African Journal of Microbiology Research Vol. 6(32), pp.6199-6203, 23 August, 2012
Available online http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.888
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Genotyping of *Mycobacterium tuberculosis* complex based on restriction enzyme analysis of the *hsp65* gene

Nwokoye N. N.[1]*, Nwaokorie F. O.[2], Iwalokun B. A.[3], Onubogu C. C.[1] and Idigbe E. O.[1]

[1]National TB Reference Laboratory, Microbiology Division, Nigerian Institute of Medical Research Yaba, Lagos State, Nigeria.
[2]Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research Yaba, Lagos State, Nigeria.
[3]Biochemistry Division, Nigerian Institute of Medical Research Yaba, Lagos State, Nigeria.

Accepted 30 June, 2012

Several genotypes of *Mycobacterium* with varied degree of virulence and drug resistance exist globally. Grouping of the genotypes into principal genetic groups is critical for tuberculosis (TB) control. This study determined the suitability of polymerase chain reaction- restriction enzyme analysis (PCR-REA) technique as an epidemiological typing tool and a technique for drug resistance surveillance of TB. Seventy-five *Mycobacterium tuberculosis* isolates from National TB Reference Laboratory, Nigeria Institute of Medical Research (NIMR) Yaba, Lagos, Nigeria were identified and their susceptibility to isoniazid and rifampicin determined using Genotype MTBDRplus technique. Isolates were further confirmed by polymerase chain reaction (PCR) with primer specific to 65 kDa heat shock protein (*hsp65*) of *Mycobacterium* species, Fingerprinting was done by restriction enzyme (Bst Ell) analysis. Of the 75 isolates studied, 5(6.7%) were multi-drug resistant (MDR)- TB, 11(14.7%) and 7(9.3%) were mono-resistant to rifampicin and isoniazid respectively. PCR-REA profile revealed 3 different patterns. Majority (96.2%) of the susceptible strains and 100% each of mono-resistant and MDR -TB yielded 2 fragments of 250 and 120 bp. Our findings demonstrated that *hsp65* kDa based molecular genotyping technique is a rapid, simple and easy-to-interpret method and may be used as a predictor of *M. tuberculosis* genetic variation.

Key words: Genotyping, *Mycobacterium tuberculosis*, heat shock protein, restriction enzyme analysis, drug susceptibility.

INTRODUCTION

Several genotypes of *Mycobacterium* exist globally (Kim et al., 2005). These genotypes have varied degree of virulence and drug resistance and their distributions differ from one geographical region to another (Kim et al., 2005). Identification and grouping of the genotypes into their principal genetic groups is critical in understanding tuberculosis (TB) epidemiology which is a crucial aspect of TB control.

Both phenotypic and genotypic methods are available for species identification of *Mycobacterium.* Phenotypic methods, although well established, are slow in generating critical information necessary for tracking *Mycobacterium tuberculosis* strains, discriminating between epidemic and sporadic strains and determining both the source of infection and mechanism of transmission of drug resistant strains (Guarin et al., 2010). This information is needed for improved treatment, care and support of *M. tuberculosis* infected patients (Kim et al., 2005; Wang et al., 2010). The sequencing of the genome of *M. tuberculosis* H37RV has enabled the use of molecular biology tools in determining and differentiating *M. tuberculosis* infections. These genotypic methods otherwise known as genotyping or molecular fingerprinting offer higher sensitivity, specificity and precision in diagnosing and discriminating *M. tuberculosis* strains (Allix-Beguec et al., 2010; Oelemann et al., 2011). They include spoligotyping, MIRU-VNTR typing, insertion

*Corresponding author. E-mail: nkirunwokoye@ymail.com. Tel: +2348136501810.

sequence 6110 (IS6110) genomic analysis and other molecular target typing (Gomgnimbou et al., 2012; Barry et al., 2012; Groenheit et al., 2011). The use of these methods is interrelated and depends on the epidemiological challenges to be addressed coupled with need to globalize epidemiological data (Hafner et al., 2004).

In recent times, a DNA fingerprinting technique that targets the highly conserved *hsp65* was developed for rapid differentiation of mycobacterial isolates (Kim et al., 2005; Hafner et al., 2004). This technique known as polymerase chain reaction – restriction enzyme analysis (PCR-REA) employs a simple method of DNA extraction followed by amplification of the 439 bp fragment of *hsp65* gene by PCR and final cleavage of the amplified gene with restriction enzymes. PCR-REA has been shown to be a promising tool in investigating the genetic variations among the *M. tuberculosis* (MTB) and the non-tuberculous *Mycobacterium* (NTM) in an endemic population (Hafner et al., 2004; Khosravi and Seghatoleslami, 2009).

Despite the usefulness of this tool, studies on its application for differentiation of mycobacterial isolates on the basis of their drug resistant patterns are lacking. In view of this, the present study was carried out to determine the suitability of PCR-REA of MTB heat shock protein as an epidemiological tool for TB and drug resistance surveillance of TB in Nigeria.

MATERIALS AND METHODS

Study size and ethical clearance

This was a prospective study conducted over a period of 6 months (January to June, 2011) on 75 viable *M. tuberculosis* isolates (one isolate per patient) from the National TB Reference Laboratory, Nigerian Institute of Medical Research (NIMR) Lagos, Nigeria. *Mycobacterium* species belonging to the *M. tuberculosis* complex (irrespective of their drug susceptibility pattern to isoniazid and rifampicin) where included in the study while non-*Mycobacterium tuberculosis* complex, were excluded. Permission to carry out the study was obtained from the Institutional Review Board of the Nigerian Institute of Medical Research (NIMR).

Study center

The study center was the National TB Reference Laboratory (NTBRL), Nigerian Institute of Medical Research (NIMR). The laboratory centers primarily on research and diagnosis of tuberculosis. It serves as a National TB Reference Laboratory for the country and overseas the activities of all other Zonal and State TB Laboratories in the southern part of the country including the South-south, South-west and South-east Zones. Patients from these zones with complicated TB cases are usually referred to NTBRL for rapid and improved laboratory investigations. It also supports the National TB and Leprosy Control program (NTBLCP) of the Federal Ministry of Health in driving TB control via diagnostic interventions. A total of 75 isolates were identified and their drug susceptibility pattern to isoniazid and rifampicin was determined using Genotype MTBDRplus technique (Huyen et al., 2010). DNA extraction was done by boiled lysate technique according to Khosravi and Hashemi (2007).

The identity of the isolates were further confirmed by PCR with primer F (TB 11: 5' –ACCAACGATGGTGTGTCCAT and R TB 12: 5' -CTTGTCGAACCGCATACCCT) specific for *Mycobacterium* and targeted towards the *hsp65*. The reaction volumes for PCR was 25 µl and composed of 50 mmol KCl, 10 mmol Tris-HCl (pH 8.3), 1.5 mmol $MgCl_2$, 0.2 mmol of each deoxynucleotide triphosphate, 10 picomole of each primer, 1.25 units of Taq polymerase, 14 µl of sterile distilled water and 5 µl of DNA template. The reaction conditions were as follows: An initial denaturation at 95°C for 60 s; 45 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR products were analyzed on 1% (w/vol) agarose gel stained with 0.5 mg/ml of ethidium bromide and visualized using a photo-documentation system (UVP, Inc USA).

Fingerprinting was carried out using restriction enzyme-Bst Ell analysis. Briefly, 10 µl of product was added to Bst Ell enzyme mix (6 µl of sterile distilled water, 2 µl of restriction enzyme and 2 µl of corresponding buffer) and incubated for 2 to 3 h in a 60°C water bath. A total of 20 µl digested products were analysed on a 2% agarose gel run by horizontal electrophoresis with 100 bp of DNA ladder. The gel was stained with 0.5 µg/ml ethidium bromide and DNA fragments visualized under UV light transilluminator (UVP, Inc.). The base pair size of each DNA fragment was determined by comparison of migration distances of different strains with the molecular markers visually. A standard strains of H37Rv (National TB Reference Laboratory, NIMR, Lagos Nigeria) was used as positive controls, while buffers served as negative controls.

RESULTS

The agarose gel electrophoresis of the PCR product is shown in Figure 1a and b. All the clinical isolates and the positive control (H37RV) were amplified with amplicon size of 489. The restriction enzyme analysis profile after digestion with the Bst Ell enzyme revealed 3 different patterns based on molecular weight and number of the DNA fragments obtained. The MTB were differentiated into 3 different groups, those with 2 fragments were placed in Group 1 while those with 1 and no fragment were placed in Group 2 and 3 respectively (Figure 2 and Table 1).

Majority (69.3%) of the MTB strains were susceptible (Table 2) while 6.7% were resistant to both drugs (Multi-drug resistant-MDR) –TB. About 96.2% of the susceptible strains and 100% each of the mono and MDR -TB yielded 2 fragments of 250 and 120 after enzyme digestion.

DISCUSSION

A band size of 436 bp established successful amplification of *hsp65* kDa gene sequence. This agrees with the study in iran (Khosravi and Hashemi, 2007), and supports the opinion that *hsp65* gene is highly conserved within the MTB species and may be of great value in taxonomic evaluations.

It is of interest to note that while other published studies (Khosravi and Hashemi, 2007; Telenti et al., 1993)

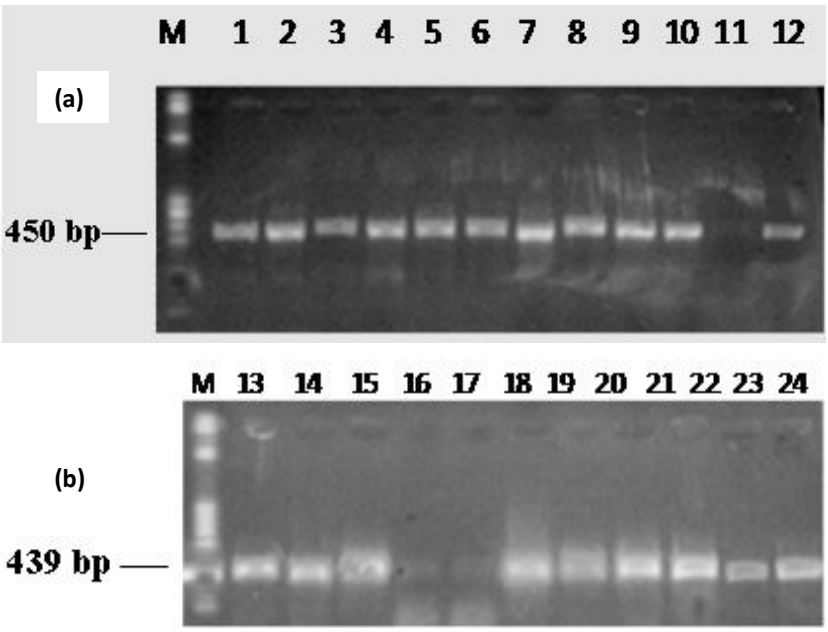

Figure 1. Agarose gel electrophoresis of PCR product showing; Lane M, DNA Molecular weight marker (a) Lane 1-10 MTB strains with visible amplification at 489 bp, Lane 11; negative control, Lane 12; Positive control H37Rv. (b) Lane 13-24 MTB strains.

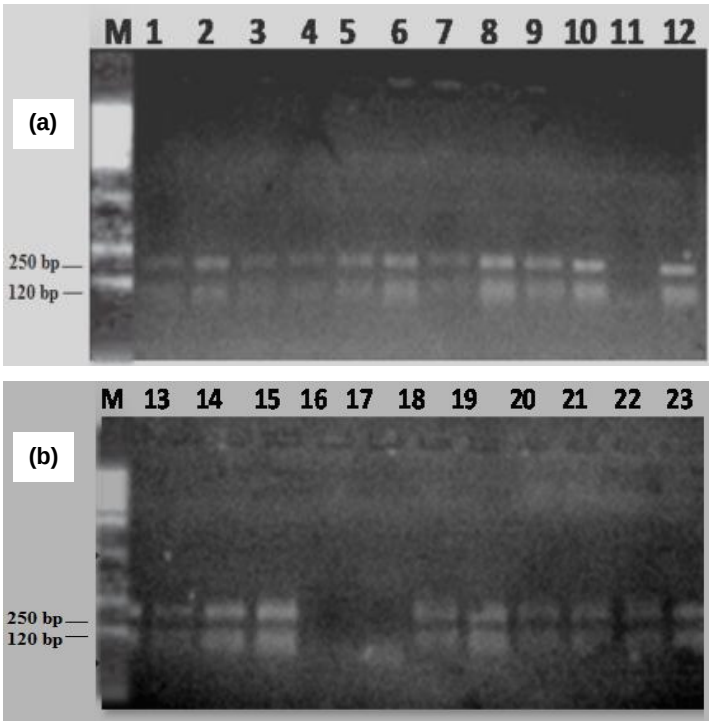

Figure 2. Agarose Gel Electrophoresis showing RAE profile of MTB isolates after digestion *with* Bst Ell restriction enzyme. Lane M DNA Molecular weight marker (a) lane 11-negative control, lane 12, Positive control H37Rv, lane 1-10 MTB clinical strains. (b) Lanes 13-23, MTB clinical strains.

Table 1. Restriction enzyme analysis (REA) Patterns obtained with the MTB clinical isolates.

Strain/Group	No. of strains	Bst Ell digest (bp)
H37RV	1	250/120
1	73(97.3)	250/120
2	1(1.3)	118
3	1(1.3)	No band

Restriction enzyme (Bst Ell).

Table 2. Restriction enzyme and drug susceptibility patterns of *Mycobacterium tuberculosis.*

REA Groups (#)	Drug susceptibility pattern (#)			
	MDR (n = 5)	RIF-resistance (n = 11)	INH-resistance (n = 7)	Susceptible (n = 52)
1 (n = 73)	5 (100%)	11(100%)	7 (100%)	50 (96.2%)
2 (n = 1)	0	0	0	1 (100%)
3 (n = 1)	0	0	0	1 (100%)

REA: restriction enzyme analysis, MDR: multi-drug resistant, RIF: Rifampicin, INH: Isoniazid.

obtained one restriction pattern (of only 3 fragments) with Bst Ell enzyme for MTB strains, the MTB strains from this study revealed 3 different patterns (of 2, 1 and no fragments) with Bst Ell enzymes indicating geographical difference among the MTB as well as intra-species variation within the MTB strains circulating in Lagos Nigeria. This result clearly demonstrates that these strains collection represents a genetically heterogeneous group, quite distinct from one another. The isolates in Group 1 (2 restriction fragments) were more prevalent suggesting predominance of their clonal spread in the environment. All the drug resistant strains were also resident in Group 1 signifying the high degree of virulence among strains in this group. In previous studies, factors incriminated as being responsible for successful clonal expansion of *M. tuberculosis* strains include adaption and survival capability, greater virulence and pathogenicity (Allix-Beguec et al., 2010; Ani et al., 2010). However, the *hsp65*-PCR-REA genotyping technique employed in this study could not further discriminate our Group 1 strains on the basis of multidrug resistance (defined as resistance to the two first-line anti-tuberculosis drugs: isoniazid and rifampicin), mono-resistance (to either isoniazid or rifampicin) or drug sensitive stains as defined by the MTBDRplus genotyping assay.

Therefore, our findings revealed limitation of *hsp65*-PCR-REA in the understanding of molecular epidemiology of DR-TB strains in Lagos, despite showing that these strains are genetically heterogeneous. In order to ascertain the level of heterogeneity among isolates with varied drug susceptibility pattern, a multi-enzyme digestion system might be needed coupled with the use of more discriminatory methods such as spoligotyping, MIRU-VNTR or whole genome sequencing which will help to understand the local and international epidemiology of DR-TB.

Conclusion

Based on our findings, we concluded that heat shock protein-65 kDa based molecular genotyping technique is a rapid, simple and easy-to-interpret molecular method with usefulness as a predictor of *M. tuberculosis* genetic variation. However, it cannot be used as an epidemiological tool for drug resistance surveillance of TB since it failed to discriminate between the MDR, mono-resistance as well as the isoniazid and rifampicin sensitive strains.

ACKNOWLEDGEMENTS

We acknowledge the grant support provided for this study by the International Association of National Public Health Institute (IANPHI). We also thank the staff of the National TB Reference Laboratory, Nigerian Institute of Medical Research Yaba, Lagos for their support.

REFERENCES

Allix-Beguec C, Fauville-Dufaux M, Stoffels K, Ommeslag D, Walravens K, Saegerman C, Supply P (2010). Importance of identifying *Mycobacterium bovis* as a causative agent of human tuberculosis. Eur. Respir. J. 35:692-694.

Ani A, Bruvik T, Okoh Y, Agaba P, Agbaji O, Idoko J, Dahle UR (2010). Genetic diversity of *Mycobacterium tuberculosis* complex in Jos, Nigeria. BMC Infect. Dis. 10:189.

Barry PM, Gardner TJ, Funk E, Oren E, Field K, Shaw T, Langer AJ (2012). Multi-State outbreak of MDR-TB identified by genotype cluster investigation. Emerg. Infect. Dis. 18:113-116.

Gomgnimbou MK, Refrégier G, Diagbouga SP, Adama S, Kaboré A, Ouiminga A, Sola C (2012). Spoligotyping of *Mycobacterium africanum,* Burkina Faso*.* Emerg. Infect. Dis. 18:117-119.

Groenheit R, Ghebremichael S, Svensson J, Rabna P, Colombatti R, Riccardi F, Couvin D, Hill V, Rastog N, Koivula T, Kallenius G (2011). The Guinea-Bissau Family of *Mycobacterium tuberculosis* Complex Revisited. PLoS ONE 6.

Guarin N, Budvytiene I, Ghafghaichi L, Banaei N (2010). Comparison of real-time polymerase chain reaction and conventional biochemical methods for identification of Mycobacterium chelonae-Mycobacterium abscessus group to the species level. Diagn. Microbiol. Infect. Dis. 67:333-336.

Hafner B, Haag H, Geiss HK, Nolte O (2004). Different molecular methods for the identification of rarely isolated non-tuberculous mycobacteria and description of new hsp65 restriction fragment length polymorphism patterns. Mol. Cell Probes 18:59-65.

Huyen MNT, Tiemersma EW, Lan NTN, Cobelens FGJ, Dung NH, Sy DN, Buu TN, Kremer K, Hang PT, Caws M, O'Brien R, Soolingen DV (2010). Validation of genotype MTBDRplus assay for diagnosis of multidrug resistant tuberculosis in South Vietnam. BMC Infect. Dis. 10:149.

Khosravi AD, Hashemi A (2007). Application of restriction enzyme analysis technique based on 65KDa heat shock protein gene for fingerprinting and differentiation of *Mycobacterium tuberculosis* clinical strains isolated from tuberculosis patients in Ahwaz, Iran. Pak. J. Med. Sci. 23:216-219.

Khosravi AD, Seghatoleslami S (2009). Genotying and identification of mycobacteria by fingerprinting techniques. Jundishapur J. Microbiol. 2:8-91.

Kim H, Kim S, Shim T, Kim M, Bai G, Park Y, Lee S, Chae G, Cha C, Kook Y, Kim B (2005). Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene (hsp65). Int. J. Syst. Evol. Microbiol. 55:1649-1656.

Oelemann CM, Gomes HM, Willery E, Possuelo L, Lima KVB, Allix-Beguec C, Locht C, Yves-Oliver L, Salmoniere G, Gutierrez MC, Suffys P, Supply P (2011). The forest behind the tree: Phylogenetic exploration of a dominant *Mycobacterium tuberculosis* strain lineage from a high tuberculosis burden country. PLOS ONE 6.

Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31:175-178.

Wang H, Yue J, Han M, Yang J, Zhao Y (2010). Rapid method for identification of six common species of Mycobacterium Based on multiplex SNP analysis. J. Clin. Microbiol. 48:247-250.