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Use of PCR Method Based on IS6110 for Detection of *Mycobacterium tuberculosis* Complex in Cattle

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Abstract: Bovine tuberculosis caused by *Mycobacterium bovis* is a highly infectious zoonotic disease. When transmitted to humans the disease symptoms cannot be distinguished from infection caused by *Mycobacterium tuberculosis* the agent of TB in humans. Transmission of the disease to humans is through direct contact with diseased animals and consumption of unpasteurized milk and milk products. The diagnostic techniques for the detection of *Mycobacterium* include polymerase chain reaction(PCR) test. The PCR technique was applied to the diagnosis of tuberculosis in cattle. After slaughter and visual inspection, tissues such as lymph nodes from tuberculin reactors were tested by the same technique. Specific oligonucleotide primers internal to the IS*6110* insertion element were used to amplify a 245-bp fragment. A total of 40 cattle were tested and PCR analysis was using lymph node. A highly sensitive PCR (IS*6110)* was positive in 23 of 40(57.7%) reactor cattle. The high sensitivity and specificity in addition to the potential for rapid detection makes this test a useful tool to detection of *Mycobacterium tuberculosis* complex.

Key words: Bovine tuberculosis · *Mycobacterium tuberculosis* complex · IS6110 · PCR

INTRODUCTION

Bovine tuberculosis has a high incidence throughout the world, especially in developing countries. As an infectious disease, it can create important public health problems as a zoonosis [1] and causes significant economical losses [2]. In addition to the economical losses it causes, because of the zoonotic property of the disease and its chronic progressive nature, eradication efforts have been undertaken in several countries. In those countries that operate bovine tuberculosis eradication programs, human infections caused by *Mycobacterium bovis* have been reduced significantly [3].

With over 40 States currently recognized in Asia, there are 7 nation that have operational test-and-slaughter programmes against bovine TB (BTB). Practically, this translates to only 6% of some 450 million bovids of the continent being included in test and slaughter programmes [4].

Biogeography of TBT in Iran: To describe the epidemiology of BTB in Iran, the annual Iranian Veterinary Organisation (IVO) records from the mid 1980 were collated and reviewed. Along with this information, other published data sources from IVO for the period 1940 through to 1970 were also scrutinized [5]. This provided a relatively extensive record of epidemiology of BTB in Iran, despite some of the older records being incomplete (Fig. 1). In 1952 the first serious attempt to comprehend the epidemiology of BTB in Iran was made and a control programme against BTB was initiated. In 1967, legislation a compulsory test-and-slaughter programme for BTB was introduced for all cattle in Iran.

During the period of 1958-1976, the highest recorded prevalence of BTB occurred [5].

During the late 1970 however the normal services of IVO were severely affected by nation-wide strikes in 1977 and the subsequent major events (establishment of the Islamic Republic of Iran and declaration of war on Iran by Iraq). This enabled a resurgence of infection resulting in tuberculin positive reactors increasing from 0.43% in 1977 to 2.74% in 1984 [5]. In 2001 the government BTB control programme was reviewed. This confirmed the intradermal comparative tuberculin test using avian and mammalian PPD tuberculinsas the diagnostic test for the BTB programme. A number of alternative methods of

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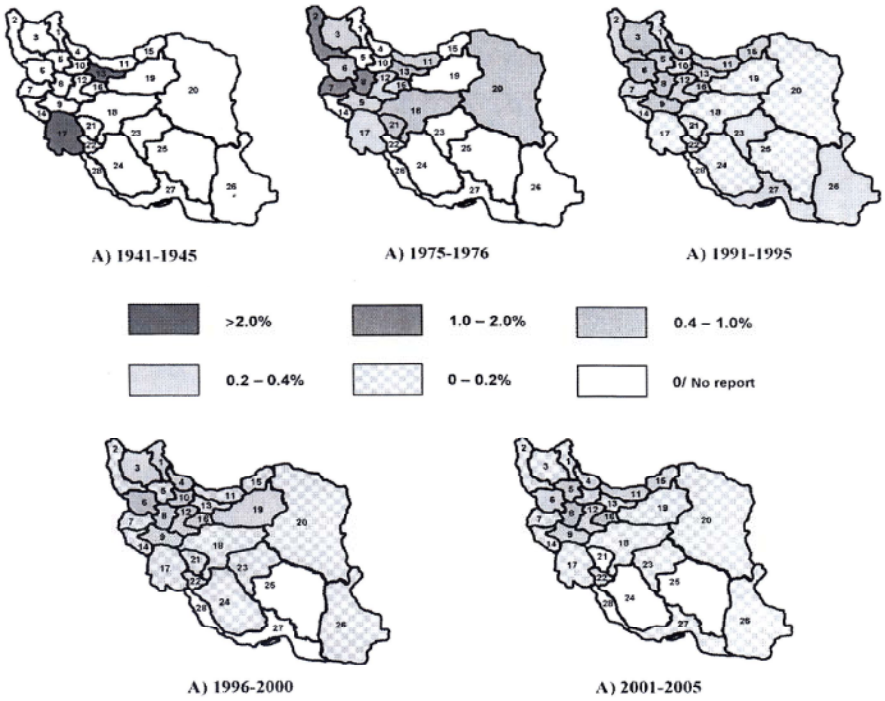

Fig. 1: Prevalence of bovine tuberculosis in Iranian provinces.

interpreting the skin test responses have been adopted, recognising that false-positive reactions may be caused by sensitisation by other mycobacteria and by local inflammation. It is important to recognise that there is a balance between sensitivity and specificity and achieving high concurrent values may not be possible.

The tuberculin test in its various formats has been widely used as a diagnostic test because of its ease of use at herd level. Despit the successes of the test-and-slaughter programme in Iran, a number of weaknesses have been identified [5]. After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques. Rapid nucleic acid methodologies, such as the polymerase chain reaction (PCR), may also be used although these are demanding techniques and should only be used when appropriately validated.

Traditional mycobacterial culture remains the gold standard method for routine confirmation of infection but usually require 4 to 8 weeks to obtain good growth.

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis,*however every isolate needs to be confirmed. It is necessary to distinguish *M. bovis* from the other members of the 'tuberculosis complex'. *Mycobacterium bovis* can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials. The Ziehl-Neelsen stain is very rapid but lacks specificity. Rapid identification of isolates to the level of *M. tuberculosis* complex can be made by Gen Probe or polymerase chain reaction (PCR) targeting 16S-23S rRNA, the insertion sequences IS*6110* and IS*1081* and genes coding for *M. tuberculosis* complex-specific proteins, such as MPB70 and the 38 kDa antigen b have been used. Specific identification of an isolate as *M. bovis* can be made usingPCR targeting a mutation at nucleotide positions 285 in the *oxy*R gene,169 in the *pncA* gene,675/756/1311/1410 and 1450 of the *gyr*B gene and presence /absence of RDs (Regions of Difference) [6]. The introduction of PCR and nucleic acid hybridization has greatly reduced identification time [7] and the use of PCR has improved the level of detection in clinical specimens. Rapid diagnosis by PCR with a number of different targets [8], including the IS*6110* insertion sequence, has been previously described [9]. IS*6110* has only been detected in species belonging to the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) [10] and a newly described species *M. canetti*. [11] which present this sequence in multiple copies. The aim of this work was to evaluate the possible application of the PCR technique to the diagnosis of tuberculosis in cattle. The results indicate that these methods could become useful diagnostic tools especially for detection of *Mycobacterium tuberculosis* complex in cattle.

MATERIALS AND METHODS

Lymph nodes (mediastinal, mesenteric and retropharyngeal) were collected from 40 reactor cattle Specimens were collected from Tehran slaughters. At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal lymph nodes and may be the only tissue affected. In addition, the lung, liver, spleen and the surfaces of body cavities arecommonly affected Early nodular pulmonary lesions can often be detected by palpation.

In some carcase we have not any symptoms or signs suggestive for tuberculosis. The lesions are usuallynon-odoriferous These reactor cattle had been slaughtered between June 2011 and September 2011.Sterile material and equipment was used for collection and handling of samples. All specimens from each animal were pooled, ground and homogenized using sand in a pestle and mortar. The samples were decontaminated with 5ml N-acetyl-L-cyctei/sodium hydroxide(5g/l N-acetyl-L-cycteine in 3.5M NaOH and 0.05M sodium citrate) for 15 min [12]. Approximately 5ml supernatant was neutralized in a universal tube with Hydrochloric acid followed by centrifugation at 3500 rpm for a further 15 min. The residue was cultured onto one slant of Lowenstein-Jensen (LJ) medium supplemented with glycerol (LJG) and another slant supplemented with pyruvate (LJP). The inoculated slopes were incubated at 37 °C for eight weeks.Genomic DNA was extracted directly from samples according to the Van Soolingen method [13]. The target DNA for amplification was a 245-bp fragment of IS6110 used to identify *M. tuberculosis* complex.

The primers used were the oligonucleotide up INS-1: (5'CGTGAGGGCATCGAGGTGGC) and down INS-2: (5'GCGTAGGCGTCGGTGACAAA) (Table 1).

PCR amplification was carried out in 50 µl reaction mixtures containing (final concentrations) 2.0 mM MgCl2, 50 mM KCl, 200 mM deoxynucleoside triphosphate, 200 nM of each primer, 5 µl of template DNA solution, 2.5 U of DNA Taq polymerase (Fermentas) and 32 µl double distilled water. The reactions were performed in an automated thermal cycler.The conditions were set as follows: denaturation at 94°C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min. A 1-min extension period at 72 °C was added after 35 cycles [14]. PCR products were analyzed by electrophoresis on1.5% agarose gels containing 0.1 mg of ethidium bromide/ml in TBE buffer (0.089 M Tris-HCl, 0.089M boric acid, 0.002 M EDTA). The gels were visualizedunder UV light with a visualization system.

RESULTS

During a 3-month period, 40 reactor cattle were tested by PCR using DNA extracted from lymph nodes(mediastinal, mesenteric and retropharyngeal). PCR was carried out using these DNA samples to confirm the diagnosis of M. tuberculosis complex infection. Gel electrophoresis analysis of PCR products is shown in Fig. 2. The 245-bp fragment of IS6110 was amplified in all purified DNA from lymph node aspirates (Fig 2). A highly sensitive PCR (IS*6110)* was positive in 23 of 40(57.7%) reactor cattle. Our study shows that 12 samples were direct Ziel-Nelson smear positive, 9 were culture positive

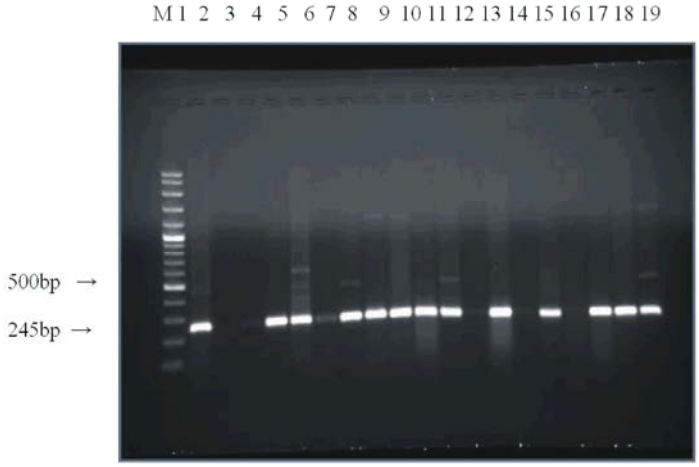

Fig. 2: Analysis of PCR amplified 254-bp fragment by 1.5% agarose gel elctrophoresis. M: Marker (Gene Ruler 100-bp DNA ladder), Lane 1: Positive control, Lane 2: Negative control, Lanes 3-19: PCR products of acid-fast bacilli isolated from lymph nodes. Arrows indicate the position of the fragments of 245 bp (diagnostic for *M. tuberculosis* complex).

Table 1: Set of primer to detect M. *tuberculosis complex* DNA sequences

Genomic marker	Holding species	PCR-product size	Sequence 5′→3′	Reference
IS6110	M. tuberculosis complex	254	INS1: CGTGAGGGCATCGAGGTGGC	
			INS2: GCGTAGGCGTCGGTGACAAA	[14]

Table 2: Results of culture, smear and PCR analyses for 40 specimens

Method of diagnosis	Negative	positive	Sensitivity
Culture	31	9	22%
smear	28	12	30%
PCR	17	23	57%

that they were grews better in pyruvate(LJP) than glycerol(LJG) and 23were sensitive to PCR for *Mycobacterium tuberculosis complex* (Table 2). On the other hand, direct PCR from lymph nodes was positive for *M. tuberculosis complex* in (23) 57.7% of the assayed samples.

DISCUSSION

Mycobacterium bovis is the causal agent of bovine tuberculosis (TB), it infects approximately 50 million animals all over the world causing economic losses of approximately 3Billion dollars per year [15]. The diseaseis zoonotic, human populations may be infected by direct contact with diseased animals and by the consumption of non-pasteurized milk and its derivatives. In 2003, the World Health Organization (WHO) estimated that approximately 8.8 million persons developed tuberculosis and 1.7 million persons died of this disease [16]. In Mexico, 28% of milk is marketed without pasteurization and is used for the preparation of cheese and other dairy derivatives [17] which implies a high risk to public health [18].

In Iran tuberculosis is prevalent at epidemic proportions, in animals, humans and maybe in the wildlife. 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 (Annual Reports of Iranian Veterinary Organization 2006). Despit the successes of the test-and-slaughter programme in Iran, a number of weaknesses have been identified [5]. The accuracy of the tuberculin test depends on several factors, including the use of high-quality tuberculins, the format of the tuberculin test used (e.g. single intradermal comparative tuberculin test, single intradermal tuberculin test, the caudal fold test), the efficiency of the testing procedure, the mode of interpretation of the test result as well as the immunological responsiveness of the animal at the time of test. Furthermore, a negative result to a tuberculin test does not mean that the animal is not infected with *M.bovis* while, on the other hand, a positive result represents an immunological response, in the form of a delayed hypersensitivity reaction to mammalian PPD tuberculin that is most commonly due to acurrent infection or a previous exposure to *M. bovis* but may less commonly be due to an infection or exposure to other bacteria that share antigens similar to those of *M. bovis*. McIlroy [19] reported that upon diagnosisof tuberculin-positive animals, tuberculous lung lesions were evident in 70% of reactive cattle, while *M. bovis* was isolated from nasal or tracheal mucus samples in just 19 % of confirmed cases. These discrepancies illustrate the need for more sensitiveand accurate methods to assist in the control of this zoonosis.

The laboratory diagnosis of *M. tuberculosis complex* is currently based on acid-fast staining and cultures on solid and/or liquid media, but its sensitivity is low [20]. Cultures on solid media require up to 8 weeks of incubation to achieve the maximum Sensitivity [21]. PCR has been successfully applied by us and other groups to detect members of *M. tuberculosis* complex. DNA amplification of specific sequences of DNA by the PCR technique has been reported to provide rapid diagnosis of many diseases and is especially useful for the direct detection of *M. bovis* in bovine tissue samples [22-24]. The sensitivity of the IS6110-PCR assay is high. In study, the sensitivity of the culture, the direct Ziel-Nelson smear and the PCR were 22%, 30% and 57%, respectively. According the results of this study it is concluded that PCR is more sensitive than culture and direct smear.The high sensitivity in addition to the potential for rapid detection of M.. *tuberculosis* complex, makes this test a useful tool for detection of *M.tuberculosis* complex [22]. The efficiency of three methods to diagnose *M. tuberculosis complex* in reactor cattle (lymph nodes samples) was compared. PCR showed the highest efficiency compared to bacteriological and microscopic examination. That confirm the advantage of PCR for diagnosis of *M. tuberculosis* complex over other procedures.False-positive and false-negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of this test. Variability in results has been attributed to the low copy number of the target sequence per bacillus combined with a low number of bacilli. Variability has also been attributed to decontamination methods, DNA extraction procedures,

techniques for the elimination of polymerase enzyme inhibitors, internal and external controls and procedures for the prevention of cross-contamination.

In the study, the agreement between PDD tuberculin and PCR was determined as excellent. IS6110 is not specific for *M.* bovis but is specific for all organisms in the *M. tuberculosis complex,* which includes *M. tuberculosis, M. bovis, M. microti* and *M.africanum* [25] and a newly described species *M. canetti.* [11]. However, because *M. tuberculosis* has been recovered from many animal species, infection with that organism might be more frequent in countries with a high incidence of human tuberculosis. However, identification of any *M. tuberculosis* complex infection would warrant a public health concern. The results presented in this study indicate that the PCR method improves tuberculosis diagnosis, with the advantage that method is rapid and more sensitive than other methods. PCR could be applied directly to be used as a routine diagnostic test in Iran.

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