CHOICE OF BLOOD AGAR AS A SUITABLE MEDIA FOR *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT: *Mycobacterium tuberculosis remains the most infectious pathogen worldwide causing on average 2 million deaths annually. A comparative study was carried out to check the growth rate and colony morphology of Mycobacterium tuberculosis (MTB) between Lowenstein Jensen (LJ) medium and blood agar in slants. 100 sputum smear positive samples were collected and processed according to Modified Petroff method. These were than inoculated in parallel on Lowenstein Jensen medium and blood agar slants and incubated at 37^{0}C. Growth was checked on daily basis. 99% isolates recovered on LJ medium slants while 84% on blood agar slants. Growth of MTB on Blood agar slants appeared on 19th day as compared to 21st day on LJ medium slants. DNA was isolated from all recovered strains, 123bp region of IS6110 was amplified, and sequenced. BLAST results of sequenced strains showed 100% homology with MTB strains. Blood agar also appeared as suitable medium for culturing of MTB as it has recovered a significant number of MTB cultures. This study indicates that in resource limited setting blood agar can also be used for the early detection and isolation of MTB.*

Key words: *Mycobacterium tuberculosis*, blood agar, LJ medium

INTRODUCTION

Tuberculosis is still one of the main public health problems in most developing countries. It remains the most relevant infectious disease worldwide, and its etiological agent, *Mycobacterium tuberculosis* (MTB), infects one-third of the world population [1]. Global estimation indicates that approximately 9 million new cases of active tuberculosis are notified and 2 million people die of tuberculosis every year [2]. Incidences are arising in most parts of the world, especially in developing countries where the epidemic of human immunodeficiency virus (HIV) has had the effect of increasing the number of tuberculosis cases [3]. Pakistan is among the 22 high burden TB countries and it is the 4th among MDR TB countries [2]. The prevalence and continual rise of drug-resistant TB (MDR-TB & XDR-TB) stands to derail the progress made in TB control over the last decade. Early identification of drug-resistant *Mycobacterium tuberculosis* is the first and most crucial step in the fight against tuberculosis. The existence of an available and cheap method for susceptibility testing may help to prevent and reduce dissemination of MDR tuberculosis and may increase the efficacy of tuberculosis control programs as well. Diagnosis of MDR TB by conventional methods is difficult because of the low growth rate of the causative agent [4]. Therefore, rapid and efficient methods are needed for the control of this disease (3–6, 13). Although the molecular advancements in the field of diagnosis of MTB has greatly enhance the TB Control strategies but those are costly and resource limited countries cannot adopt those immediately. So they are far from the approach of poor people due to TB disease burden [5].

In the last decade, the Bactec MGIT 960 automated system was introduced for early diagnosis and DST of *Mycobacterium tuberculosis* from various clinical specimens. However, this system is costly, its consumables are not easily available, and it requires technical expertise [6]. Parallel to the progress in automated systems, much work has also been published on the development of rapid but economical methods which are equally effective and comparable to automated systems, especially for resource- limited countries [7].

The aim of present study is to establish an economic and rapid method for culturing of *Mycobacterium tuberculosis* on blood agar for routine diagnostic testing in resource limited setting.

MATERIALS AND METHODS

Sample Collection

100 Sputum samples received from different cities of Punjab in the month of May-June 2015 at provincial TB reference Lab, Institute of public health Lahore were selected for this study. Selected samples were sputum smear positive by Zeihl Neelson staining method which was performed according to cappuccino and Sherman [8].

Culture Inoculation

Samples were processed according Modified Petroff Method [9]. Slants of LJ media and Blood agar media were prepared and 100µl of each sample was inoculated twice on each media and incubated at 37^{0}C for 8weeks. Growth was observed and recorded on daily basis and growth rate was calculated.

DNA Extraction

After appearance of growth on both media, colonies were collected and subjected to DNA extraction by Phenol-Chloroform method [10]. Briefly, low T.E buffer was added in heat killed MTB strains along with 3-4 glass beads and lysozyme, vortexed and incubated for 1 hour at 37^{0}C. Then again incubated at 65^{0}C for 15 minutes after adding 10% SDS and proteinase k solution. Centrifuged by adding phenol-chloroform solution, and transferred the supernatant to a new tube, isopropanol was added and placed at -20^{0}C for 30 minutes. 70% ethanol was then used to remove the impurities and DNA pellet was dissolved in T.E buffer. Isolated DNA was confirmed on 0.9% agarose gel.

Confirmation by Amplification of IS6110

For the purpose of confirmation of growth that appeared on LJ media and blood agar media, isolated DNA were

amplified using MTB specific primers for the 123bp region of IS6110. Primers used were IS-F 5'CCTGCGAGCGTAGGCGTCGG3' and IS-R 5'CTCGTCCAGCGCCGCTTCGG 3' and amplification was done as follows: denaturation: 95^0C for 1 minute, annealing: 68^0C for 1 minute, extension: 72^0C for 1 minute [11].

Product Purification

In order to remove primer dimers and any non-specific bands, PCR products of IS6110 were then purified and confirmed on 1.5% agarose gel.

Sequencing

PCR amplified DNA were then sent to 1st Base Asia Laboratories, Singapore for Sequencing. Sequences were obtained and they showed 100% homology with strains of MTB.

RESULTS

Demographics

In this study, samples of 53% male and 47% female patients were collected. Mean age of patients was 33 years. Sputum smear positive samples confirmed by ZN staining method were collected from 5 major cities of Punjab including Lahore, Multan, Sargodha, Sialkot, and Bahawalpur. Most of the patients (62%) were from Lahore (Figure: 1).

CULTURE RESULTS

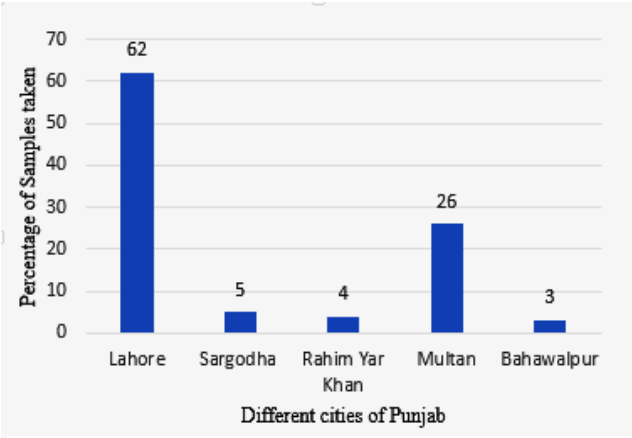

Figure 1: Graph showing percentage of samples taken from 5 big cities of Punjab.

Colonies appeared early on blood agar as compared to LJ media. On average, growth were observed on blood agar at 19th day of incubation and on LJ media at 21st day of incubation. On blood agar, MTB colonies appeared as small, non-pigmented, rough and creamy white coloured while on LJ media, colonies were rough, buff and tough (Figure: 2). Rate of positive growth on LJ media and blood agar were as 100%, and 84% respectively. With the increased incubation period, number and sizes of colonies appearing on both media, were increased. 10% samples were contaminated on blood agar as compared to LJ media on which there was only 1% contamination rate was seen. 6 samples failed to grow on Blood agar slants. Number of colonies were also less on blood agar slants as compared to LJ media slants.

Figure 2: MTB colonies on A) Blood agar and B) LJ media.

DNA Isolation

DNA from all positive samples were successfully isolated manually by phenol-chloroform method. H37Rv strain was used as positive control. Extracted DNA were confirmed on 0.9% agarose gel electrophoresis.

PCR of IS6110

In order to confirm that all the colonies appeared on LJ media and blood agar were of *M.tuberculosis*, amplification of MTB specific marker element IS6110 was carried out. A product of 123bp was obtained from all samples.

Size of product was confirmed by using 100bp molecular marker (Figure: 3).

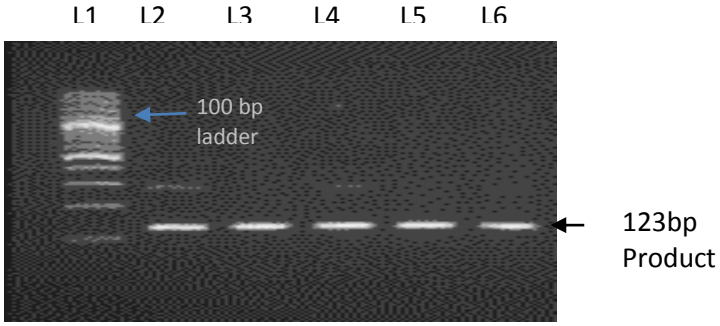

Figure *3*: Gel electrophoresis results of PCR of IS6110. L1=100bp molecular marker, L2-L6= PCR product

Product Purification & Sequencing

PCR products were purified by using Gene Get purification kit and sent for sequencing. Sequences were obtained and their BLAST results showed 100% homology with MTB strains.

DISCUSSION

Mycobacterium tuberculosis remains the most deadly pathogen across the world. Pakistan ranks 5th among high burden TB countries and 4th among high burden drug resistant TB countries. Due to developing country with resource limited setting, culturing and incubation of

Mycobacterium tuberculosis remains the big dilemma. Despite of introduction of Molecular assays which have fastens the diagnostic speed, still in resource limited setting, diagnosis of Mycobacterium tuberculosis relies on conventional methods of culturing. Blood agar is the most suitable media for most of the pathogens but it proves less useful for the most infectious pathogen which is MTB. Our results seems to be little bit contradictory to many other studies that showed blood agar as more efficient for culturing MTB as compared to LJ media. In our study, number of strains recovered, were greater on LJ media as compared to blood agar similarly more number of colonies appeared on LJ media as compared to blood agar but the difference is not statistically significant. A study from France reported opposite results, they obtained higher number of colonies on blood agar as compared to LJ media [12]. Contamination rate was higher on blood agar as compared to LJ media. Time taken for the appearance of macroscopic colonies of MTB was greater for LJ media than the blood agar but the difference is not statistically significant and this is in accordance with the study of Drancourt *et.al.,* 2003 from France [12]. There was no statistically significant difference for the specimens exhibiting different no. of AFB per field on blood agar. Robert Koch was the first to isolate M.tuberculosis, he used heat coagulated serum media and blood agar for the isolation of MTB. But unfortunately, as the time passed, we forgot Robert Koch's original media and egg-based media superceded the blood agar which became as gold standard for the culturing of MTB. With time, the use of blood agar as a primary medium for the culturing of MTB was forgotten. Although LJ media is more suitable and efficient in supporting the growth of MTB, but blood agar can also be used in some laboratories that routinely used blood agar for the isolation of other micro-organisms.

A limitation of this study is that we used blood agar and it is it is not a selective medium, so, it allows the growth of contaminants also. It can be made more effective, by the addition of suitable antibiotics for the inhibition of other non-specific bacteria.

CONCLUSION

MTB colonies usually took 4-6 weeks before the appearance of visible growth on egg-based media, so, the main step in culturing MTB is to develop a method which will be safe, fast and inexpensive for the early detection of MTB in resource limited countries. So, it was concluded from this study that Blood agar slants can be used as a good substitute for the primary culturing of MTB in resource limited countries.

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