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Identification of Bovine and Human Tuberculosis in AFB Positive and Negative Patients

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Abstract.- This study was performed on both pulmonary and extra pulmonary tuberculosis. The *Mycobacterium tuberculosis* (MTB) complex was identified by targeting insertion sequence (IS6110) and 984 bp fragment was observed while *M. bovis* was identified by targeting the same insertion sequence and amplified fragment size was 500 bp. The patient's sputum, fine needle aspiration cytology (FNAC) of lymph node, pus, peritoneal effusion and pleural effusion samples coming from different rural areas of district Gujranwala and Hafiz Abad were included in the study. These were examined for AFB and analyzed for mycobacterial DNA (insertion sequence) by PCR. The samples were initially diagnosed for MTB complex and later subjected to *M. bovis* identification. None of them was positive for human immune deficiency virus. Out of 324 samples, PCR of MTB complex was positive in 184/324 (56.79%) TB cases. PCR was positive in 64/324 (19.75%) in AFB (acid fast bacillus) negative TB samples, while 120/324 (37.03%) samples were both positive in PCR and AFB. Out of 324 samples, 24 were found positive for *M. bovis* in PCR among them 16 were negative in AFB test. The sensitivity of PCR in tuberculosis(56.79%) was high than that of AFB (37.03%). The sensitivity of PCR was high in extrapulmonary TB (70.00%) than in pulmonary TB (52.45%). To conclude, molecular diagnostic techniques are important adjuvant to the traditional microbiological techniques for better and rapid diagnosis of tuberculosis.

Key words: PCR, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, FNAC.

INTRODUCTION

About 3 million deaths and 9 million new tuberculosis (TB) cases are reported every year throughout the world, of these 98% of TB deaths and 95% of TB cases are in the developing countries. The 75 % of infected individuals are between the age of 15-45 years, which is economically the productive age group (Harries *et al*., 1996). One third of the world population is affected with a tubercular micro-organism (Kochi, 1991) and Pakistan stands seventh according to prevalence (WHO, 2007)

Three species of Mycobacteria *viz.*, *Mycobacterium tuberculosis*, *M. bovis* and *M. africanum* are responsible for tuberculosis (Robbins *et al*., 1981). One third of world population is infected with TB organism (Raviglione *et al*., 1995). Since tuberculosis is a major health threat, particularly in developing countries, it has been declared as global emergency by the World Health Organization in 1995 and national emergency in 2001 by the Government of Pakistan (WHO, 2003).

M. tuberculosis is a rod shaped nonmotile bacterium 2-4 µm× 0.2-0.5 µm (Vollum *et al*., 1970). It is an obligate aerobe with long generation time and facultative intracellular parasite especially of macrophages (Levinson, 2010). Middle Brookes medium (agar based) and Lowenstein-Jensen (L-J) medium (egg based) are being used for its growth; the addition of sodium pyruvate to L-J medium enhances the growth of *M. bovis*, a character which differentiates *M. tuberculosis* from *M. bovis* cultures (Todar, 2002). *M. tuberculosis* is being transmitted via aerosol delivery of the infectious foci (Harries *et al.*, 1996), while *M. bovis* is being transmitted by aerosol delivery of the sputum of infected animals, their dried dung and milk (Ayele *et al*., 2004) (OIE, 2000). The *M. tuberculosis* (MTB) complex, consisting of *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* has been identified with the insertion sequence IS*6110* which is considered as specific to the members of the MTB complex **(**Haddad *et al*., 2004).

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The importance of *M. bovis* is due to its zoonotic nature particularly in rural areas where human and animals are in close contact (Madkour and Munir, 2004). These bacteria are commonly known as tubercle bacilli or acid-fast bacilli as they form granuloma or tubercle in diseased tissue (Robbins *et al*., 1981). The prevalence of bovine TB in livestock particularly during lactation can infect young calves and human (OIE, 2000). The primary tuberculosis develops in a person who is not previously exposed to TB whereas secondary tuberculosis is caused by reactivation of the focus in a previously sensitized individual (Kumar, 2010).

It is difficult to completely cure patients suffering from TB of animal origin with conventional anti-tuberculosis treatment particularly when *M. bovis* has developed resistance to pyrazinamide **(**Somoskovi *et al.*, 2001). Moreover, culture of *M. bovis* takes many weeks to complete. Pakistan stands seventh according to prevalence of TB (WHO, 2006). There is a need for reliable and rapid test for early diagnosis. The study was aimed at developing a rapid and sensitive test for reliable diagnosis and determining the exact prevalence rate of bovine tuberculosis in human population of some selected areas of Punjab.

MATERIALS AND METHODS

Patients

The TB suspect patients between 15-45 years of age, either sex visiting different hospitals *viz.*, Basharat Hospital Kamoke, Civil Hospital Kamoke, Civil Hospital Gujranwala and Civil Hospital Hafiz Abad from rural areas of Gujranwala district were included in the study. A total of 335 suspected individuals exhibiting signs of fever, cough, weight loss, sputum production, chronic diarrhea, hemoptysis of more than four weeks duration were investigated initially by chest X-ray images, complete blood picture, urine examination, Mantoux test, sputum test for acid fast bacillus (AFB) and fine needle aspiration cytology (FNAC). The medical history of suspected individuals was recorded on specific clinical data consent forms. The patients included in this study did not have to any TB treatment intervention earlier. The individuals found to be positive for TB on these initial investigations were registered for the clinical study.

This study was approved by Ethical Review Committee, School of Biological Sciences, University of the Punjab, Lahore.

Sample collection

The samples of sputum (3 ml), other body fluids such as pus (3ml), pleural fluid (5ml), peritoneal fluid (5ml) and material (3ml) from lymph nodes of each patient were collected in sterile tubes.

The sputum samples were collected as early morning sputum specimen after deep cough, one at the premises of the hospital and the other on the next early morning. The sputum samples were treated with 2% NaOH in 0.5% NALC (N-acetyl-L-cysteine) -NaOH for direct and concentrated smears preparation and centrifuged for 15 min at 3000 rpm (3360xg). Supernatant was discarded and 1-2 ml of sterile phosphate buffer of pH 6.8 (1 to 2 ml) was added to the pellet and then again centrifuged for 15 min at 3000 rpm (3360xg).

Aspiration of body fluids like peritoneal and pleural was carried out under carefully monitored aseptic techniques after fully explaining the procedure to the patients (Agarwal, 2005). Standard sterile disposable needle of 22-24 gauge 1-1½ inch fitted over 10 cc disposable plastic syringe was used for this purpose. Local anesthetic (Lignocaine, 2%) was applied at the site of intervention. The color, consistency and amount of fluid were recorded.

All the TB suspect patients with enlarged lymph node or swelling were examined for fine needle aspiration cytology (Agarwal, 2005). Standard sterile disposable needles of 22-24 gauges 1-1½ inch fitted over 10 cc disposable plastic syringes were used. The needle was pricked into the lesion, and material was procured by cutting action of the needle. The consistency, color and amount of material was recorded.

Each sample was divided into two parts, one used for AFB smear test, while the other was stored at -40°C until used for molecular analysis.

Acid fast bacillus (AFB) smear test

The smears of sputum, peritoneal and pleural fluids and content of lymph nodes were fixed, dried

in the air, heat fixed and stained with Ziehl-Neelsen stain (ZN stain) and studied under the microscope to detect acid fast bacilli (Watt *et al*., 1996; Iqbal *et al*., 2010).

Molecular identification of MTB complex

For identification of MTB complex and *M. bovis*, the genomic DNA was isolated from different types of samples including sputum, FNAC material, pus from cold abscess and body fluids (peritoneal and pleural aspiration fluids) using Gentra DNA isolation kit (Gentra Inc, USA).

The frozen 300 µl sample was thawed and put in 1.5 ml eppendorf tube containing 1000 µL cell lysis solution. The sample was incubated at 65°C for 30 min. The tubes were centrifuged for 3 min at 13000 rpm (14,560xg). Supernatant was shifted to separate eppendorf tube and 200 µL protein precipitation solutions (as provided by kit) was added for 5-10 min. The samples were centrifuged at 12000 rpm (13,440xg) for 3 min. The protein pellet was discarded. Two volumes of isopropanol was added to the supernatant and incubated at -70°C for 20 min. The tubes were centrifuged at 12,000 rpm (13,440xg) for 10 min and supernatant was discarded. The pellets were dried and then dissolved in 50-100 µL distilled water before proceeding for PCR.

The 324 pulmonary and extrapulmonary samples were subjected to PCR by using primers specific for identification of MTB complex named as IS6110 (accession numbers FR878060.1, CP001642.1 and CP001641.1) (Kidane *et al.*, 2002; Hsiao *et al*., 2003). A product of 984 bp was amplified by using primers

IS5 F 5’ cggagacggtgcctaagtgg 3’ and
IS6 R 5’ gatggaccgccagggcttgc 3’

This amplified PCR product was then further subjected to nested PCR by same primers to further validate the results.

The bacterial genomic DNA containing *M. bovis* specific DNA fragment (accession U87961) of 500 bp was amplified by using the following primers.

JB21F 5’ tcgtccgctgatgcaagtgc 3’ and
JB22 R 5’ cgtccgctgacctcaagaag 3’

This amplified PCR product (500 bp) was then further subjected to nested PCR by the same primers set under the same conditions.

The 50 µL PCR reaction mixture contained 5 µl 1x PCR reaction buffer, 5µl $MgCl_2$ (4 mM), 5µl dNTPs (0.2 mM), 2µl forward primer (200 pmol) and 2µl reverse primer (200 pmol).

The PCR cycling conditions were checked with three different melting temperatures *i.e.* 50°C, 55°C and 60°C for both primer sets *i.e.* MTB and *M. bovis*. The PCR mixture was subjected to initial denaturation at 94°C for 4 min followed by 30 cycles each of denaturation at 93°C for 30s, melting temperatures (50°C, 55°C, 60°C) for 45s, extension at 72°C for 30 s and final extension at 72°C for seven min. The reaction was stopped by cooling the mixture to 4°C. The amplified PCR products were checked on 1% agarose gel.

RESULTS

A total of 335 TB patients were registered in this study. Samples of 11 patients could not be obtained, so 324 samples were processed for AFB smear and PCR amplification. Out of 335 patients 244 (73%) were found to have pulmonary tuberculosis whereas 91 (27%) suffered from extrapulmonary TB (Table I). Only 85 (35%) patients out of 244 of pulmonary TB were sputum smear AFB positive (Table I). Out of 244 pulmonary TB samples, 128 (52.45%) were PCR positive for MTB complex. Out of 128 PCR positive samples, 43/244 (17.62%) were AFB negative. The number of samples which were positive both for AFB and PCR was 85/244 (34.83%) (Table I).

The total number of extrapulmonary TB samples (FNAC, pleural and peritoneal fluids) was 91 but only 80 were used for PCR amplification since the remaining 11 were patients of Carries spine (bone TB) (Table I). Out of 80 extra pulmonary TB cases 56 (70.0%) were found to be positive in MTB PCR and cases found negative in AFB and positive in PCR were 21/80 (26.25%) (Table I). However 35/80 (43.75%) were positive both for AFB and PCR (Table I).

Table I. - Analysis of MTB complex in different human tissue samples.

Samples	PCR positive	AFB and PCR positive	AFB negative and PCR positive
TB Samples	184/324 (56.79%)	120/324 (37.03%)	64/324 (19.75%)
Pulmonary	128/244 (52.45%)	85/244 (34.83%)	43/244 (17.62%)
Extrapulmonary	56/80 (70%)	35/80 (43.75%)	21/80 (26.25%)
Lymph node	36/40 (90%)	24/40 (60%)	12/40 (30%)
Pleural effusion	10/27 (37.03%)	5/27 (18.51%)	5/27 (18.51%)
Cold Abscess	9/10 (90%)	5/10 (50%)	4/10 (40%)

Table II. - Molecular identification of *M. bovis* (n = 324). Eleven out of 335 suffered from caries spine, and hence have not been included in the analysis.

Samples type	PCR positive	AFB and PCR positive	AFB negative and PCR positive
TB Samples 324	24/324	8/324	16/324
Pulmonary 244	5/244	2/244	3/244
Extrapulmonary 80	19/80	6/80	13/80
Lymph node 40	13/40	4/40	9/40
Pleural effusion 27	4/27	1/27	3/27
Cold Abscess 10	2/10	1/10	1/10

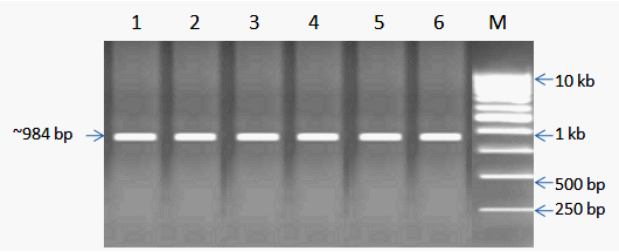

Fig. 1. PCR detection of *M. tuberculosis* complex by using primer set IS5 F and IS6 R. Lanes 1-3, patient samples (ElF16); 4-5, nested PCR of samples 1 and 2; 6, positive control (confirmed MTB culture); M, 1 kb DNA marker (Fermentas).

Out of 324 samples in 64 (19.75%) were AFB test negative and MTB complex positive (Table I) indicating the fact that PCR is more reliable diagnostic technique than AFB test. However, cases which were both AFB and MTB PCR positive were 120 out of 324 (37.03%). The comparative sensitivity of PCR in AFB negative cases was more in extrapulmonary TB than pulmonary TB (Table I). In case of *M. bovis* out of 324 samples 24 samples were found PCR positive (Fig. 2, Table II). Sixteen out of 324 samples were AFB negative, but PCR positive, whereas 8/324 were AFB as well as PCR positive (Fig. 2, Table II).

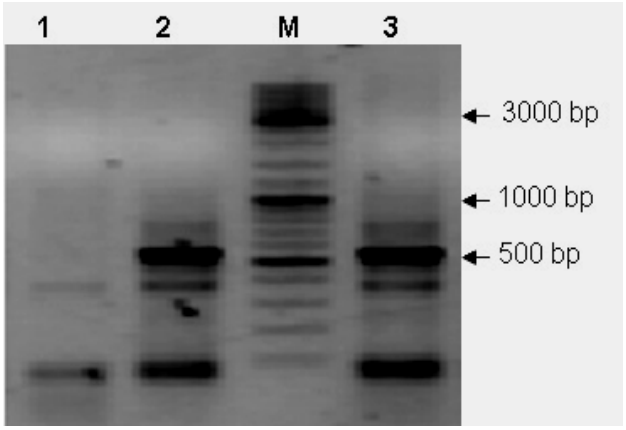

Fig. 2. PCR detection of *M. bovis* by using JB21-22 primers (500 bp). Lane 1, negative control for *M. bovis;* Lane 2, 3, experimental samples ElF16 and PM31 positive for *M. bovis;* M, marker DNA (Fermentas, #SM1173).

DISCUSSION

M. tuberculosis is human specific, whereas *Mycobacterium bovis* is zoonotic in nature. *Mycobacterium bovis* is characteristically resistant to pyrazinamide, part of 4 drug regimen used in TB treatment, and also resistant to isoniazid mostly in HIV positive and immune-compromised cases (Harries *et al*., 1996).

Most of the infected people and deaths are in developing countries and in the younger age group (Raviglione *et al*., 1995). All types of TB (pulmonary, extra pulmonary, multidrug resistant, smear positive and smear negative TB) is increasing day by day in Pakistan. The development of multidrug resistance and bovine TB cases are important factors in treatment failure.

Diagnosis of TB is a great challenge in developing countries. Sign and symptoms like fever, cough, weight loss, loose motions, and abdominal pain of more than 4 weeks duration are

important parameters in TB diagnose (Chesnutt and Prendergast, 2007). History of TB contact, overcrowding in houses, younger age group, and co morbid diseases, animal handling along with poor economical conditions are important factors in spread of this disease. Sputum for AFB, X-ray chest, Mantouxe test, changes in blood CPC, ESR and histopathology of specimens are considered to be important diagnostic tests (OIE, 2004).

Sputum smear is believed to be the most significant diagnostic tool for TB. This test has high specificity but very low sensitivity. In Pakistan only 34% chest TB cases are sputum smear positive (WHO, 2003). To visualize the TB bacilli with a 100x microscope objective, there is need of >10,000 organism per ml of sputum. The dead microorganisms, lesser number of MTB bacilli in the specimen and inadequate techniques are responsible for the negative results (Harries *et al*., 1996). Lack of bacteriological and molecular result in TB investigation are diagnostic challenge especially when clinical presentation is suggestive but bacteriological proof is lacking. Pahwa *et al*. (2005) compared the various diagnostic techniques in clinically suspected cases of tubercular lymph nodes to find a suitable, cost-effective but sensitive and specific method for diagnosis. They compared the culture with PCR and found that the sensitivity and specificity of PCR was 89.5% and 86.1%, respectively. PCR was able to detect 80% of smear-negative but culture-positive cases.

Chest X-Ray is known to be one of the important diagnostic tools for TB diagnosis. The lesions seen on X-Ray considered to be of TB some time closely resembles with atypical TB lesion or other bacterial infections (Assefa *et al*., 2011). AFB negativity along with resemblance of X-Ray lesion with atypical lesions further makes the physician uncomfortable in diagnosing TB resulting in under or over diagnosis. Mantouxe test a test commonly used in TB diagnosis depends upon immune response of the patients to the TB microorganism. The test is usually negative in TB patients of extreme age group, with anemia, HIV, hypoproteinemia, advance diabetes, carcinomas, on corticosteroid and immune suppressant medication, reducing the importance of this test as good diagnostic tool. Change in ESR has prognostic instead of diagnostic value and may be higher in many other diseases as well.

There is strong need to consider the importance of *M. bovis,* while diagnosing TB because of its characteristic resistance to pyrazinamide and its contribution in drug resistance TB and bad outcome of the treatment. In developed world the researcher emphasize the importance of typing and sub typing the *M. bovis* in human samples (Kubica *et al*., 2003)

The introduction of PCR is very important diagnostic tool in the diagnosis and typing of TB. PCR should be done on clinical samples like sputum, FNAC, pleural effusion peritoneal effusion, pus and biopsy material as living or dead bacteria may be present in these samples. Blood samples do not contain the sufficient quantity of bacterial DNA necessary for PCR for TB. When lymphoid tissue are involved then a special technique buffy coat monocytic PCR can be done from blood samples (Mirza *et al*., 2003).

The molecular diagnostic techniques in addition to conventional AFB test and culture based procedures can aid in rapid screening for determining drug resistance (van Rie *et al*., 1999). Molecular analysis may also be useful in drug surveillance studies to further improve standard of the data in these studies. However, implementation for both rapid diagnosis and surveillance requires proper quality control guidelines.

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