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Comparison of Coventional Isolation, Phage-Based Assay and PCR for Detection of *Mycobacterium tuberculosis* Complex

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Abstract: In the present study, conventional bacterial isolation, bacteriophage-based assay (FAST Plaque TB) and Polymerase Chain Reaction (PCR) were evaluated for detection of *Mycobacterium tuberculosis* complex. A total of 47 mycobacterial isolates consisting of 38 isolates of *Mycobacterium tuberculosis* complex and 9 isolates of mycobacteria other than *M. tuberculosis* complex were used in this study. In addition, nine reference strains of Mycobacterium consisting of 7 *Mycobacterium tuberculosis,* one strain *of Mycobacterium flavescens*, one srtrain of m*ycobacterium duvalii* were also used in this study. Conventional isolation is laborious, cumbersome and time consuming where it takes as long as 2 months for definitive diagnosis. The phage assay is sensitive and it takes only two working days. PCR is a rapid assay and definitive diagnosis of tuberculosis infection could be made possible within the same working day. The described bacteriophage-based assay could be used as rapid, sensitiveand specific method to support the currently available conventional methods used for detection of *Mycobacterium tuberculosis* in developing countries.

Key words: Conventional isolation, *mycobacterium tuberculosis*, PCR

INTRODUCTION

Tuberculosis (TB) is the major cause of deaths in an estimated 8.5 million cases of clinical tuberculosis[1]. TB is the single leading cause of mortality of people living with Human Immunodeficiency Virus (HIV). Since 70% of those co-infected patient live in Sub-Saharan Africa, this region bears the Overwhelming burden of the global epidemics of (HIV)-associated TB[2]. Although Acid-Fast acillus (AFB) smears are simple and inexpensive test that play and important role in the initial diagnosis of tuberculosis, it is essentially to identify isolated acid-fast cultures quickly as well as accurately. The differentiation of *M. tuberculosis* from mycobacteria other than *M. tuberculosis* is most important from a clinical point of view, since tuberculosis is an infectious disease. Management and treatment of a patient with tuberculosis is different from that of a patient infected with other mycobacteria. Traditionally, a battery of conventional tests including determination of colony morphology and pigment production, growth rateand biochemical reactions have been used to identify mycobacteria to the species level[3,4]. It offers a sensitive low cost methods but it requires long time which can delay and impede treatment and control efforts. New sensitive techniques have been developed for identification of clinical isolates of mycobacteria. These assays include capillary gas chromatography (GLC) of low-molecular-weight fatty acids, high-performance liquid chromatography[5,6], the radiometric BACTEC 460TB system which offers a NAP[7] as well as nucleic acid amplification for identification of the *Mycobacterium tuberculosis* complex and nucleic acid probes for identification of *M. tuberculosis* complex[8-10] but the high cost of these methods and requirement for sophisticated equipment have resulted in restriction for application of these methods in developing countries.

Gardner and weiser isolated the first mycobacteriophage in 1947, there are now over 250 known mycobacteriophage[11,12]. David *et al.* (1979) described the lytic cycles of mycobacteriophages D29 in both *M. tuberculosis* and the rapidly growing *M.smegmatis*[13]. Mycobacteriophage-based techniques have been reported as potentially useful tools for detection of viable bacilli as well as for antimicrobial susceptibility testing[14,15]. This method is based on the ability of infected *Mycobacterium tuberculosis* to protect internalized mycobacteriophage from chemical inactivation and support their replication. Mycobacteria are mixed with the phages, which are allowed to adsorb

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and infect the viable TB bacilli. All unabsorbed extracellular phages are then inactivated using phagecidal chemical which is Ferrous Ammonium Sulphate (FAS). Following treatment with FAS the only phage remaining are those within host mycobacteria that have resulted from successful infection and replication. Detection of phage is by plating in agar containing *M. smegmatis* bacteria. As infected mycobacteria lyse they release progeny phages which will infect adjacent *M.smegmatis* bacteria which will in turn lyse eventually causing clear zones (plaques) on a lawn of *M.smegmatis*. The aim of this study is to evaluate the use of bacteriophage-based assay (FASTPlaqueTB) as a rapid method for detection of *Mycobacterium tuberculosis* complex from the culture by comparing it with PCR amplification technology.

MATEIALS AND METHODS

Mycobacterial strains and clinical specimens: Nine reference strains (*Mycobacterium flavescens*, *Mycobacterium duvalii* and 7 *Mycobacterium tuberculosis* strains as follow; H37Rv, 2 MDR strains and 4 mono-resistant strains to rifampicin, ethambutol, streptomycin and isoniazid), were provided by the Ministry of Health, National Health Laboratory, Khartoum, Sudan. Clinical specimens mostly sputum were obtained from 90 different patients admitted to AL Shaab Medical Teaching Hospital and Abu Anga Hospital at Omdurman, Khartoum state, with symptoms of pulmonary tuberculosis.

All the specimens were decontaminated by the petroff's sodium hydroxide (NaOH) methods[16]. 0.5 mL of the residual sediments were inoculated into Lowenstein Jensen (LJ) medium. After the detection of a positive culture the presence of Acid-fast Bacilli (AFB) was confirmed by Ziehl-Neelsen (ZN).

Preparation of DNA: A loopful culture of each strain was suspended in 500 µL distilled water, inactivated at 80°C for 10 min and pelleted by centrifugation. The pellet was resuspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), mixed by vortexing for 2 min with glass beads. Two il of the supernatant was used as a template DNA in the PCR amplification[17].

Amplification of insertion sequence IS 6110 by PCR: Amplification of the insertion sequence IS6110[18]: was performed with a pair of primer (P1 and P2). P1 included forward sequence sequence 5: CCT GCG AGC GTA GGC GTC GG. P2 included backward sequence 5: CTC GTC CAG CGC CGC TTC GG. Two µL of Template DNA and

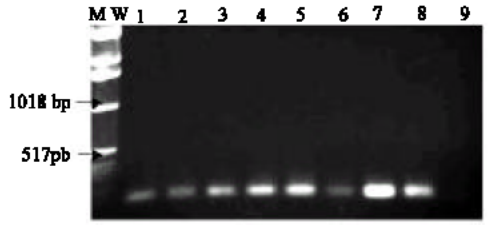

Fig. 1: Agarose gel electrophoresis visualizing the 123 bp PCR product from *Mycobacterium tuberculosis* complex Lane MW; 1 Kb ladder; Lane 1: positive control; Lane 2-8: clinical isolates of Mycobacterium tuberculosis complex; Lane 9: negative control

1.0 µL of each primer (25 µM) were added to PCR mixture (Amersham, UK), which contained 1 unit of Taq DNA polymerase, 2.0 µL dNTP (10 mm) and 2.5 µL PCR buffer, the volume was adjusted to 25 with distilled water. Theamplification was done with a 25 µL reaction mixture using a personal thermal cycler (Roche Diagnostic system USA). PCR cycling profile were conducted with an initial 5 min denaturation step at 95°C coupled to a repeating cycles of I-minute at 95°C, 1 minute at 65°C and 2 min at 72°C for 35 cycles, followed by a 10 min final extension step at 72°C. Amplified DNA was electrophoresed onto a 1.8% agarose gel. The 123 bp product was stained with ethidium bromide and visualized under UV light transillumination (Fig. 1) The molecular size of the product was estimated by comparing the migration distance with those of the Standard 1 KB DNA ladder.

Phage assay procedure: The *FASTPlaque*TB reagents were prepared according to the manufacturer's instructions (Biotec Laboratories Limited, UK).

Sample preparation: Half a loopful of a mycobacterial culture was transferred from growth on LJ slopes to a 10-mL plastic container containing 6 glass beads in 5 mL FPTB Medium Plus. Organisms were vortexed for 20 sec. The homogenate was allowed to stand for 15 to 20 min to allow larger clumps to settle. The supernatant was used as a test suspension.

Phage assay: Half ml of the test suspension was transferred aseptically to reaction vessels then mixed gently and incubated for 24 h at 37°C. Hundred µL of Actiphage solution was added to the pre-incubated specimen, mixed gently and incubated at 37°C for 1 h. Following incubation, 100 µL of Virusol (FAS) was added to each sample. The content of the tube was mixed by inverting and rolling of the reaction vial to ensure that the

Virusol came into contact with the entire inner surface of the vessels to aid efficient exogenous phage inactivation. The samples were allowed to stand at room temperaturefor 5 minutes. Five mL of FPTB Medium Plus was added to each sample and mixed by inverting the vial once to neutralize the Virusol activity, then one mL of Sensor cell (*M. smegmatis)* was added to each sample. The molten agar was removed from the water bath and 5 mL was aseptically poured to a sterile petri dish. The entire contents of the reaction vial were immediately poured into the petri plate and mixed by swirling in both directions. Once the plate has set (approximately 30 min at 20-25°C), the plate was inverted and incubated at 37°C overnight.

The test is considered positive if 100 plaques or greater are present. Plates exhibiting complete lysis are also considered positive. The test is considered negative if no plaques or less than 100 plaques are present.

Positive and negative assay controls were tested with each batch according to the manufacturer's instructions.

Statistical analysis: The sensitivity and specificity for phage assay were calculated by comparing with the proportion method. The following formulae were used for calculations. Sensitivity was true resistance/(true resistance+false negatives)×100; specificity was true susceptibility/(true susceptibility +false positives)×100.

RESULTS

From the collected 90 sputum samples, 51 showed visible colonies on L J medium incubated at 37 °C for up to 8 weeks. Out of these, 4 specimens developed contamination on LJ medium and were discarded and the other 47 isolates were confirmed as acid-fast bacilli by ZN staining.

The growth rate of the isolates ranged between 3 days to 5 weeks. Most of the isolate showed visible growth after 2 weeks. 9 out of the 51 isolates Showed visible colonies in 3 days and were identified as rapid grower, the growth rate of the other 38 isolates ranged between 2 weeks to 5 weeks and were identified as slow grower.

Forty seven mycobacterial isolates were subjected to conventional PCR and the results were as fellow 38 were identified as *M. tuberculosis* complex and 9 Mycobacterium Other Than Tuberculosis (MOTT). Forty seven mycobacterial isolates and the nine reference strains were used to compare the performance of the bacteriophage based assay (FASTPlaqueTB) with the PCR methods and the results were as fellow; All the 38 isolates which were identified as *M. tuberculosis* complex by the PCR and the seven reference *M. tuberculosis* strains were positive with phage assay. The nine MOTT isolates, *Mycobacterium flavescens* and *Mycobacterium duvalii* were negative with the phage assay. The overall sensitivity and specificity of phage assay compared to PCR was 100%. The detection time for phage assay was two working days compared to one working day for PCR.

DISCUSSION

Rapid and accurate diagnosis allows proper management of a disease. The ability of the current methods to rapidly detect *Mycobacterium tuberculosis* complex are either time consuming or costly. Therefore, a rapid, reliable, simple and cost effective method would be highly desirable, especially in developing countries where prevalence of tuberculosis is high. The fastplaque TB test which the phage amplification technology forms its basis, have been reported as potentially useful tools for the diagnosis of tuberculosis by many researchers[19-23]. High specificity was reported even in the presence of a high number of non-tuberculous mycobacterial isolates[24]. Furthermore, they also reported good performance of the *FASTPlaque*TB in detecting *M. tuberculosis* in body fluids and aspirates with a sensitivity and specificity of 93.5 and 98.4% respectively[25]. This study evaluated the *FASTPlaque*TB assay as a diagnostic method by comparing it to PCR methods. The detection time for PCR was consistently one working day and for the phage assay was two working days. Molecular methods for detection of TB are proving rapid and sensitive, but the high cost of these methods and requirement for sophisticated equipment currently renders them inappropriate for routine use in developing countries with a high burden of disease. Phage amplification technology requires no special equipment and the results can be read visually. The overall sensitivity and specificity of phage assay compared to PCR was 100%. The phage assay is a simple technique, which does not require any expensive instrumentation and can be used in most of the routine mycobacteriology laboratories. An additional advantage is the safety during the assay procedure, as large percentage of the bacilli are rendered non infective by mycobacteriophages. This is in contrast to culture techniques where a substantial increase in the number of infective particles is observed.

CONCLUSION

This method is easy to perform and presents a low-cost, rapid means of detection of *Mycobacterium tuberculosis* complex cultured on solid media and could provide an alternative to currently available detection methods. In addition, the limited capital outlay and

training required make this an assay suitable for use in developing countries, such like Sudan.

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