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DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS* IN SPUTUM AND BLOOD SAMPLES OF HUMAN

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ABSTRACT

Mycobacterium bovis (*M. bovis*) is the major cause of gastrointestinal tuberculosis in humans. The study was conducted in Lahore to compare 100 blood and 100 sputum samples from suspected patients of active tuberculosis, using the Duplex PCR and conventional methods for the detection of *M. bovis* and *Mycobacterium tuberculosis (M. tuberculosis)*. In *M. tuberculosis,* pncA gene and species-specific 500-bp fragment of *M. bovis* was targeted in Duplex PCR. In sputum samples 37 % revealed the presence of *M. tuberculosis* while 05 % samples were positive for *M. bovis* using duplex PCR. Blood samples, 39 % and 04 % were positive for *M. tuberculosis* and *M. bovis* respectively through PCR. Acid fast bacilli (AFB) have been instituted in 23 % sputum 08 % blood samples using Ziehl-Neelsen staining. On culturing of 11 % sputum and 09 % blood samples were found positive. The sensitivity and specificity of Duplex PCR was found statistically significant in comparison to conventional methods for the differential diagnosis of tuberculosis caused by *M. tuberculosis* and *M. bovis*. Therefore Duplex PCR is a better choice of diagnostic test in the clinical setups where clinical urgencies necessitate a reliable, sensitive and specific test with results in a short time period.

Key words: Blood samples, Duplex PCR, *Mycobacterium bovis, Mycobacterium tuberculosis,* Sputum samples.

INTRODUCTION

One of major global health problem is tuberculosis that causes death from most frequently witha single infectious agent, being annually responsible for approximately two million deaths and eight million new cases (WHO, 1999). According to the Centers for Disease Control and Prevention (CDC), nearly one-third population of the world is prone to infect with the *Mycobacterium tuberculosis*, which kills almost 2 million people per year (CDC world TB day, 2005).

Tuberculosis becomes active disease, 75% of cases effect the lungs i.e. pulmonary tuberculosis. The main symptoms include 3 week prolong cough that productive, chest pain and coughing up blood (WHO, 2006). In 24% of non-HIV-infected patients (Prieto et al., 1990) and in 77% of human immunodeficiency virus (HIV) detected with affected lungs, disseminated and extrapulmonary forms of the disease (Clough, 1917).

The epidemic of HIV infection in developing countries, particularly those in which M. bovis infection is present in animals and the conditions favor zoonotic transmission, could make zoonotic TB a serious public health threat to the persons at risk (Cosivi *et al*., 1995; Grange *et al*., 1994; Daborn *et al*., 1997).

The diagnosis of Tuberculosis includes history, physical examination and radiological findings of either consolidation or cavitations in lung apices. Along with this Acid-fast smears & cultures of sputum are also required. The use of direct smear microscopy is an inexpensive and rapid test for diagnosis of tuberculosis (Kesarwani *et al*., 2004), but it does not permit differentiation between species of M.tuberculosis complex .Culture is the gold standard, but it requires a lot of time, whereas clinical urgency necessitates immediate laboratory back up. The PCR amplification of the Mycobacterium tuberculosis DNA is a rapid, reliable method for rapid diagnosis (McAdam *et al*., 2007).

In this study a Duplex Polymerase Chain Reaction was optimized and its comparison with the conventional methods was carried out, which defined the causative organisms of tuberculosis (M.bovis and M.tuberculosis) from samples of sputum and blood taken from patients of active tuberculosis. The validity of PCR used for patients with active tuberculosis on samples of blood and sputum, especially in patients with extrapulmonary tuberculosis, was checked, as this form of disease is not diagnosed via routine sputum or blood tests.

MATERIALS AND METHODS

One hundred sputum and blood samples each were collected from suspected TB patients on basis of

clinical sign like pulmonary tuberculosis, chronic coughing that were admitted in different hospitals of Lahore along with the other data. The sputum samples were collected in 5ml sterile plastic bottles and placed in 15ml sterile bottles to avoid leakage of infectious samples and the blood samples were collected from same patients in sterile vacutainers.

Following tests were applied to the collected samples of blood and sputum, collected from the same patient:

Conventional Methods including Ziehl-Neelsen stainingdescribed by Quinn *et al*. (1994),Culture/ Isolation on Lowenstein and Jensen (LJ) media withthe addition of glycerol for the growth of M. tuberculosis where as for the growth of M.bovis, samples were cultured on specific media known as Stone brink's medium containing 1% sodium pyruvate.

The growth obtained after culture were subjected to the following biochemical tests for the confirmation of *M.bovis* and *M.tuberculosis*.(a)Nitrate reduction test (b)Niacin accumulation test as described by Quinn *et al*., 1994.

For duplex PCR the DNA was extracted from the blood and sputum samples by using FERMENTAS DNA Purification Kit. 5 µl of the Genomic DNA extracted was then subjected to 0.7% agarose gel electrophoresis for determination of quality (Santha *et al*., 1990). The DNA extracted from the blood and sputum samples were subjected to duplex PCR by using two set of primers, as described by (Shah *et al*., 2004). Amplification was carried out in a 50-µl reaction mixture containing 4µl PCR buffer; 5µl MgCl2; 5 µl of 200-mM dATP, dGTP,dCTP, and dTTP; 0.5 U Taq polymerase (Fermantas, MD); 1.6µl each of both primers Forward (F) and Reverse (R) , and 5µl genomic DNA solution. The PCR conditions consistedof an initial denaturation at 94°C for 4 minutes, 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute), Extension (73°C for 1 minute), and final elongation at 74°C for 10 minutes. For Mycobacterium tuberculosis;

Forward (ATGCGGGCGTTGATCATCGTC);
Reverse: pncAMT-2 (CGGTGTGCCGGAGAAGCGG).
For Mycobacterium bovis;
Forward: JB21 (TCGTCCGCTGATGCAAGTGC);
Reverse: JB22 (CGTCCGCTGACCTCAAGAAG).

All the PCR reactions were carried out in an automated thermocycler (Eppendorf, USA). After amplification, the PCR mixture was analyzed by the gel electrophoresis in 1.5% agarose gel. For each Multiplex PCR test distilled water was used as negative control and for positive control culture of M. tuberculosis and M.bovis were obtained from Veterinary Research Institute, Lahore.

The statistical analysis was made by using software win episcope 1.Culture was used as gold standard.

RESULTS

Out of 100sputum samples 37 were positive for *M. tuberculosis* while 05 samples were positive for *M.bovis* on the basis of duplex PCR. On Ziehl-Neelsen staining showed positive 23 Acid Fast Bacilli out of 100 sputum samples. Blood culture was perfumed in 100 sputum samples 11(11%) samples were positive on culture that was confirmed on basis of biochemical tests as shown in Table 1.

Table 1: PCR, ZN Staining and Cultures of Sputum and Blood of human subjects

Techniques	Sputum Samples			Blood Samples		
	M.Tuberculosis	***M.bovis***	**Total**	***M.Tuberculosis***	***M.bovis***	**Total**
Duplex PCR	37 (37%)	5 (5%)	42 (42%)	4 (9.30%)	39(90.69%)	43 (100%)
ZN Staining	-----	----	23 (23%)	----	----	8(18.60%)
Culture	11 (11%)	----	11 (11%)	----	----	9(20.93%)

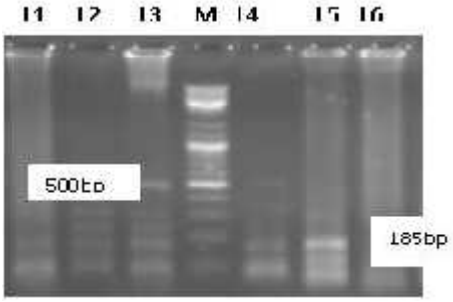

Figure 1: Duplex PCR of Human Sputum Samples L1 to L6: Experimental Samples, L2, L3; Positive for M. bovis and M. tuberculosis, M: DNA marker, L4, L5 Positive for M. tuberculosis, L6 is Negative

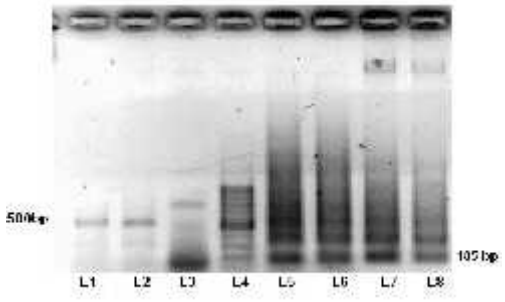

Figure 2: Duplex PCR of Human Blood Samples L1 and L2: 500bp products (positive for M. bovis). L3: Negative for both *M. bovis* and *M. tuberculosis*. L4: DNA ladder (100bp), L5, L6 and L7:185 bp products with additional bands of 500bp (positive for *M. tuberculosis*), L8:185 bp positive for *M. tuberculosis*

In case of blood samples out of 100 samples 39(90.69%) were positive for *M. tuberculosis* and 04(9.30%) were positive for *M. bovis* on the basis of Duplex PCR. While out of total 43 positive samples 08(18.60%) were positive on Ziehl-Neelsen staining and blood culture were perfumed in 43 positive samples; M. tuberculosis were recovered from 09(20.93%) samples showed growth on LJ media, that were confirmed on basis of biochemical test as shown in Table 1.

The sensitivity of Duplex PCR was 100% while the specificity was 70.79%. Positive predictive value was 29.73% while the negative predictive value was 100% at 95% confidence interval (CI) by using software win episcope 01.

DISCUSSION

Tuberculosis is a major infectious disease in Pakistan. The epidemiological impact of bovine tuberculosis on human health is an important issue especially in the developing countries like Pakistan. Tuberculosis due to *M.bovis* has a significant value as in the recent years there is increased incidence of tuberculosis due to *M.bovis* in AIDS patients (O'Reilly *et al*., 1995).M. tuberculosis DNA in PBMC samples from 9 of 11 (82%) HIV- infected patients and in 7 of 21(33%) non-HIV-infected patients with different forms of tuberculosis detected and also suggested that PCR detects *M. tuberculosis* DNA in PBMC only in case with active tuberculosis infection by Folgueira *et al*., 1996.

In the laboratory the diagnosis of tuberculosis is based on the traditional methods of Ziehl-Neelsen acid fast staining and culture of the causative organism, *M.tuberculosis*. The presentation of pulmonary tuberculosis caused by *M. bovis* and *M .tuberculosis* is the same in concert with its clinical, radiological, and pathological findings.

The Duplex PCR proposed in this study is based on one step amplification of two mycobacterial genomic fragments (Shah *et al*., 2004); *M.bovis*-specific 500-bp fragment and *M.tuberculosis* specific pncA gene. Purified genomic DNA from all the sputum and blood samples was subjected to Duplex PCR using a mixture of four primers, JB21, JB22, pncATB-1.2 and pncAMT-2 (1:1:1.5:1.5). As expected, there were 500-bp and 185-bp amplified products obtained, using the total genomic DNA.

Out of 100 sputum samples, 37 were *M.tuberculosis* positive while 5 were *M.bovis* positive, and out of 100 blood samples 39 were positive for *M.tuberculosis* whereas 5 were positive for *M.bovis* by Duplex PCR. It had been confirmed that species-specific 500 bp fragment is well conserved in *M.bovis*. The results of the current study are in full accordance with Shah *et al*. (2004). In the present study our data propose that blood culture is not considered a useful technique in mycobacterial infection which is according to the study of Folgueira *et al.,* 1996. The yield of culture is associates the severity of immune- compromised and dissemination of tuberculosis as studied by Jones *et al.,* 1993. The published report by Bouza *et al*., 1993 and Shafer et al., 1989depict a very less positive rate, as in the present study.

The Duplex PCR used in the study successfully differentiated *M.bovis* from *M.tuberculosis*strains as in all cases a single product of 500 bp was detected in *M.bovis*, whereas a product of 185 bp was detected in case of *M.tuberculosis* with or without an additional product of 500 bp. The amplification signal is given only by *M.bovis* and *M.tuberculosis*(Shah *et al*., 2004).Isolation of *M.tuberculosis* requires 6-8 weeks on the conventional egg containing media (Ghatole*et al*., 2005).In the current study *M.bovis* was found positive in 09 suspected tuberculosis patients by Duplex PCR; 6 of them were having signs of extra pulmonary tuberculosis, and 3 were having signs of pulmonary tuberculosis.And the results shown in the present study confirmed the previous studied of Emler *et al*., 1995 and Schluger *et al.,* 1994 that shown that a prodigious improvement in sensitive diagnosis of tuberculosis from blood samples.

The sensitivity and specificity of Duplex PCR was found to be more than the conventional methods, including ZN staining, and Culture. The sensitivity of Duplex PCR was 100% while the specificity was 70.79%. Positive predictive value was 29.73% while the negative predictive value was 100% at 95% confidence interval (CI). This Duplex PCR is hence more better for the patients, for not only it is more sensitive and gives earlier result, but also it is better diagnostic test for patients of extra pulmonary TB, where other test options include biopsy from site of lesion or other invasive or radiological methods, which is more painful for the patients and is also less sensitive approach for diagnosis.

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