NBT to detection buffer. The probed DNA fragment sized from 21226 bp to 1904 bp were compared visually on the membranes and allocated patterns were given to each isolates for DR and PGRS probes using Arabic numbers or alphabetical order respectively (Arenaz *et al* 1998, Skuce *et al* 1996).

RESULTS

No gross pathologic changes were observed in specimens from buffalos**.** One specimen was positive for mycobacteria in culture. It was susceptible to TCH, weakly accumulated niacin and grew better in pyruvate than glycerol. All isolates in this study produced IS6110 in PCR test (Figure 1).
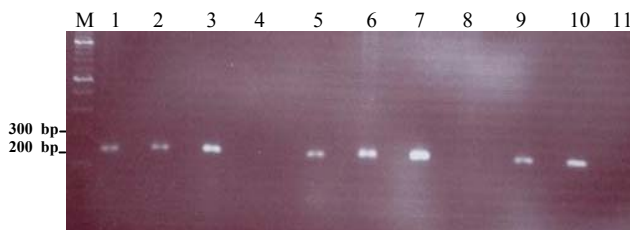

Figure 1. Electrophoresis of PCR product for *IS6110*. Lane M: Size marker no. XIV: prepared by restriction digest of specifically constructed plasmid (Roche Germany), *Lane* 1: IR 202, *Lane* 2: IR-131, *Lane* 3: IR 116, *Lane* 4: Negative control, *Lane* 5: IR 54, *Lane* 6: IR 9, *Lane* 7: Buffalo-16, *Lane* 8: D4 (*M.avium* strain D4), *Lane* 9: Sub 83-28, *Lane* 10: positive control, *Lane* 11: Negative control.

Table 1. RFLP pattern obtained by PGRS and DR probes for isolates of *M. bovis, M. bovis* BCG and *M. tuberculosis* complex from Western Azarbaijan and other provinces.

ROW	Name of Strains	Origin	DR Pattern	PGRS Pattern
1	Buffalo -16	West Azarbaijan	1	A
2	BCG 1173 P2	Pasture Institute	2	B
3	IR 9	Isfahan	2	B
4	IR 202	West Azarbaijan	2	C
5	Sub 84 – 34	Tehran	2	B
6	Sub 83 – 28	Lorestan	3	B
7	IR 131	Ardebil	------------	B
8	IR 116	Unknown	2	D
9	IR 54	Ardebil	4	B
10	IR60	Ardebil	2	-----------
11	IR69	Isfahan	2	B
12	Sub 84-55	Tehran	2	B

Using biochemical test and PCR, all Mycobacteria recovered from cattle were identified as *M. bovis*. Ten isolates of *M. bovis* were differentiate in 3 genotypes by PGRS. Probe DR also identified 3 DNA banding patterns for 10 isolates of *M. bovis*. They were differentiated by RFLP using PGRS and DR probes (Figure 2 and 3). The isolate from buffalo was distinct from bovine isolates by PGRS and DR probes. The *M. bovis* isolate isolated from Western Azarbaijan cattle was distinct from *M. bovis*

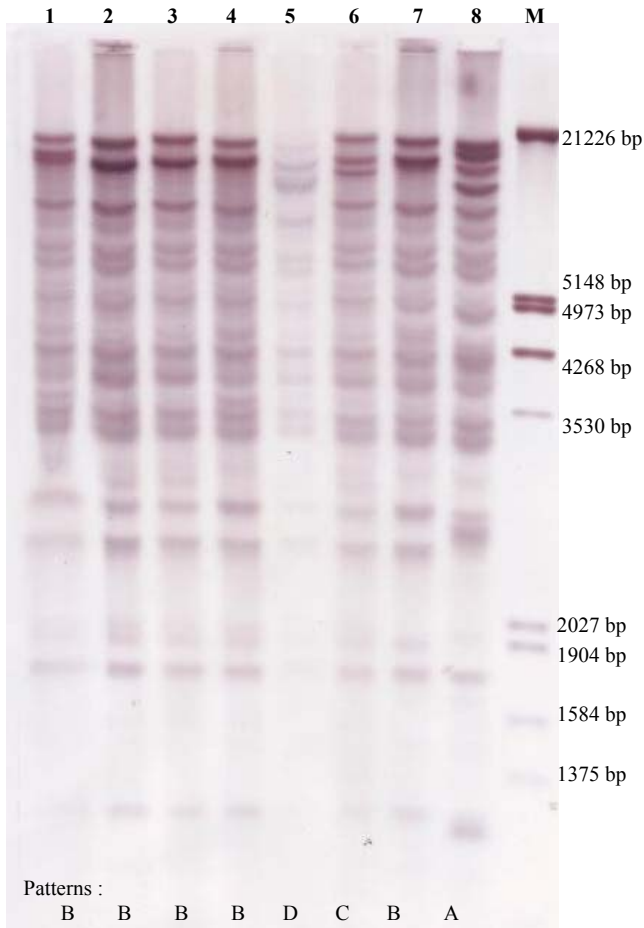originated from other Iranian provinces by PGRS probe (Table 1) and (Figure 2).

polymorphism of isolates in different group obtained by DR and PGRS probe is demonstrated in Table 1 and Figure 2.

Figure 2. Hybridization of *Pvu*II digested genomic DNA with PGRS probe, Left to Right: *Lane* 1: IR 54, *Lane* 2: IR 69, *Lane* 3: Sub 84-55, *Lane* 4: Sub 83-28, *Lane* 5: IR 116, *Lane* 6: IR 202 (Strain separated of W. Azarbaijan), *Lane* 7: BCG 1173 P2, *Lane* 8: Strain separated of Buffalo, *Lane* M: Size marker Dig Labeled λ-DNA with *Eco* RI and *Hind III* (Roche Germany).
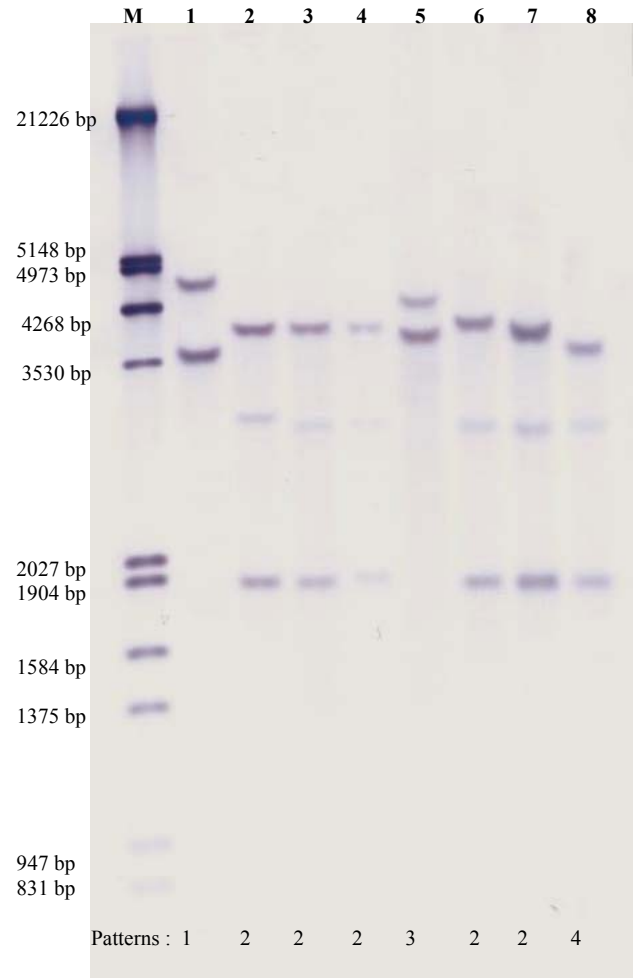

Figure 3. Hybridization of *Pvu*II digested genomic DNA with DR probe, Left to Right: Lane M: Size marker Dig Labeled λ-DNA with *Eco* RI and *Hind III* (Roche Germany), Lane 1: Strain separated of Buffalo, Lane 2: BCG 1173 P2, Lane 3: IR 202 (Strain separated of W.Azarbaijan), Lane 4: IR116, Lane 5: Sub 83-28, Lane 6: Sub 84-55, Lane 7: IR 69, Lane 8: IR 54.

Using PGRS probe the 12 isolates were clustered in 4 groups named as A-D. Group A contained an isolate from buffalo and it was distinct form other isolates. This isolate was also different from a bovine isolate in the same region, Western Azarbaijan (group C). Group B, consisting of 8 isolates, was the major group. Isolates in this group were closely related to *M. bovis* BCG. A single isolate in group D was from cattle in Gillan. The information on the

DISCUSSION

The prevalence of bovine tuberculosis in Iran has declined from 5% before initiating the test and slaughter programme in the 1971 down to 0.12% in recent years. In W. Azarbaijan, the prevalence is 0.06% (Annual Reports of Iranian Veterinary Organization 2006). There is no comparative data on the prevalence of the disease in buffalos in the same

period. To control the infection with *Mycobacterium bovis* in farmed animals, the reservoirs should be identified and removed (Gutierrez *et al* 1995). Buffalos may have a role in maintaining the infection in other farmed animals (Michel *et al* 2002). Infection with *M. bovis* has already been reported in 6.91% of Buffalos in Pakistan (Jalil *et al* 2006) and India (Singh *et al* 2004). However still no information is available on the prevalence of tuberculosis in this animal in Iran. In W. Azarbaijan, buffalos and cattle are often bred in the same field and share water, food supplies and barns. These are all risk factors in transmission of bovine tuberculosis (De Vos *et al* 2001).

The results of this study might suggest a prevalence of 1/140 (0.7%) in buffalo. The true prevalence might be lower as animals with a high index of suspicion of disease were screened in this study. It would be possible to detect pathological changes in specimens if bacterial culture be done from tuberculin positive buffalos in Iran. Based on the results of niacin accumulation and TCH test, the isolate cultured from buffalo in W. Azerbaijan was distinct from *M. bovis*. It also showed distinct RFLP patterns (Figure 2 and 3). The results of RFLP showed that differentiation power of RFLP can be improved if both PGRS and DR probes are used (Table 1 and Figure 2 and 3). The prevalence of bovine tuberculosis among the Iranian population of cattle have also been a historical debate since imported livestock from the western countries has been blamed for spreading of disease throughout the countries. The PGRS probe showed relationship existed between *M. bovis* BCG type isolates and the majority of isolates, isolates in Group B. Finding similarity between the Iranian isolates of *M. bovis* and those of western origin had previously reported by others (Cousins 2001). Though DR probe could detect some difference between these isolates, however, not considerable polymorphism was found by this probe among the isolates in group B. Epidemiological trace back of reactor and diseased

cattle is necessary for effective control and eradication of bovine tuberculosis. The application of molecular epidemiological techniques, such as RFLP and spoligotyping can and should be used in conjunction with the current traditional trace back approaches or environments which may aid the spread and maintenance of tuberculosis. We suggest a more substantial study to understand epidemiological interactions between buffalo and cattle on infection with *M. bovis* in Iran.

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