The Diagnostic Value of Polymerase Chain Reaction (PCR) in Bronchioalveolar Lavage

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Objectives: **In this study we investigated the diagnostic value of polymerase chain reaction (PCR) which is fast and highly specific in terms of isolating *the Mycobacterium tuberculosis* (*M. tuberculosis)* from the bronchoalveolar lavage obtained by bronchoscopy when tuberculosis is suspected in the patients with negative sputum smears or when the sputum can not be collected.**

Method: **Our cases consist of patients who can not expectorate or have three respective negative sputum samples taken at the morning after starvation. Fiberoptic broncoscopy was applied to all patients-under local anestesia-at the hospital. Lavages were taken from bronchi, which were appropriate to the lesion. Lavage samples were sent to the laboratory without delay. Results were evaluated 2 days later.**

Totally 47 cases from 3rd Thoracic Diseases Clinic of Yedikule Thoracic Diseases and Thoracic Surgery Education and Research Hospital were included in this study. Thirty-five of the cases had a higher possibility for tuberculosis, and the remaining 12 cases had no evidence for tuberculosis (control group). In these cases *M. tuberculosis complex* DNA specific IS6110 field has been investigated by PCR and the results were compared with the microbiological culture, lavage AFB.

Results: **Twenty-one of 35 patients in the study group had positive lavage PCR results. Fourteen patients had negative lavage PCR results. Four of 21 lavage PCR-positive patients were found to be ARB positive by lavage. Nine of 21 lavage PCR positive patients were found to be ARB positive by culture. We had no false positive results. All control cases were negative in terms of AFB smear, culture, and lavage PCR.**

Lavage PCR sensitivity and specificity calculations were compared with ARB culture and lavage results. Considering the culture positivity as gold standard, the sensitivity and specificity of PCR were 60% and 100% respectively.

Conclusion: **PCR can be used as a supportive diagnostic test providing rapid and accurate results, if tuberculosis is suspected clinically and radiologicaly in patients with three consecutive AFB smear-negative sputum specimens or when the sputum can not be collected.**

Key words*: Mycobacterium tuberculosis, polymerase chain reaction, bronchioalveoar lavage*

Every year, 17 million active new cases occur and 3 million people die due to tuberculosis worldwide. The report of World Health Organization (1995) estimates that 30 million people will probably die in 10 years and 300 million people will be infected by *M. tuberculosis* (1). In many developed countries, tuberculosis problem acquired new dimensions by the increase of AIDS cases. The important factors affecting the tuberculosis epidemiology are preventive measures, successful chemotherapy, education, social aids and early diagnosis of patients with tuberculosis (2). Early recognition of cases and beginning the antituberculosis treatment quickly are essential in tuberculosis control (3).

In diagnosis of pulmonary tuberculosis, isolation of alcohol-acid resistant bacilli is essential. Direct microscopic examination is the leading bacteriologic examination because it is easily done, has a low cost and gives the results quickly. But it has a low sensitivity and there must be at least 5000-10000 bacilli /ml in order to be able to see acid fast bacilli. Sensitivity of the test changes between 50% and 80% according to characteristics of the patient population examined (presence of cavity, HIV, etc.). The specificity of the test is above 99% (4).

There must be at least 1 million bacilli in the clinical samples for establishing *M. tuberculosis* with nucleic acid probes. It is very dificult to have clinical samples with that amount of bacilli so today nucleic acid probes are not used in order to find out the presence of mycobacterium in the samples. But the nucleic acids of a few mycobacterium can be amplified by nucleic acid amplification methods like PCR and then they can be distinguished by specific nucleic probes. Today studies are continuing about feasibility of PCR examination of clinical specimens (sputum, bronchioalveolar lavage, pleural fluid, blood, CSF) for presence and typing mycobacterium and the results are optimistic. In these studies, a specific DNA sequence (IS6110, hsp 65 gene or 16S rRNA gene) of *M. tuberculosis* complex bacilli is frequently used (5).

The specific IS6110 field is one of the most frequently detected common DNA fragments of *M. tuberculosis complex*. Therefore, we have searched for the fragmentation of IS6110 fielf of *M. tuberculosis complex* with the PCR technique (6).

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We assessed the diagnostic yield of polymerase chain reaction (PCR) which is one of the nucleic acid-based amplification tests used in the diagnosis of pulmonary tuberculosis and which is evaluated by amplification of DNA sequences specific to *M. tuberculosis.*

Material and Method

As the tuberculosis incidence increases in world, easy applicable, cheap, rapid laboratory methods with high specificity and sensitivity became an important necessity in daily practice. We studied with PCR method, a DNA amplification method, which can detect even one mycobacterium genome in clinical specimen to show the presence of *M. tuberculosis* in the lavage fluids obtained with fiberoptic bronchoscopy. Totally 47 cases from 3rd Thoracic Diseases Clinic of Yedikule Thoracic Diseases and Thoracic Surgery Education and Research Hospital were included in this study. These data were processed between April 1999 and October 2001. Thirty-five of the cases had a higher possibility for tuberculosis, and the remaining 12 cases had no evidence for tuberculosis (control group). The ages of the cases in our study were ranging from 14 years to 67 years (average age; 39.38±14.97 years). Thirty nine (82.98%) of them were male and 8 (17.02%) were female (Table I).

Table I. Sex predilection for control and tuberculosis groups

	Control Group		Tuberculosis group	
	n	%	n	%
Male	10	83.3	29	82.9
Female	2	16.7	6	17.1

χ^2=- , *p=0,970*

The cases were divided into 2 groups.

Group I: 35 of the cases who had a higher possibility for tuberculosis were included in this group.It was not possible to obtain sputum from them or the sputum specimens were AFB(-). They were clinically thought to have tuberculosis as the conventional radiographies and computed tomographies were showing cavitary, exudative, acinar, milliary, reticular lesions which were thought to be in favor of activity. The cultures resulted as either positive or negative. They showed definite radiological and clinical regression with test antituberculosis treatments and samples were taken before starting the treatment.

Group II: 12 cases without tuberculosis were included in this group. (Control group). It was not possible to obtain sputum from them or their three consecutive AFB smears were negative. They were clinically thought not to have tuberculosis. There were sequelae, fibrotic lesions on conventional radiographies and thoracic CTs. They had definite radiological and clinical regression after a nonspecific antibiotic treatment or no response to test antituberculous treatment. Samples were taken from these cases. In these cases, diagnosis were verified by cytopathologic examination of samples and after a period of one year follow-up.

All of the subjects were taken medical history, and physical examination, PA and lateral chest x-ray, thoracic CTs, hemogram, erythrocyte sedimantation rate (ESR), routine biochemical tests, and urine analysis were also performed.

We used PCR to amplify the field os IS6110 with the hybridisation technique. PCR study was done in three steps:

1- DNA isolation

-NaOH as well as material within the Eppendorf tubes at the rate of %2 was mixed in mixer adding N-Acetyl-L-Cystein.

-After 15 minutes, 80 mcl 2 M (pH 7.4) TRIS add per 2% NaOH 100 mcl and mixed.

-The mixture was centrifuged at 12000 G for 15 minutes.

-The top part of it was thrown away and the sediment at the bottom was added 200 mcl distilled apyrogenic water and stirred.

-200 mcl chloroform was added and stirred.

-Kept in the sterilizator at 80°C for 15 minutes.

-This material was centrifuged at 12000 G for 1.5 minute and upper layer was saved for PCR.

2-Reproduction of DNA

Taq DNA polymerase , dATP, dTTP, dGTP, dCTP, reaction buffer , the primers that codify the field of IS6110 of *M. tuberculosis* complex DNA and water were loaded in a tube and 5mlt of phase which was saved in DNA isolation was added to this.The material prepared for PCR was put into the thermocycler (Minicycler MJ. Research Inc.USA) programed for 40 cycles. DNA was reproduced through the steps of denaturation in thermocycler, connection and longevity.

3-To show the reproduction

-Sterilizer was set to 68 °C, mixture of prehydridization (N-loril-sarkosil, 10% sodium dedoksil sulphate, blocking reagent, 20 X SSC, distilled water) and probe (marked as digoxigein) was taken from deep- freezer.

-Membrane was prepared, loaded (2ml) and kept, under UV for 3 minutes.

-Prehibridization mixture was kept at 68 °C for 15 minutes and quickly frozen in deep-freezer.

-Probe was kept at 37 °C for 2.5 hours.

-Buffer II (5 ml blocking reagent, 45 ml buffer I) was taken out of the freezer and let to melt. Sterilizator was set to 68°C.

-At the room temperature it was shaken twice for twenty minutes in the solution of 2 X SSC (sodium chloride + sodium citrate) + 0.1 % SDS.

-It was kept at room temperature in the solution of 0.2 x SSC + 0.1 SDS twice for 30 minutes.

-Buffer I (maleic acid, sodium chloride, distilled water, sodium hydroxide, pH 7.4) was shaken at the room temperature for a minute.

-Buffer II was also shaken at the room temperature for 30 minutes.

-Buffer II (15 ml) + Dapcon (3 ml) was also shaken at the room heat for 30 minutes.

-Buffer I was also shaken at the room temperature twice for 15 minutes.

-Buffer III (1M pH 9.5 tries, sodium chloride, 1M magnesium chloride and distilled water) was also shaken at the room temperature for 2 minutes.

-We waited until seeing the color changed dark in the mixture of buffer III(10ml) + NBT (45mcl) + X-phosphate (35 mcl) at 37 °C.

-Buffer I was also shaken for 5 minutes.

-The buffer (2M (pH 7.4) TRIS, 0.5 MpH 8.0 EDTA, distilled water) was also kept in the refrigerator.

The results were analysed comparing the positive and the negative controls on the membrane.

Results

28 (71.4%) of the 35 patients in the study group were cases with three acid-fast bacilli (AFB) smear-negative sputum specimens at baseline and for 7 (28.6%) of the cases it was not possible to obtain sputum. In the control group, of the 12 cases, 9 (75%) were cases with three AFB smear-negative sputum specimens and in 3 (25%) cases it was not possible to collect sputum. In the control group no positive results were obtained with PCR for bronchoalveolar lavage fluids. Bronchoalveolar lavage of 21 (60%) of the 35 cases were PCR (+) and bronchoalveolar lavage of 14 (40%) cases were PCR (-) (Table II, III).

When the bronchoalveolar lavage of control and study groups were compared for AFB, 29 (82.9%) of 35 cases were AFB (-), 6 (17.1%) cases were AFB (+). When all the cases were taken into consideration, the sensitivity, specificity and total diagnostic values were calculated as 17.1%, 100% and 38.3%, respectively. In the study group, the sensitivity of lavage AFB was 13.3% and specificity was 100 (Table IV).

Table II. The PCR results in the groups

	PCR positive	PCR negative	Total
Group 1	1	14	35
Group 2	(-)	2	12
Total	21	26	47

There was no significant differences between groups. $p>0.05$

Sensitivity: 17.1%

Specificity: 100.0%

Positive predictive value: 100.0%

Negative predictive value: 29.3%

Total diagnostic value: 38.3%

In the study group cultures were positive for 12 (34.2%) of the 35 cases and negative for the 23 (65.8%) cases. In the control group there was no positive culture result. When all cases were taken into consideration the sensitivity of culture was 34.4% and specificity was 100% (Table VI).

There was a significant difference between two groups. $p<0.05$

When in all cases the results of lavage PCR was evaluated, sensitivity of PCR was 60%, specificity was 100%, positive predictive value was 100%, negative predictive value was 46.3% and total diagnostic value was 70.2 according to diagnostic value PCR was found to be superior to lavage AFB and culture (Table VI), (Fig. 1).

There was a significant difference between two groups. $p<0.05$

Sensitivity: 60.0%

Specificity: 100.0%

Positive predictive value: 100.0%

Negatif predictive value: 46.2%

Total diagnostic value: 70.2%

Twenty-one of 35 patients in the study group had positive lavage PCR results. Fourteen patients had negative lavage PCR results. Four of 21 lavage PCR-positive patients were found to be ARB positive by lavage. Nine of 21 lavage PCR positive patients were found to be ARB positive by culture. We had no false positive result.All control cases were negative in terms of AFB smear, culture, and lavage PCR.

Lavage PCR sensitivity and specificity calculations were compared with ARB culture and lavage results.Considering the culture positivity as gold standard, the sensitivity and specificity of PCR were 60% and 100% respectively.

Discussion

In pulmonary tuberculosis, sputum examination can not always show the agent and it is not always possible to grow it in a culture. In our study, early cultures (after approximately 45 days) were positive only in 12 (34.2%) of the 35 pulmonary tuberculosis cases. The most important advantage of the invasive methods is that they provide quick and exact results so in order to reach a quick result and exact diagnosis, we studied with PCR method, a DNA amplification method, which can show even one mycobacterium genome in clinical specimen to show the

Table III. PCR results in lavage and culture specimens.

Group 1	PCR positive	PCR negative	Total	Group 1	PCR positive	PCR negative	Total
Lavage positive	4	2	6	Culture positive	9	3	12
Lavage negative	17	12	29	Culture negative	12	11	23
Lavage negative	17	12	29	Total	21	14	35

Table IV. Comparison of AFB lavage findings between groups.

AFB lavage	Control Group		Tuberculosis group	
	n	%	n	%
Negative	12	100.0	29	82.9
Positive	-	-	6	17.1

χ^2=- , *p=0.315*

Tablo V. Comparison of culture results between groups.

Culture	Control Group		Tuberculosis Group	
	n	%	n	%
Negative	12	100.0	23	65.7
Positive	-	-	12	34.3

χ^2=-, *p=0.021*

Tablo VI. PCR results of groups

Lavage PCR	Control Group		Tuberculosis group	
	n	%	n	%
Negative	12	100.0	14	40.0
Positive	-	-	21	60.0

χ^2=*13,01, p=0.0001*

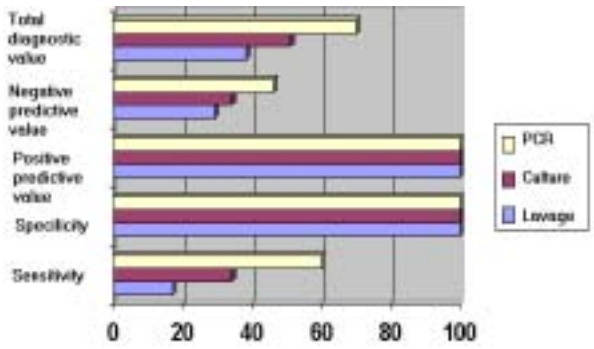

Figure 1. Comparison of results.

presence of *M. tuberculosis* in the lavage fluids obtained by fiberoptic bronchoscopy (7). In a trial with 31 cases (1996) it was established that the diagnostic contribution of microbiologic examination of bronchioalveloar lavage alone is 28% (8). Danek and Bower (9) in a study with 41 cases examined the bronchial lavage, brush and postbronchoscopic sputums and cultures. They got only 24% positive results for bronchial lavage and 63% positive results for culture. Kaya carried-out a study on 55 pulmonary cases in Heybeliada Sanatorium Thoracic Diseases and Thoracic Surgery Center (1999) and found that the contribution of lavage to diagnosis was 18% (10). In our study while the diagnostic contribution of bacteriologic examination of lavage taken by fiberoptic bronchoscopy was 17.1%, this rate was 34.3% with the culture. Our results which are similar to the other studies, show that the help of only lavage fluid obtained by bronchoscopy is very low and waiting for the result of culture takes long time so different methods are necessary. PCR is more sensitive than direct examination and faster than the culture. The sensitivity of the method is beneficial especially in extrapulmonary tuberculosis (tuberculous pleurisy, tuberculous meningitis) containing few mycobacterium genome (11,12). Witde et al. (13) found the sensitivity of PCR as 81% and specificity of culture as 52.8%. In a study with 15 tuberculous pleurisy cases, Lance et al.(14) found that 9 (60%) of the cases were PCR positive . Babu et al. (15) divided 60 cases into two groups, 14 of 20 cases with tuberculous pleural effusions were PCR positive and none of the 40 cases was PCR positive in the control group and found the specificity and sensitivity as 70% and 100%, respectively. They concluded that in pleural effusion diagnosis, PCR is a quickly resulting and sensitive diagnostic method (15). Takagi et al. (16) in their study on pleural biopsy samples calculated the sensitiviy and spesificity of PCR as 89% and 100% respectively. Bahadir et al. (17) investigated the diagnostic value of polymerase chain reaction for tuberculous pleurisy in Yedikule Toracic Diseases and Thoracic Surgery Education and Research Hospital, found the sensitivity of PCR as 89.5% and specificity as 52.9%. Brisson-Noel et al.(18) worked on 514 clinical samples using sputum, bronchial lavage fluid, pleural fluid and found the rate of negative culture results in positive PCR results as 12.3% (18). In

our study, 52.2% of the culture negative cases were PCR positive. Claridge et al (19) in a study with 5000 cases including sputum, bronchial lavage fluid, BAL, pleural fluid, urine sample found that 623 sample smears were positive, PCR was positive for 181 (85%) of 218 culture positive samples, PCR was positive for 62% of the cases with smear negative and culture positive samples. They found that the rate of false positive results was very low, sensitivity was 83.5%, specificity was 99% and positive predictive value was 94.2%. Eisanach et al. (20) were the first who carried-out a study with PCR technique in pulmonary tuberculosis. In their study (1991) with 162 cases, they obtained sputum samples from 94 cases with pulmonary tuberculosis, 42 cases with nontuberculousis and 26 cases with nonmycobacterial infections. Fifty-one (54,2%) of 94 cases were PCR positive, smears and cultures were positive in 42 PCR positive cases, one of them was smear negative and culture positive, 4 of them were smear positive and culture negative (20). Cartyvels et al. (21) in the study they carried out using Amplicor PCR for sputum, bronchial lavage fluid and tracheal aspiration, they found that PCR sensitivity was 68%, specificity was 97,4% and sensitivity in smear negative cases was 46% (21). PCR is a very sensitive test so it is easily contaminated and many false positive results occur (5). The reasons of false positive results are contamination during preparation, contamination of specimens with dead organisms, impairing the sterility of bronchoscope upper respiratory tract secretions, faults during transportation of the sample (22,23). In treated patients mycobacterium can be seen even after the culture became negative. They can be easily established by PCR (5). For this reason before treatment PCR is more sensitive than the smear for diagnosis of tuberculosis and after the start of antituberculous treatment it is more sensitive than the culture (24). Yuen et.al. (24) compared the PCR and culture results after a 4 week treatment, they saw that 32% of the cultures and 70% of PCR remained positive. They concluded that PCR is superior to the other conventional methods in demonstration of organisms which do not live and grow in culture after treatment (24). PCR is more sensitive than the culture and lavage smear, it saves 4-6 weeks needed by culture for the cases with negative smear and positive culture, it can also differentiate the agent early in the culture negative cases (11,23,25). Direct examination of the smear should be the first step and PCR should not be used as the first procedure. When the financial and technical conditions are sufficient, it is beneficial for quick diagnosis of the active tuberculous cases with negative smears. Even one mycobacterium genome can be identified by PCR. It is more useful for extrapulmonary tuberculous patients because of low amount of bacilli (11,25) the disadvantages of PCR are high cost, complexity, failure in discrimination between the living and non-living bacilli, need for trained personnel (26).

Conclusion

Lavage PCR is more sensitive than AFB smears and culture, reduces the period of 4-6 weeks (approximately 45 days) necessary for the culture and is able to detect the agent in culture negative cases.Using appropriate primers and probes, it was possible to detect the *M. tuberculosis* DNA within 2-3 days with a sensitivity of 100%.

PCR can be used as a supportive diagnostic test providing rapid and accurate results, if tuberculosis is suspected clinically and radiologicaly in patients with three consecutive AFB smear-negative sputum specimens or when the sputum can not be collected.

It is recommended to use PCR as a complementary test, rather than substituting the standard microbiological analyses, as a rapid diagnostic test method. PCR should not be used as the first diagnostic process and direct examination-smear should be performed at first.

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