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PHAGE AMPLIFICATION TECHNOLOGY AND ANTI-TUBERCULOUS DRUG SUSCEPTIBILITY TESTING IN NIGERIA

Otive-Igbuzor, E. J.

Tuberculosis Unit Nigerian Institute of Medical Research,
6, Edmond Crescent, P.M.B. 2013, Yaba, Lagos, Nigeria.
Correspondence to: E. J. Otive-Igbuzor (e-mail: ejirotive@yahoo.com)

The emergence of multi-drug resistant tuberculosis (MDR-TB) defined as combined resistance to the two most effective anti-tuberculosis drugs, rifampicin and isoniazid, threatens to create a public health hazard of unprecedented proportion. The fact that MDR-TR is more difficult and expensive to cure creates the need for prompt diagnosis. Conventionally, the proportion method on Lowenstein Jensen (L J) medium is used in most developing countries as the 'gold standard' in the drug susceptibility testing of *Mycobacterium tuberculosis* (MTB) and it takes 3-4 weeks to give results from an MTB culture.

The use of phage as a diagnostic is fast gaining ground today. It involves targeting viable MTB cells from culture with a specific mycobacteriophage. After a one hour incubation, it is treated with an antivirus to destroy the phage that are not protected with the bacilli. Upon addition of cells of growing, non-pathogenic *Mycobacterium smegmatis* (sensor cells), progeny phage from the MTB cells infect the sensor cells, thus amplifying the effect of the phage. When plated in an agar medium overnight, plaques occur in the cell lawn indicating the presence of viable MTB in an original sample. A comparison is made between the number of plaques produced in a drug-free control and a sample incubated in the presence of the drug. While the presence of plaques beyond a cut-of point indicates drug resistance, the absence of plaques indicates that the drug destroyed MTB cells. Overall accuracy from several trials so far conducted is put at 97-98% compared with the 'gold standard'. With the phage amplification method, antituberculosis drug susceptibility results are obtained from MTB culture within 48 hours as opposed to the L J proportion method, which gives resulted in 3 to 4 weeks. Also, phage, as a diagnostic, is much more applicable in Nigeria laboratories than newer, rapid methods which requires specially dedicated instrumentation and are therefore very expensive. Phage amplification technology requires no special equipment and the results can be read visually.

Key words: Tuberculosis, drug susceptibility, phage, treatment, FASTPlaque-TB, rifampicin

INTRODUCTION

The World Health Organization (WHO) in 1993 declared tuberculosis (TB) a global emergency (1). Coupled with it deadly alliance with HIV/AIDS (2), TB has emerged as a public health hazard of unprecedented proportion especially for the developing world which bears 95% of its burden (3). Eight million new cases and 2-3 million deaths from TB are recorded annually (4). Nigeria is one of the 22 countries, which bears 80% of all incident cases of TB worldwide. She is also one of the 16 countries in which the progress of TB control is of greatest concern (5). Average annual case notification is put at 25,000 in the year 2000 (6), however, 26,641 cases were recorded (7). In an epidemiological study of TB in Lagos, Nigeria, a significant increase from an incident rate of 21% in 1982 to 42% in 1992 was reported (8). Also, a study by Wokoma in 1999 showed a 7.74% increase in the incidence

rate of TB in Port Harcourt, between 1993 and 1999 (9). These were hospital-based studies. It is important to note that disease surveillance in Nigeria is neither intensive nor extensive and so cannot provide exact numbers of new cases or deaths occurring from TB in a year. Due to the stigma attached to TB in the Nigerian society, several cases go unreported. Of the cases reported, up to half or more are not diagnosed and therefor not treated. Smear microscopy has a sensitivity of 30-70% depending on whether direct or processed (concentrated) smears are examined (10).

TB is a curable disease, which ravaged the globe for over two centuries after its emergence. The discovery of streptomycin in 1946 offered a respite to TB sufferers because prior to its discovery, TB patients were subjected to mutilating surgical procedures such as thoracoplasty in which large sections of the chest wall were excised in order to close cavities. TB came to be known as "captain of all men of death" as it continued to kill several famous people because there was no cure (12, 13). Several other anti-TB drugs were later discovered and they included rifampicin, pyrazinamide, ethambutol, ethionamide, thioacetazone, kanamycin, viomycin, capreomycin and cycloserine. Of these, rifampicin and pyrazinamide are bactericidal in vivo while the others are bacteriostatic (13). For the treatment of TB, the WHO has listed five essential drugs, which include isoniazid (H), rifampicin (R), pyrazinamide (Z), streptomycin (S) and ethambutol (E). These are the most effective drugs against TB and are referred to as first line drugs. The second group consists of capreomycin, cycloserine, ethionamide, kanamycin, and a host of others. They are referred to as 'second line' drugs and are only used in cases of relapse, treatment failure or chronic cases (14,15). Usually a treatment regimen is made up of two phases namely, the initial phase during which the first-line drugs are used in combination to ensure rapid smear conversion from positive to negative, and a continuation phase (15). In Nigeria, the full regimen is rifampicin (R)/isoniazid (H) (combined tablet), pyrazinamide (Z) and ethambutol (E) daily for two months followed by thiazina (isoniazid plus thiacetazone) or ethambutol daily for 6 months. This is standardized according to the patient's age and body weight as well as the type of TB (16).

DRUG RESISTANCE IN TUBERCULOSIS

The greatest threat to chemotherapy today is the

frequency and rapidity with which bacteria develop resistance to drugs (17). Several workers have demonstrated drug resistance. In a study by Fattorini and co-workers, the activity of sixteen antimicrobial agents was tested against drug resistant strains of *Mycobacterium tuberculosis* (MTB). Of the first line drugs, isoniazid was ineffective against all strains while resistance to streptomycin; rifampicin, pyrazinamide and ethambutol were 80.4%, 71.7%, 39.1% and 8.7% respectively. Amongst second line anti-TB drugs, resistance to ciprofloxacin, ofloxacin, sparfloxacin, amikacin and kanamycin was 20%. About 10% of strains were resistant to capreomycin and cycloserine and 4.3% were resistant to ethionamide (18). In another study by Karamat and co-workers, four first line anti-TB drugs were tested on 300 isolates form clinical samples at the Armed Forces Institution of Pathology, Rawalpindi. Of these, 52.66% were resistant to one drug at least. Of the resistant isolates, 26.33% were resistant to isoniazid, 24.0% to rifampicin, 28.0% to streptomycin and 23.33% to ethambutol with or without resistance to other drugs.

Besides MTB resistant to single drugs, there have been dramatic outbreaks of multidrug resistant tuberculosis (MDR-TB). It is described as a man-made disease because it is caused by improper treatment, inadequate drug supplies as well as erratic and indiscriminate use of drugs. The availability of drugs over the shelf in most developing countries including Nigeria has led to uncontrolled self-medication (20). Multi-drug resistance in TB has been described as combined resistance to rifampicin and isoniazid. Patients with MDR-TB face chronic disabilioty and death and they represent an infectious hazard for society (21). In the study by karamat and co-workers (19), multi-drug resistance was found in 41 isolates (13.66%). Studies have shown that rifampicin resistance is a good predictor of MDR-TB. This conclusion was drawn after studies in several parts of the world pointed to the fact that MTB strains resistant to rifampicin are often invariably resistant to isoniazid at least and are thus considered MDR-TB (22). Some of such studies include those conducted in Estonia, Ethiopia, India (Delhi) and Latvia in which of the rifampicin resistant strains, 100%, 100%, 95% and 96% respectively were MDR-TB (22,23).

ANTI-TUBERCULOSIS DRUG SUSCEPTIBILITY TESTING

The management of MDR-TB requires a quick diagnosis as well as rapid and accurate susceptibility results to ensure early administration of a new regimen,

usually based on a quinolone, for retreatment of the patient. This is a major problem in developing countries including Nigeria.

In Nigeria, TB culture is limited to the National Reference Laboratory, which so far does not have a good liaison with peripheral laboratories where TB diagnosis by direct smear microscopy (16) is done under the National TB Control Programme. Even when such samples are sent to the reference laboratory, it takes 3-4 weeks after the availability of culture (which itself takes 6-8 weeks) for drug susceptibility results to be available. This is because under currently available infrastructure, it is only feasible to carry out drug susceptibility testing using the conventional proportion method on Lowenstein Jensen (LJ) medium.

The proportion method, which can also be done on the agar based Middlebrook 7H10 and 7H11 media is regarded as the 'gold standard' in drug susceptibility testing of TB (24) and is recommended by the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) (22). Though susceptibility testing can be performed directly on sputum samples through this method to give results within 3-4 weeks, there is often the problem of inoculum standardization. Besides the proportion method, there exist other methods, which include the resistance ratio and absolute concentration method, which are not commonly used.

The need for prompt treatment of MDR-TB has led to a search for more rapid method of susceptibility testing. There exists today, semi-automated methods like the Bactec 460 system (Becton Dickinson) which gives results within 7-21 days, the fully automated MB/Bac T (Organon Teknika) and the Mycobacterial Growth Indicator Tube (MGIT) (Becton Dickinson) which also has manual use. While the Bactec 460 system detects growth by the evolution of radiolabeled $^{14}CO_2$, the other two use non-radioactive materials. The results obtained by testing sputum samples directly can be quite confusing and misleading because of insufficient growth of the control as well as contamination (22). Special skills and experience are required to read the results correctly. It will also be tantamount to a gross waste of reagents to perform susceptibility tests on organisms, which may turn out to be something other than MTB. It is easier and more economical to perform susceptibility tests after culture results are available. In most developing countries including Nigeria, the cost of these machines, their servicing, cost of reagents and

personnel expertise prohibit their use.

The WHO Global Tuberculosis Programme (GTB) recognizes the need for new, rapid and affordable diagnostic in developing countries to replace the slower conventional methods. The GTB is committed to the promotion of new diagnostic of proven value in TB control programmes (26).

PHAGE AMPLIFICATION TECHNOLOGY

The use of mycobacteriophage in the disagnosis of TB is fast gaining ground today. Research into the use of phage in diagnosis dates back to 1947, when Gardner and Weiser identified phages that are specific for the mycobacteria (27). In 1960, Redmond and Cater isolated phages that specifically infect *M. tuberculosis* and *M. bovis* (28). The fact that phages are specific in their infection of hosts is the backbone of this technology. In the procedure, a specific phage that infects members of the MTB complex is used to target viable MTB in a specimen. If it is a positive sample, the phages infect the MTB cells (27). Any phage not incorporated within MTB cells are destroyed on treatment with a specific virucide. To confirm the presence of phage in the MTB cells, rapid growing *Mycobacterium smegmatis,* a non-pathogenic mycobacterium, which is also susceptible to infection by the phage is added. Using the pour plate method, this mixture is inoculated into molten agar, which is allowed to solidify, and the plate is incubated overnight. The phage particles within the MTB cells replicate and lyse the cells to release progeny phage. These then infect the cells of *M. smegmatis,* thereby amplifying the presence of the phage. Since *M. smegmatis* is a rapid grower, growth is seen in the medium overnight and plaques are seen where lysis of the cells occurred. A comparison of the number of plaques observed is made between culture suspensions incubated in both the presence and absence of the antibiotic. The number of plaques visualized from a given sample is related to the number of viable tubercle bacilli in the original sample (22,29,30-33).

FASTPLAQUETB-MDRI™

The FASTPlaque TB-MDRI™ (FPTB-MDRi) is a commercially available kit (Biotec Laboratories, Ipswich, UK) for the rapid detection of MTB resistance to rifampicin, a proven indicator of MDR-TB (22,23). Results of the trials conducted so far have been very impressive with 100% sensitivity and overall accuracy of 97%-98% compared with the LJ proportion method, which is used in most developing countries including Nigeria.

PHAGE AMPLIFICATION TECHNOLOGY VERSUS CONVENTIONAL METHODS

In comparison with the LJ proportion method, phage amplification technology is rapid; giving conclusive results within 48 hours after culture is available. With the FASTPlaque TB-MDRi, further studies have indicated that it may be possible to achieve a 24-hour test by the use of a shorter rifampicin incubation period (31). Availability of susceptibility results in a shorter time will reduce morbidity and mortality from MDR-TB and treatment can be promptly initiated. It will thus check the spread of MDR-TB in the community. Phage amplification technology does not involve complicated procedures requiring extra-ordinary expertise. It involves simple microbiological skills like centrifugation, pipetting and pour plate culture method. The results are read visually and it requires no specially dedicated instrumentation hence it is less expensive than the new automated methods and it is applicable under currently available infrastructure in most laboratories in developing countries. One of the attractions of the FASTPlaque TB-MDRi™ Kit (Biotec Laboratories, Ipswich, UK) is that it contains all materials-reagents, freeze-dried virus, *M. smegmatis*, virucide, and the drug, medium and reagent bottles needed for the test. It is thus very convenient. The FASTPlaque TB-MDRi™ assay has been evaluated and compared with conventional methods in several studies. In a study conducted at the TB Reference Laboratories of the South African Institute of Medical Research (SAIMR) in Johannesbourg (LAB I) and Cape Town (LAB II), the ability of the FPTB-MDRi kit to correctly identify rifampicin susceptibility on strains of *Mycobacterium tuberculosis* culture on solid media was evaluated and compared with conventional methods. By conventional methods, 81 out of 191 strains were found to be rifampicin resistant and 110 strains out of 191 were rifampicin susceptible. Respectively, sensitivity, specificity and overall accuracy of the FASTPlaque TB-MDRi for LAB I were, 100%, 97% and 98% and for LAB II they were 100%, 94% and 97% (22). In another study at the TB Unit of SAIMR, Johannesbourg, the ability of FPTB-MDRi to detect rifampicin resistance within 48 hours was evaluated using samples with known Bactec 460 automated culture results. In this study, the use of FPTB-MDRi was compared to the Bactec 460 radiometric method in determining TB drug susceptibility patterns to rifampicin. Also, the possibility of reducing the time for reporting

results from 48 hours to 24 hours was explored. The study showed that FPTB results are available within 48 hours (sooner than the automated Bactec 460 method). With a reduced rifampicin incubation time, the overall time for reporting results could be reduced to 24 hours. Overall accuracy was 90% (31).

COST-EFFECTIVENESS OF PHAGE AMPLIFICATION TECHNOLOGY

TB is a disease symptomatic of poverty. MDR-TB is particularly common among those who have had previous anti-TB chemotherapy, ethnic minority groups, migrants, refugees, substance abusers, HIV positive patients and the homeless (34). Cost is therefore a major consideration that determines the applicability of diagnostics in the developing world including Nigeria. This is exemplified by the fact that despite the availability of rapid sophisticated methods for the diagnosis of TB and the detection of MDR-TB, none of these are being used in National TB control programmes in most third world countries. Any new test must therefore compare favourably with the conventional methods otherwise their high cost would prohibit their use.

The cost of full diagnosis of TB (AFBX3, x-ray, culture and sensitivity) is about N2000 (approximately $15). This is highly variable depending on where the tests are done and the method used. The FASTPlaqueTB-MDRi kit costs $400 per determination of 50 tests, if one buys in small quantities from Biotec Laboratories, Ipswich, UK (35). It is difficult to work out what the cost per test would eventually be in Nigeria with the availability of discount on bulk purchases but it is unlikely to go beyond WHO recommendations of less than $10 for a drug susceptibility test. It places very little demand on the scarce resources of Nigerian laboratories because it is manual, requiring no specially dedicated instrumentation.

It has been suggested that it may be possible to reduce the cost of this technology by propagating the phage and *M. smegmatis* in a local laboratory. Studies have however shown that a particular gene in *M. smegmatis* when over expressed induces resistance to certain phages (36). Coupled with the problem of standardization, it may be better to use the commercially available kit which passes through a quality control test to ensure high performance standards before it is plunged into the market.

The ability of phage amplification technology to promptly detect MDR-TB will reduce morbidity and mortality

from the disease. Also, the number of contacts that the patient has a chance to infect will be greatly reduced. Thus the number of people requiring anti-TB drug susceptibility testing as well as treatment with the more expensive second line drugs in the future will be reduced. These future savings could be used to pay for the diagnostic (26). With increased budgetary allocation to the National Tuberculosis and Leprosy Control Programme (NTLCP) and donor support, the cost of diagnosis and treatment on the individual patient would be greatly reduced.

CONCLUSIONS AND RECOMMENDATION

The emergence of MDR-TB is indeed a major threat to the treatment of TB and an important impediment to National TB Control Programmes. In order to avoid its fatal effects on individual patients and its spread within communities, there is need for prompt diagnosis. FPTB-MDRi gives susceptibility results from MTB cultures within 48 hours and is therefore recommended as a substitute for the conventional LJ proportion method, which takes about one month to provide susceptibility results. This would ensure that appropriate treatment is initiated in good time.

Cost has emerged as a major consideration and an important factor determining the applicability of new diagnostics in the National TB Control Programmes of developing counties including Nigeria. There is an urgent need for all tiers of government to prioritize TB control because of its chronic and debilitating effects on the workforce and the general population as well as its deadly alliance with HIV/AIDS, which has its highest prevalence in developing countries. Prioritization of TB control goes beyond an expression of the political will to do so. It involves actual dedication and disbursement of funds to the National TB Control Programme. This has become necessary because majority of TB patients are poor and so cannot afford exorbitant testing and treatment. Ensuring that such patients undergo and receive free or highly subsidized testing and treatment would be an indirect investment into the workforce of nations. The right to quality/affordable healthcare is entrenched in several international human rights documents as well as the Nigerian constitution.

There is the need for the NTLCP in Nigeria to embark on laboratiories inspection and to identify and equip strategically located centers where TB testing can be done nationally. This would do little to reduce the spread of MDR-TB as the onus of culture and

drug susceptibility testing lies with the National TB Reference Laboratory in Lagos which so far has no strong liaison with peripheral laboratories. It is therefore recommended that a network of laboratories be established so that samples from cases of treatment failure can be promptly submitted to the Reference Laboratory for further diagnosis. Amongst other things, provision of vehicles and biohazard containers/ice chests for the transportation of samples from peripheral centers to the National Reference center is a top priority.

The World Health Organisation as the regulatory body for National TB Control Programmes has a great role to play in promoting the evaluation of new diagnostics as well as recommending their use. It is thus recommended that the WHO assists National Reference Laboratories in developing countries where TB prevalence is highest to evaluate new kits in other to ascertain their adaptability to particular situations. The WHO also has a role to play in supporting laboratory personnel in charge of TB diagnosis to attend training courses to improve their skills especially in new technologies.

Though considered rapid compared with the L.J proportion method, FPTB-MDRi relies on culture. There is the therefore need for the manufacturers to engage in continuing research in order to produce kits that could detect MDR-TB rapidly and directly form sputum and other samples.

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