POLYMERASE CHAIN REACTION DETECTION OF MYCOBACTERIUM TUBERCULOSIS FROM SPUTUM

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ABSTRACT

Tuberculosis has remained to be a major public health problem in Nepal. The risk of spread of infection and emergence of drug-resistant strain has created the need for a rapid, sensitive and specific diagnostic test. In addition, clinically suspicious cases that do not give positive result in conventional laboratory test need more sensitive test for diagnosis.

In order to evaluate the possibility of incorporation of Polymerase Chain Reaction (PCR) in the diagnosis of tuberculosis, we performed a comparative study of PCR to detect *Mycobacterium tuberculosis* in sputum specimens, against Ziehl-Neelsen (Z-N) stain and culture as a standard method.

A total of 103 specimens were subjected to Z-N staining, culture and PCR for detecting *Mycobacterium tuberculosis*. Of these, 19 were positive by Z-N stain, 26 by PCR and 25 by culture. Four stain negative specimens showed positive result in both culture and PCR. Two specimens of stain and culture positive were PCR negative. Five specimens showed positive result only with PCR. Two culture positive specimens gave negative results by both Z-N stain and PCR. Sensitivity, specificity, positive predictive value and negative predictive value of PCR which were 84%, 93.5%, 80.8% and 94.9% respectively.

This study showed that there is no need for PCR test for the smear positive cases. However, PCR could be a possible diagnostic tool for the confirmation of the smear negative cases that show clinical symptoms of TB.

Key Words: *Mycobacterium tuberculosis*, Z-N stain, PCR, sensitivity, specificity.

INTRODUCTION

The incidence of tuberculosis (TB) worldwide is increasing in an alarming way. In April 1993, World Health Organization (WHO) declared TB as a global emergency.¹ TB kills 2 million people each year.¹ Eight million becomes sick every year and nearly 3 million TB cases per year occur in South-east Asia.¹ The global epidemic is growing and becoming more dangerous.

The breakdown in health services, the spread of HIV/AIDS and the emergence of multi-drug resistant TB are contributing to the worsening impact of this disease.

WHO estimates that between years 2000 and 2020, nearly 1 billion people will be newly infected, 200 million people will get sick, and 35 million will die from TB – if control is not further strengthened.¹

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TB is a major public health problem in Nepal. Almost half of the over 20 million population are infected with TB. Of these, up to 90,000 people have active TB and there are 44,000 new cases of the disease every year.²

Pulmonary TB occurs when the primary infection does not heal completely and there is multiplication or reactivation of organisms in the lung due to poor health, malnutrition or defective immune responses. Tuberculous meningitis occurs when tubercle bacilli reach the meninges in the blood and miliary infection can occur if a site of primary infection ruptures through a blood vessel and bacilli are disseminated throughout the body. There is bone tuberculosis when the commonly infected site, the spine, may lead to the collapse of vertebrae.³

Confirmatory diagnosis of TB requires the identification of *M. tuberculosis* in the patients' samples. Conventional laboratory diagnosis starts with direct examination of smear for acid-fast bacillus by Ziehl-Neelsen (Z-N) stain or auramine stain, followed by culture isolation and biochemical testing of the isolated mycobacterium for the species identification.³

Microbial examination of smear of acid fast bacillus by Z-N stain is time tested and remains extremely valuable in testing sputum for *M. tuberculosis* while other specimen like bronchial lavage, blood and aspirates may also be used. The diagnostic yield of this technique is limited primarily because the number of organisms required for a positive smear is between 5,000 and 10,000 bacterial cells per ml for reliable detection. The immediate diagnosis of tuberculosis by direct sputum examination ranges from 40 – 75%. Isolation of the organism by culture and subsequent identification by biochemical test is time-consuming (takes 4-6 weeks) and has only 40-60% sensitivity.^{3,4,5}

To overcome this limitation, a procedure based on the amplification of mycobacterial DNA by use of Polymerase Chain Reaction (PCR) has been developed.⁶ The technique of PCR enables the amplification of specific sequence of target nucleic acids. It is not only simple and fast, but also very sensitive and specific to amplify even a single molecule of DNA.⁷ Even if PCR tests are expensive currently, they will help in the overall savings to the patient by providing a timely diagnosis.

PCR is based on the repetitive cycling of three simple reactions (steps), which take place within one small tube containing thermostable DNA polymerase and other reaction components. Of the 3 steps, the first is the denaturation of the native double-stranded DNA by heating at 95°C. The heat breaks the hydrogen bonds that hold the two complimentary strands of DNA together, allowing them to separate. The second step of

the cycle, annealing of primers, allows the two short DNA primers to anneal to their respective complimentary sequences on the separated strands of DNA at reduced temperature, usually between 42^o-60°C. The third step of the cycle entails the actual synthesis of the new strand of DNA at 72°C. Each primer that has annealed to the native DNA template is extended by Taq polymerase, building a complimentary sequence of DNA along each side of the separated strands of the source DNA. By repeating these three steps for 30 cycles, the target DNA can be amplified up to 2³⁰. Detection of PCR product after amplification is achieved by agarose gel electrophoresis, in the presence of ethidium bromide, and the band identified can be confirmed by Southern blot and hybridization.^{7,8}

Control of the disease should not only mean to treat the clinically proven cases of TB but more important to identify early those individuals who are in the early stages or at risk of developing the disease. An important key to successful control of TB is, thus, timely diagnosis. The timely identification of persons infected with *M. tuberculosis* and the rapid laboratory confirmation of tuberculosis are two key ingredients of effective public health measures to combat the resurgence of tuberculosis and the outbreaks of nosocomially transmitted tuberculosis and the potential for the emergence of drug resistant strains.

The study was performed to find out the effectiveness of PCR over routinely used conventional methods. The routinely used acid-fast smear, culture and radiological examination have been and still remain the methods of diagnosis of human TB in the developing country like Nepal today. As they have some disadvantages like low sensitivity & specificity, time consumption etc, the PCR was studied as a possible counterpart.

MATERIALS AND METHODS

One hundred and three sputum samples were collected from patients who were referred for Acid-Fast Bacilli (AFB) staining in sputum at Tribhuvan University Teaching Hospital from September 1999 to February 2000. Early morning sputum specimen was collected from the patient in a clean, leak-proof, dry container. The mucoid specimens were accepted for the study.

Examination of Bacilli by Z – N Staining

Smear was prepared and then stained by Z-N method³:

If any definite rod shaped AFB were seen, the smear was reported as AFB positive and given an indication of the number of bacteria present as recommended by the ALA (American Lung Association) Scale.

CULTURE OF BACILLI

Sputum specimens were inoculated on the culture media after pretreatment with 4% NaOH to homogenize and to kill the undesirable microorganisms other than the acid-fast bacilli. 0.1 ml of decontaminated sputum was inoculated into each of the two culture tubes containing 3% Ogawa medium. The tubes were examined on 7th day for rapid growers and weekly thereafter for slow growers. If any colony were seen at any stage, acid-fastness of the bacilli was determined by Z-N staining. Negative report was given when no colonies appeared after observing weekly for 8 weeks.

POLYMERASE CHAIN REACTION

Mycobacterial DNA extraction

DNA was extracted as described in DNA Extraction Kit (Cat No K-3020, Bioneer Co. Korea). The procedure involved hydrolysis and solubilization of sputum, washing and lysis. Five hundred micro-liters of sputum was taken and solubilized with hydrolysis buffer. It was vortexed and centrifuged for 10 minutes at 13,000 rpm at 4°C. The supernatant was discarded and the pellet was re-suspended with wash solution. The tube was centrifuged and washed twice. Then the supernatant was completely discarded and the pellet was re-suspended with lysis buffer. Mineral oil (liquid paraffin) was overlaid and the mixture was boiled for 20 min and centrifuged. The supernatant was subjected to PCR.

For positive extraction control, bacterial suspension of *Mycobacterium tuberculosis* was used and for negative extraction control, distilled water was used.

PCR Reaction

Five micro-liters of the DNA extract was put together with the two primers in the AccuPower™ PCR Premix (Cat No K-2014, Bioneer Co. Korea) and sterile distilled water was added to make the final volume of 20 µl. Two sets of primers for nested PCR were purchased from Bioneer Company, Korea (Cat No N-5821). For the first PCR, primers were obtained from the position 555 to 1111 of IS6110 *M. tuberculosis* sequence (KBN 5 & 6, Bioneer Co. Korea) to give 557 bps products. The first PCR product was subjected to second nested PCR using the primer obtained from position 590 to 874 of IS6110 sequence (KBN 7 & 8, Bioneer Co. Korea) to give 285 bps bands.⁹

IS6110 is used as the target sequence. It is specific for strains of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*). This transposon is generally present in high copy number (10-20) in *M. tuberculosis*. The specificity

and repetitive nature of IS6110 make it an ideal target for amplification by PCR¹⁰. The lower limit of detection reported by PCR varies between 1 and 100 bacilli.¹¹

The AccuPower™ PCR Premix contained T_{aq} DNA polymerase, each dNTPs (dATP, dCTP, dGTP, dTTP), Tris-HCl (pH 8.0), KCl, MgCl₂, stabilizer & tracking dye. The reaction was done in the thermal-cycler (Perkin Elmer-Cetus, USA) with initial 5 min denaturation, and then cycle of 1 min, each in denaturation at 94°C, annealing at 60°C, and extension at 72°C. The reactions were repeated for 30 cycles, ending in 5 min for final extension at 72°C. Since it was nested PCR, 2 µl from the first PCR product was taken and the reaction was repeated with a second set of nested primer at the same temperature and procedure.

To ensure the PCR reaction, previously extracted DNA of known positive result was used as positive PCR control, and to verify the contamination of PCR reagents with PCR products, water was used as negative PCR control.

Agarose gel electrophoresis, Visualization & Photography

Two percent agarose gel was prepared with Tris-acetate buffer (pH 8.0). The electrophoresis was performed at 50V for 1 hour. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml). The gel was observed under short-wave UV transilluminator and the image was photographed using Polaroid-type camera with Type 667 film (ISO 3000/36°). The positive result was obtained by comparing it with the positive control and the appropriate DNA size marker (Cat No D-1030, Bioneer Co. Korea).

RESULTS

In the PCR, all the positive extraction controls and positive PCR controls gave expected band (285 bps) throughout the

Fig. 1

Fig1. Polymerase Chain Reaction products obtained after amplification of DNA extract from 7 selected sputum specimen. M: Marker, +P & -P: Positive & Negative PCR control, +E & -E: Positive & Negative extraction control, Lane 1, 2, 3, 4, 14, 15, 16 - specimen serial number

experiment, thus, validating the extraction procedure and the PCR reaction condition. Also all the negative extraction controls and the negative PCR controls gave no bands, hence, excluding any possibility of contamination (Fig. 1)

Of the 103 specimens, 19 were positive by Z-N stain, 25 by culture, and 26 by PCR (Table I). All 19 Z-N stain positive specimens were also positive in culture, however, 2 of them were negative in PCR. None of stain positive specimen showed negative results in culture. However, 6 stain negative specimens were culture positive, of which four gave PCR positive result (Table II).

Two Z-N stain positive specimens did not give positive result in PCR. However, five specimens with negative result both in stain and culture gave positive in PCR (Table II).

The sensitivity, specificity, and positive and negative predictive values were calculated for each Z-N staining and PCR in comparison to culture as a standard method. For Z-N stain, the values were 76.0%, 100.0%, 100.0% & 92.8% respectively and for PCR, the values were 84.0%, 93.5%, 80.8% & 94.8% respectively (Table III).

DISCUSSION

This study evaluated the efficacy of Polymerase Chain Reaction for the detection of Mycobacterium tuberculosis on sputum specimens against Z-N stain and culture as a standard method.

A total of 103 specimens were subjected to Z-N stain, culture and PCR for detecting Mycobacterium tuberculosis. Of these, 73 cases were Z-N stain, culture and PCR negative. 19 were positive by Z-N stain, 26 by PCR and 25 by culture.

None of Z-N stain positive specimen showed negative results in culture, thus showing 100% specificity. This means that as long as the stain gives the positive result, it

is not necessary to do the further test for the purpose of diagnosis. However, due to the increase of drug resistant bacilli, culture is recommended to test for the sensitivity to anti-tuberculous drugs.

As described by many other authors,^{12,13,14} the sensitivity of Z-N stain was not as good as its specificity. In our study, six stain negative specimens were culture positive. However, considering the availability and technical easiness, the Z-N stain method could be still used as the method of choice in the first line diagnosis of tuberculosis.

Two stain and culture positive specimens did not give positive results in PCR. In addition, 2 stain negative but culture positive specimens also were PCR negative. In our study, the

Table IV : Clinical Utility of PCR in diagnosis of Tuberculosis¹⁶

sensitivity of PCR was lower (84%) as compared to that obtained by another author (94%)¹³ This may be due to the technical error during either DNA extraction step or PCR step. On the other hand, Perera et al. (1994) reported that the presence of T_{aq} polymerase inhibitor in some clinical specimen might affect the result.

Interestingly, in our study, 5 stain - and culture negative specimens gave strong positive result in PCR. Given the high prevalence of TB in Nepal, it is possible that these false positives represent non-viable organisms, latent infections or may be from the patients who were in anti-tuberculosis treatment. Another explanation is that these results may represent DNA contamination, a constant problem with PCR method. However, this is unlikely as strict procedures were employed to exclude the problem and also the negative controls or the DNA-free controls were all negative, which ruled out the possibility of contamination.

Since our study did not incorporate the clinical and radiological findings and used the culture result as the only criteria for the presence of tuberculosis disease, we cannot totally exclude the possibility of these culture negative cases as TB negative. This suggests that further study with combined clinical, microbiological and molecular biological expertise is necessary to evaluate the value of each diagnostic tool.

The findings of the study is more or less consistent with the findings of the study conducted in Spain, where there were cases which were smear negative, culture negative but PCR positive and vice versa¹⁷. However, the sensitivity of PCR in the present study was 84.0%. Similar result with PCR positive and both Z-N stain and culture negative and vice-versa were also reported in a study done in Korea. The culture sensitivity was found to be 35.3% and PCR sensitivity 94.1% in sputum from diseased cases.¹³

As the result showed, the sensitivity of PCR to be greater than that of Z-N stain, PCR is more relevant in sputum specimens than Z-N stain. Apparently, it is also applicable in specimens other than sputum like cerebrospinal fluid, pleural fluid, peritoneal fluid, etc, where the chances of detection of bacilli through Z-N stain is poor^{12,18}. The specificity of PCR was found to be low and so it should be regarded as an investigational procedure before the specificity is confirmed.

CONCLUSION

This study showed that PCR test is not necessary for the diagnosis of smear positive cases. However, PCR could be a possible tool for confirmatory diagnosis of the smear negative cases, which show clinical symptoms of TB.

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