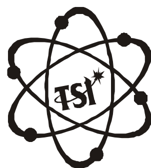
ISSN : 0974 - 7435 *Volume 6 Issue 4,5*



Trade Science Inc.

BioTechnology

An Indian Journal

Full Paper

BTAIJ, 6(4,5), 2012 [103-110]

Rapid and safe diagnosis of *Mycobacterium tuberculosis* directly from sputum and biological fluids specimens using a low cost molecular biology dedicated system

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Received: 15th April, 2012 ; Accepted: 15th May, 2012

ABSTRACT

Objective: Early and rapid detection of the causative organism is necessary in tuberculosis. We present here an integrated and dedicated molecular biology system for tuberculosis diagnosis. **Methods:** One hundred and eighty nine (189) biological specimens from patients strongly suspected by clinical parameters of tuberculosis were studied by Ziehl-Neelsen staining, cultivation on a solid medium and by a balanced heminested fluorometric PCR system (Orange G3TB) that preserves worker safety and produces a rather pure material free of potential inhibitors. DNA amplification was carried out in a low cost, with a tuberculosis termocycler-fluorometer. The double stranded DNA produced is fluorometrically detected. The whole reaction is carried out in one single tube which is never opened after adding the processed sample, thus minimizing the risk of cross contamination with amplicons. The assay is able to detect 30 bacilli per ml of sample having a 99.8 % inter-assay coefficient of variation. **Results:** PCR were positive in 36 (18,9%) tested samples (33 of them were smear negative). In our study, it yields a preliminary overall sensitivity of 97,4 %. In addition, its overall specificity is 98.7 %. The total run time of the test is four hours with two and a half real working hours. All PCR positive samples also had a positive result by microbiological culture and clinical criteria. **Conclusions:** The results obtained showed that it could be a very useful tool to increase eficiency in detecting the tuberculosis disease in low bacillus inoculum samples. Furthermore, its low cost and friendly usage make it feasible to be used in regions with poor development. © 2012 Trade Science Inc. - INDIA

KEYWORDS

Tuberculosis;
Sputum;
Cerebral spinal fluid;
Pleural liquid;
Molecular diagnosis;
Low cost;
Real-time PCR.

INTRODUCTION

The reemergence of tuberculosis is an important public health issue and the spread of drug-resistant tuberculosis has emphasized the need for rapid diagnosis. However, the standard culture methods that are currently used are quite slow.[1] Detection of mycobacterial growth on conven-

tional Löwenstein-Jensen medium requires form 4 to 8 weeks. In addition, it requires technical skills and a high complexity bacteriology laboratory. The need for speed also is an important factor when the results of tests have a possitive impact in helping to take decisions related to infection control regarding patient isolation and therapeutic management.[2]

It is in these types of situations that molecular diagnostic methods can provide more quickly the data needed and, in many cases, it is more efficient than conventional diagnostic methods.[3,4] However, in Argentina there has been serious reluctance among some clinical microbiologists as regards the development and the way molecular techniques are carried out for TB diagnosis. A false myth is that these techniques produce high false positives, althought there is not a local interlaboratory study that validates this affirmation and there is a certain conservative attitude that prevents from going ahead with this beneficial approach.[5]

Four years ago, we decided to develop a system of TB diagnosis that fulfilled these requirements. We have designed a completed, integrated and dedicated system for the diagnosis of the TBC taking into account the real needs of Latin American small cities and towns where it is most likely to find a TB spread.[6]

MATERIALS AND METHODS

Sample processing and DNA amplification

We performed our study with an integrated system (Orange TBC G3 Tuberculosis Molecular Diagnosis System, Orange SRL, Lomas del Mirador Argentina) that included: a) a device for sample processing that simultaneously preserved the bio-security of the operator and exhibited purified nucleic acids for gene amplification (Figures 1a and 1b); b) a dedicated thermocycler equipment of low cost, with an incorporated portable fluorometer, to be used in laboratories of very low complexity or health centers of primary attention, avoiding the building and technological infrastructure of high complexity and which is easy to operate by non-specialized personnel. It used a primitive but efficient heat exchanger system and

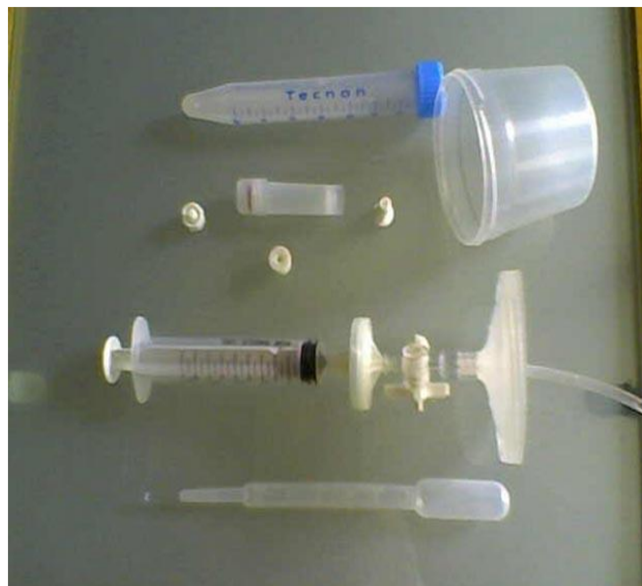

Figure 1(a) : Scheme of the complete procedure for Orange G3TB tuberculosis diagnosis.

Figure 1(b) : Scheme of the complete procedure for Orange G3TB tuberculosis diagnosis.

it had a serial communication via Internet or telephone modem that allows the Sanitary Authorities to monitor and to coordinate, in real time, epidemiological data. (Figure 2); c) a closed system of PCR balanced heminested in a single tube with a primer strategy design that significantly decreased the contamination probabilities, thus guaranteeing the precision of the results5. All reagents for amplification and fluorometric detection were included in PCR microtubes in a ready-to-use form. Time and temperature parameters for PCR reaction are inserted in a microprocessor firmware,

Figure 1(c) : Scheme of the complete procedure for Orange G3TB tuberculosis diagnosis.

Figure 2 : Scheme of DNA extraction procedure.

thus simplifying the utilization of the equipment. Five microliters of a previous sample of purified DNA were used for all the amplifications, which takes approximately two hours and a half. After amplification, PCR products are evaluated by fluorometry in the dedicated fluorometer and the data is logged in the memory and displayed on the screen. The amplified double stranded DNA is end-point fluorometrically detected in a simple but efficient low cost fluorometer designed "ad hoc". It can also be visualized with a 498 nm blue transiluminator (Figure 3). From the moment of obtaining the sample to the obtaining of the final result, there was no exhibition of biological material to the environment (see procedure scheme in Figure 4a and 4b). This implies a dramatic reduction of the rates of false positives, therefore avoiding the contamination possibilities, as well as avoiding a reduction of the false negatives when the total amount of the sample in the study is used.

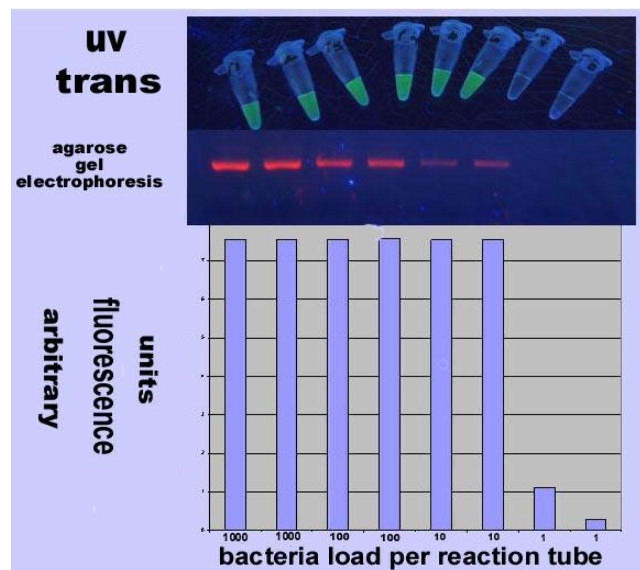

Figure 3: Low cost budget termocycler Orange G3TB for amplification of *Mycobacterium tuberculosis* genome.

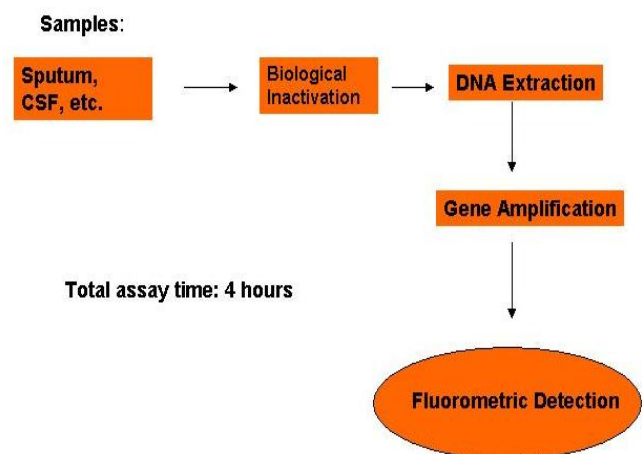

Figure 4 : Comparison of TB DNA extraction methods: Lane 1: cont neg, lanes 2,3: sample 1 and 2 extracted with the classic procedure; lanes 4,5: samples 1 and 2 processed with OrangeG3TB extraction Kit; lanes 6,7: samples 1 and 2 processed with OrangeG3TBC enhanced extraction Kit; lane 8: cont pos.

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Patients

We studied one hundred and thirty nine (139) sputum specimens (from 100 patients with symptoms suggesting clinical manifestations of pulmonary tuberculosis. One or two spot samples were obtained in the morning). In addition, 25 CSF from immunocompromised patients suspected of having meningitis tuberculosis and 25 PF from patients with symptoms of non-pulmonary tuberculosis were analyzed. All the samples were collected in the containers designed for operator safety.

Statistical analysis

Assay parameters as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and inter-assay coefficient were calculated. Positive and negative results are expressed as a proportion. In order to evaluate the differences between PCR and TB assays, the binomial Mc Nemar test was used.

RESULTS

Patients' specimens were studied by acid-fast bacillus smear direct observation (AFB), by culture and by a balanced heminested fluorometric PCR system (Orange G3TB). The target of amplification is a segment of IS6110 insertion fragment. The assay was able to detect 30 bacilli per ml of sample and it had a 99.8 % inter-assay variation coeficient. Tweenty eight sputum, 4

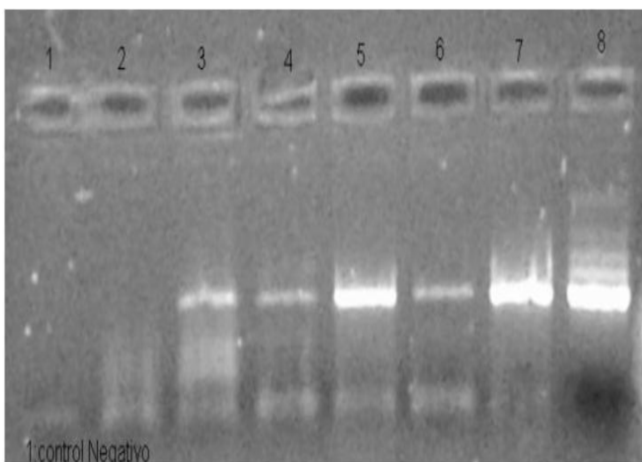

Figure 5: Direct fluorescent detection and gel electrophoresis of a curve of positive controls (c negative, 3x105, 3x103, 3x101 bacilli).

TABLE 1 : Comparison between Orange G3TB PCR and AFB direct observation patient results. Sputum, CSF and PF specimens. There is a very poor correlation between both techniques.

	AFB-Ziehl-Neelsen POS	AFB-Ziehl-Neelsen NEG	TOTALS
PCR POSITIVE	3	33	36 (18.9%)
PCR NEGATIVE	0	152	152 (80.0%)
TOTAL	3 (1.6%)	185 (97.3%)	188
Mc Nemar Test p<0.00015			

TABLE 2 : Orange G3 TBC Diagnostic System Parameters.

OVERALL SENSITIVITY	OVERALL SPECIFICITY	INTERASSAY VARIATION COEFICIENT	SENSITIVITY LIMIT (bacillus / sample)
97.4%	99.3%	99.8%	30

CSF and 5 PF specimens were positive by Orange G3TB PCR assay and only 3 of them (sputum) were positive by Ziehl Neelsen staining. Each of the 33 were negative samples by AFB smear observation and were confirmed positive by traditional culture methods as TB infected patients were detected by PCR. In order to test simplicity of our procedure samples, the processing of assays was performed by duplicates, including a team of laboratory technicians with only two weeks of training, and clinical microbiologists. The results obtained by both teams coincide 100% (one hundred percent). Samples processed by our improved extraction methodology (Orange TBC G3 biosecure procedure for tuberculosis samples followed by silica-based DNA purification) were compared with the standard TB DNA extraction method. 3 Results are shown in (Figure 5). It is clearly observed that our DNA extraction system increased significantly the sensitivity of the molecular strategy for tuberculosis detection. The assay had, in our study, an overall sensitivity, that is, a sensitivity of 97.4 %. In contrast, acid fast bacillum (AFB) direct observation had only 52.5 %. Moreover, its overall specificity is 98.7 % (TABLE 1). Their corresponding preliminary negative predictive value (NPV) was 98.9 % and 81.2 % for Orange G3TB PCR and AFB direct

observation, respectively. Positive predictive value for Orange G3TBC was 99,8 %. The total run time of the test was four hours with two hours and a half of real working time. All TB detected by PCR specimens also were positive according to microbiological culture and clinical criteria (TABLE 2).

DISCUSSION

Several new diagnostic approaches have been proposed for TB and several others will surely appear in the near future. The most important consideration to take into account for new diagnostic methods is that they should be as good as or better than the currently existing tools and, at the same time, be adequate for low-resource countries where the burden of TB is more important. For example NAA methods, especially the commercial kits that have the advantage of being well standardised and reproducible, have shown to be highly sensitive and specific in smear-positive samples; however, these values are much lower in smear-negative samples or in extrapulmonary specimens where the usefulness of these newtools would be much more desirable. [7,8]The cost is another important consideration, since at the current prices these commercial kits are still out of the reach of most TB diagnostic laboratories in low-resource countries. Other molecular procedures even call for the use of sophisticated expensive equipment and highly-skilled personnel that are available only in developed countries or in central laboratory facilities in TB endemic countries. [9] As long as these constraints are not properly addressed, expensive commercial kits making use of NAA techniques will remain restricted to developed countries or academic and research laboratories with the appropriate funding, but far away from of the TB control programmes 3. A final consideration is that any new method or approach, sophisticated or not, commercial or in-house, should be evaluated in well-designed and controlled clinical trials and tested in high-endemic, low-resource settings where the implementation and use of these methods are more needed to contribute to the improvement of tuberculosis control.[10]

New technologies for the rapid diagnosis of tuberculosis can and must be applied in developing countries where there is a high prevalence of TB. Previous TB diagnostic tests have required skilled technicians and tools and have lacked either timeliness or sensitivity. Culture tests are highly sensitive, but these tests take as long as 2–6 weeks to produce results and they demand specialized materials to support the virulent microbacteria in the culture. [7,8]While sputum smear tests are quicker, that is, they produce results in about 30 minutes, sputum smears can only detect 10–75% of TB cases and require trained microscopists. [9]In developing countries where the technical expertise and tools needed to perform these tests are limited, TB is often not diagnosed or treated early, allowing the disease to spread quickly in crowded living quarters and to build resistance to the drugs used to treat the infection. [10,11]

Nucleic acid amplification (NAA) testing is useful for rapid identification of M. tuberculosis in respiratory samples; results can be available within two and four hours. 10 In addition, this technique has a higher sensitivity than sputum acid fast bacillus (AFB) smears; it can detect as few as one Mycobacterium tuberculosis organism per 100 ml of sample.[12-14]

Several molecular techniques were used for TB diagnosis such as Gen-Probe MTD (Gen-Probe Incorporated, San Diego, CA). The enhanced MTD test (E-MTD) is an improved version of the Gen-Probe MTD that was approved in 1999 by the FDA for the diagnosis of TB in either smear-positive or smear-negative cases. A larger sample can be used, and the processing time is shorter than for the earlier MTD test, which required three hours to be completed. The Amplicor Mycobacterium tuberculosis test kit (Roche Diagnostic Systems, Inc., Branchburg, NJ) is FDA-approved for testing smear positive respiratory samples. 3 However, all the previous assays required special skills and very high building infrastructure to be carried out. It seems to be that a simplified integrated test which was described in a paper published by the New England Journal of Medicine[15] promises to help the public health sectors of low-income countries, where the occurrence of TB and multidrug-resistant pulmonary tuberculosis is high. Another similar and important test which fulfilled the disease diagnostic requirement is the one is the one that utilized loop-

mediated isothermal amplification (LAMP) techonology. It is a novel nucleic acid amplification method in which reagents react under isothermal conditions with high specificity, efficiency, and rapidity developed at Eiken.[16]

Recently, we presented the new heminested fluorometric PCR-based TB diagnostic test - called Orange G3TB-, which is fast, sensitive, and semi automated and attempts to be a real and more economic alternative to the two previous assays noted above. Our extended work with cerebral spinal fluids and pleural fluid in addition to sputum samples of the new heminested fluorometric PCR-based TB diagnostic test, showed that Orange G3TB migth be in Argentina and other southamerican countries a closer and cheaper alternative to the systems mentioned above. [17]In addition this system included a biosecure device to protect the health operator. In addition, its biosecure dispenser prevents the operator from accidental TB infection, and there is no need of a special laboratory infrastructure. An accurate diagnosis can be obtained in less than 4 hours. This novel technology increases the sensitivity and specificity for TB identification according to the results published by several authors. [18-21]Our system is semi automated, thus needing little technical training to perfom the assay. Orange G3TB also seems to have a potential use for determining infectiousness; it appears to be as specific as -and more sensitive than- serial AFB smear testing to look for TB infectivity. This was shown in our prospective study including 176 patients (a total amount of 190 samples). Two AFB smear–positive patients had also a positive Orange G3TB test. In addition, another 33 samples from 25 patients with AFB smear-negative TB were identified by Orange G3TB in sputum AFB smear-positive samples, the confirmation of AFB as M. tuberculosis within several hours can be valuable in the management of individual cases and allows the rapid mobilization of public health resources. Sputum specimens that are AFB positive and Orange G3TB positive always contain M. tuberculosis. Orange G3TB should be carried out on all AFB smear-negative respiratory specimens; a positive Orange G3TB of an AFB smear-negative respiratory sample is a diagnosis of tuberculosis.

A positive Orange G3TB result may be valuable in the early detection of approximately 88% of active tuberculosis cases that are smear-negative. It is important to note that a relatively high clinical suspicion for TB is the factor that should lead to nucleic acid amplification testing in smear-negative cases. It is certainly not appropriate to test patients with AFB negative smears in the absence of a strong clinical suspicion.

According to FDA data, the sensitivity of the tests for TB (compared with traditional culture) is approximately of 95% in patients with a positive AFB smear, but only of about 50% in smear-negative cases. However, in some patients this sensitivity could be much smaller and it could also lead to an important number of false negatives. Specificity is higher than 95% in either smear-negative or smear-positive samples. When molecular methods are used together with classical epidemiology, their utility for TB control has been noted.[22-25]

In Argentina and in many other developing countries, the main way for TB diagnosis is still the Ziehl Nielsen staining direct observation. AFB smear observation is a technique that in addition to expose testing personnel to bacilli [22,26], it has a very poor sensitivity and a low negative predictive value. Due to these technique parameter values, many patients are currently misdiagnosed and the epidemic is increasing. [27]

CONCLUSIONS

ZN staining requires = 105 bacilli/ml. The yield of microscopic examination correlates well with the extent of disease, the presence of cavitation, and the quality of specimen. Hallmark of staining is Ziehl-Neelsen stained slides is easiest & quickest diagnostic test with limited sensitivity (46-78%) but specificity is virtually 100%. Detection and identification of mycobacteria directly from clinical simples by genotypic methods (PCR, LAMP, TMA / NAA, Ligase chain reaction) and/ or, phenotypic methods such FAST Plaque TB. A number of nucleic acid amplification assays (NAAs) have been employed to detect tubercle bacilli in clinical specimens for tuberculosis (TB) diagnosis. Among these, loop-mediated isothermal amplification (LAMP) is an NAA possessing superior isothermal reaction characteris-

tics. Loop-mediated isothermal amplification, it is a novel nucleic acid amplification method in which reagents react under isothermal conditions with high specificity, efficiency, and rapidity. LAMP is used for detection of M.tb complex, M.avium, and M.intracellulare directly from sputum specimens as well as for detection of culture isolates grown in a liquid medium (MGIT) or on a solid medium (Ogawa's medium). This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. Species-specific primers were designed by targeting the gyrB gene. Simple procedure, starting with the mixing of all reagents in a single tube, followed by an isothermal reaction during which the reaction mixture is held at 63°C, 60-min incubation time. Using this system, a total of 200 sputum samples from Nepalese patients were investigated. The sensitivity of MTB-LAMP in culture-positive samples was 100 % (96/96), and the specificity in culture-negative samples was 94.2 % (98/104, 95 % confidence interval 90.5–97.9 %). The positive and negative predictive values of MTB-LAMP were 94.1 and 100 %, respectively. These results indicate that this MTB-LAMP method may prove to be a powerful tool for the early diagnosis of TB. Due to its easy operation without sophisticated equipment, it will be simple enough to use in: small-scale hospitals, primary care facilities, clinical laboratories in developing countries. 16 However, the diagnosis in developing countries using LAMP is no easy in relationship with the cost. The diagnosis in endemic countries depends more on the use of labour intensive, easy to use methodology with minimum infrastructure or equipment. This method has to have these desirable features : results within 2 hours, simple training, easy interpretation, should function well in HIV +ve patients, should allow start of treatment as early as possible.

Our objective will be now to redesign this device in a way that the process is carried out following a continuous flow within a column or injects so that it can be assembled on a industrial scale and in order to continue maintaining the characteristics of operation facility and the lowest cost.

The field of Molecular Diagnostics is not only the testing and tools in order to obtain a better diagnose and to treat the disease; rather, the goal of innovative technologies and discoveries in the diagnostics field is to treat the right patient with the right therapy and the right tools. We are currently performing a validation project that will include a total amount of 1000 patients to better characterize technique parameters.

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