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Asian Pacific Journal of Tropical Disease

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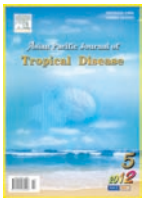

Document heading doi: 10.1016/S2222-1808(12)60075-9

Molecular typing of *Mycobacterium tuberculosis* isolates from Yaoundé reveals RIF resistance markers, clonal relatedness and mutation patterns

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ARTICLE INFO

Article history:
Received 15 July 2012
Received in revised form 27 September 2012
Accepted 28 Octorber 2012
Available online 28 October 2012

Keywords:
Tuberculosis
Diagnosis
Sequencing
RopB
Random amplified polymorphic DNA
TA cloning

ABSTRACT

Objective: To understand the spread of drug resistance and identifying diagnostic probes among the local tuberculosis (TB) strains in order to design rational control tools for tuberculosis controls. **Methods:** TA cloning and sequencing were used to characterize mutation associated with RIF resistance in 69 bp region of the gene, *rpoB*. The analysis identified two regions of mutations but no unusual insertion and deletion. No mutation was observed in RIF sensitive strains. **Results:** We employed Random Amplified Polymorphic DNA (RAPD) analysis for typing strains of *M. tuberculosis* to determine whether new strains were present among *M. tuberculosis* isolates circulating in Yaounde. Three groups (I to III) of *M. tuberculosis* were identified among 93 isolates randomly selected. RAPD analysis provided a rapid and easy means of identifying polymorphism in *M. tuberculosis* isolates, and it was found to be a valuable alternative epidemiological tool. RAPD was used to select the new site of diagnostic by PCR. Also single nucleotide polymorphisms between *M. tuberculosis* and *M. bovis* were found, suggesting that RAPD can be a useful technique for distinguishing between species. **Conclusions:** Molecular typing is defined as the integration of conventional epidemiological approach to track specific strains of pathogens in order to understand the distribution of disease in populations.

1. Introduction

In the developing countries, tuberculosis (TB), once considered to have been essentially eliminated, has rebounded and is increasingly caused by drug resistant strains[1-4]. Early detection of infectious patients and completion of treatment are essential[5].

Despite the availability of highly effective anti-TB drugs and wide spread efforts to implement prevention programs, about a third of the world's population (1.7 billion people) are still infected with *Mycobacterium tuberculosis (M. tuberculosis)* and at risk of developing clinical TB[6,7]. Each year, approximately 9 million new active cases and 3 million deaths associated with the disease are reported from around the world[8, 9]. Consequently, in 1993, the World Health Organization declared TB to be a global emergency. TB has been complicated by an increase in the incidence of drug resistant strains of *M. tuberculosis* which have been found in both developping and developed countries[10]. The most common causes of acquired drug resistance to *M. tuberculosis* are inadequate chemotherapeutic regimens and non compliance by patients during therapy. Infections with multi-drug resistant *M. tuberculosis* are often associated with extraordinarily high mortality rates and rapid progression from diagnosis to death[11].

RIF is an important antibiotic used in the treatment of TB that inhibits the *rpoB* gene product (the RNA polymerase β subunit). It has been observed that the efficacy of chemotherapy can markedly be reduced when infections are caused by *M. tuberculosis* strains that are RIF resistant. Many studies suggested that prevalence of MDR tuberculosis is linked to performance of national tuberculosis control programmes[12]. The rapid detection of RMF resistance is of particular importance, since it also represents a valuable surrogate marker for multiple drug resistance (MDR) resistance, which is a tremendous obstacle to TB therapy[13]. Molecular methods can provide accurate and rapid drug susceptibility results[14]. We used DNA sequencing to detect resistance to RIF, one first-line antituberculosis drugs. Collectively, DNA sequencing

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Foundation Project: supported by IPICS/ISP (project CAM 01).

studies have demonstrated that >95% of RIF-resistant *M. tuberculosis* strains have a mutation within the 69-bp hot-spot region (codons 511 to 533) of the RNA polymerase β subunit (*rpoB*) gene[15]. The prevalence of the mutations determined so far varies for *M. tuberculosis* strains obtained from different countries. Thus, it is important to determine the distribution of resistance mutations in each country as a prelude to the introduction of molecular tests for routine diagnosis[16].

Despite the constant evolution and innovation in the field of bacterial fingerprinting and DNA based diagnostics, the molecular identification and typing of mycobacteria associated with human and animal diseases is still frequently hampered by a lack of resolution at the inter or intraspecific level. A most striking example of these problems is found with the epidemiology of the *M. tuberculosis* complex, that is composed of *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*. The first two species are widely recognized as the causal agent of human and bovine tuberculosis, respectively, but *M. bovis* infections have also been documented in humans due to the wide host range of this organism. Imaeda *et al* 1985 showed that the members of the *M. tuberculosis* complex were originally grouped together on the basis of their phenotypic similarities and the very high levels of DNA relatedness. The genome of *M. bovis* is 99.95% identical to that of *M. tuberculosis*[17]. Cell wall components and secreted proteins show the highest variation, indicating that mutations within the genome are present.

The present study was to analyse the region within and around the *rpoB* gene using TA cloning and sequencing associated with RIF resistance which could be useful for a rapid diagnosis and to evaluate the performance of Random Amplified Polymorphic DNA (RAPD) between members of *M. tuberculosis* complex.

2. Materials and Methods

2.1 M. tuberculosis isolates

All specimens were collected at the Centre Pasteur du Cameroun (from hospitals of Yaounde, Central, CHU, General and majotary from Jamot, a hospital specialized for lung diseases in Cameroon). Ninety-three *M. tuberculosis* sputum isolates cultured on Löwenstein-Jensen medium were identified as *M. tuberculosis* complex by culture and PCR reaction using primer P3P4 (P3 CTCGTCCAGCGCCGCTTCGG and P4 GTGCGGATGGTCGCAGAGAT) specific for the tuberculosis complex[18].

A total of 50 clinical isolates of *M. tuberculosis* were used in the present study. Primary isolation, differentiation, and drug sensitivity testing were performed with Löwenstein-Jensen medium containing isoniazid 0.1 μg/mL, RIF 2 μg/mL, streptomycin 6 μg/mL, and etambutol 7.5 μg/mL. Five isolates were RIF resistant and forty five susceptible[19].

2.2. DNA extraction

DNA was extracted from the sample using a modified cetyltrimethylammonium bromide (CTAB) procedure. Samples (10 mn) boiled at 100 ℃ were added to 0.6 mL of CTAB extraction buffer (2% CTAB; 100 mM Tris-HCI, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 0.2% 2-mercaptoethanol). The suspension was incubated in a water bath at 650 ℃ for 30 min with occasional shaking. 2 μL of proteinase K (20 mg/mL) were added and incubated for 30 min at 370 ℃. The mixture then were cooled to room temperature and extracted with one volume aqueous phenol/chloroform/isoamyl alcohol (25:24:1) twice. The two phases were separated by centrifugation and one volume ethanol, 1/10 of 5 M NaCl and 1 μL of glycogen were added to the aqueous phase. The mixture was centrifuged at 12 000 x g for 30 min. The pellet was washed with 70% ethanol twice, then dissolved in 20 μL TE. The DNA solution was used in the RAPD reactions.

2.3. PCR amplification

2.3.1. Resistance analysis

A 517 bp fragment containing the *rpoB* gene was amplified by PCR with primers JCTR1 GGATCGGCGATTGGGACG and JCTR2 ATATCGCAGCCTCCCACG. PCR was carried out in 25 μL of a reaction mixture containing 2.5 μL of 10X PCR buffer, 1.5 μL $MgCl_2$ (25 mM), 1 μL JCR_1 (10 pmol/μL), 1 μL JCR_2 (10 pmol/μL), 10-15 ng of DNA, 0.4 μL Taq Polymerase (5 U/μL), 0.4 μL dNTPs (10 mM) and 25 μL ddH2O. Samples were then subjected to denaturation at 94℃ for 5 min, followed by 35 cycles at 94℃ for 1 min, 68℃ for 1 min, and 72 ℃ for 1 min. After the last amplification cycle, the samples were incubated further at 72℃ for 10 min for complete elongation of the final PCR products.

2.3.2. RAPD analysis

PCR was performed in a total volume of 25 μL. The reaction mixture contained 5-10 ng of genomic DNA, 10 pmol of primer, 500 μM concentrations of each deoxynucleoside triphosphate, 2 U of Taq DNA polymerase (Sigma), and 2.5 μL of PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 3.5 mM $MgCl_2$). PCR were conducted in a Perkin-Elmer with the following parameters: denaturation at 95℃ for 5 min, followed by 35 cycles at 94℃ for 1 min, annealing at 40℃ for 1 min, and extension at 72℃ for 1 min. Reproducibility was checked by varying annealing temperature and repeating the analysis at least twice. RAPD products were analyzed by electrophoresis in a 1.5% agarose gel in Tris-borate-EDTA (pH 8.0 buffer) and visualized by ethidium bromide staining. All bands obtained on RAPD gels were numbered and scored as present or absent on a data sheet.

2.4. Purification and cloning PCR product

PCR products were separated by 2% agarose gel electrophoresis and examined in UV light after staining in 0.5 μg/mL of ethidium bromide and purified by use of a QIAquick PCR purification kit (Qiagen). Cloning was performed used TA Cloning Kit (Invitrogen). After transformation the recombined clone was confirmed using PCR reaction with M13 reverse Primer and M13 forward primer.

2.5. Sequencing

Sequencing was performed with the sequenase DNA sequencing Kit according to the manufacturer's instructions using M13 reverse primer or M13 forward primer[20, 21] at the Marine Biological Laboratory, Woods Hole, USA.

3. Results

3.1. Drug Resistance

Specimens were identified as *M. tuberculosis* complex by PCR reaction with primer P3P4 (Figure 1). Fifty clinical isolates (Table 1), 5 MDR strains and 45 sensitive were subjected to TA cloning sequencing analysis of the hypervariable *rpoB* region (Figure 2). The results of antibiogram were confirmed, 5 RIF resistant and 45 sensitive isolates were identified. All of the positive were single-nucleotide mutations involving two codons. No silent substitutions in the 158-bp *rpoB* gene fragment examined were observed for any of the *M. tuberculosis* strains analyzed. The codons affected by point mutations were codons 516 (3 isolates) and 531 (2 isolates). No strains exhibited more complex mutations. No mutations were observed outside of this 69 bp hypervariability region. Three strains contained a GAC GTG (Asp-to-Val) mutation in codon 516, and in the other strain codon 531 had the mutation TCG TTG (Ser to Leu). In total, five RIF resistant isolates were identified in the present study, with all involving changes in one codon. None of the 45 RMP-sensitive control isolates had any mutations.

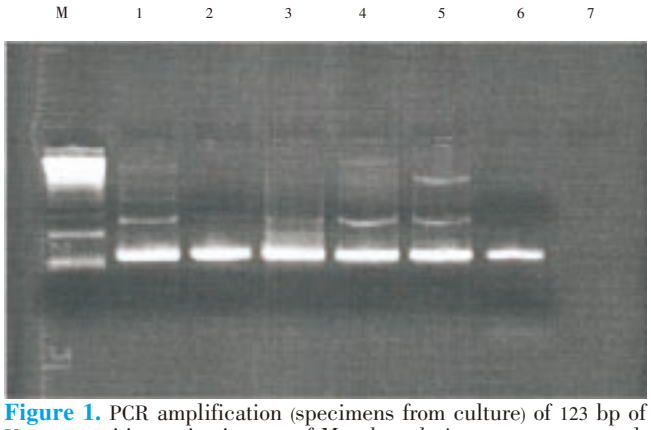

Figure 1. PCR amplification (specimens from culture) of 123 bp of IS6110 repetitive region in gene of *M. tuberculosis* on 1.5% agarose gel. Lane M, 100 kd ladder, 7 Negative control, 1 to 6 different isolates.

Table 1
Drug sensitivity of some *M. tuberculosis* isolates collected at the Centre Pasteur du Cameroun du Cameroun.

No of strains 50	INH	RMP	EMB	SM
3	R	R	R	R
1	R	R	R	S
1	R	R	S	R
45	S	S	S	S

[a] INH, isoniazid; EMB, ethambutol; RMP, RIF; SM, treptomycin; *R, resistant; S, sensitive.*

3.2. RAPD analysis

For the RAPD analysis, we tested 10 different primers (Table 2 and Figure 3). The primers JCT1 5′-TGCCGAGCTG-3′ and JCT9 5′-GTCAGTGCGG-3′ yielded the best results. These oligonucleotides were selected based on high-intensity bands, hypervariability, and good definition of amplified DNA fragments and were used for molecular characterization of 93 different isolates of *M. tuberculosis*. Typical electrophoregrams obtained with these primers are shown in Figures 4 and 5. The bands were scored in comparison to the 100 bp ladder, which is found in lane (M) on the gel. Bands ranged in length from 200 bp to 1200 bp, with the majority of individuals exhibiting bands in the 500 bp region and the 900 bp region.

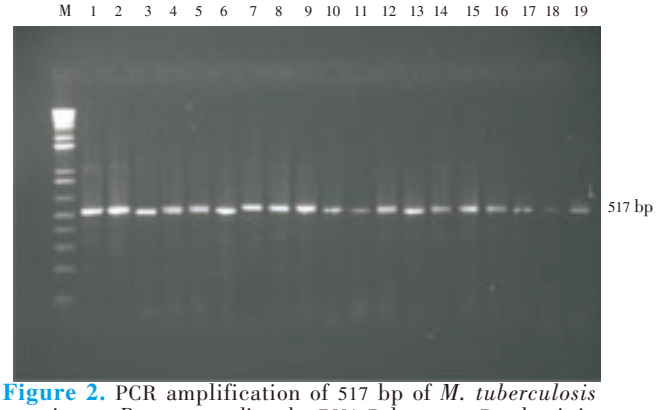

Figure 2. PCR amplification of 517 bp of *M. tuberculosis* containg *rpoB* gene encoding the RNA Polymerase B subunit in RIF resistant. Lane M, 100 kd ladder, 1-16 different isolates.

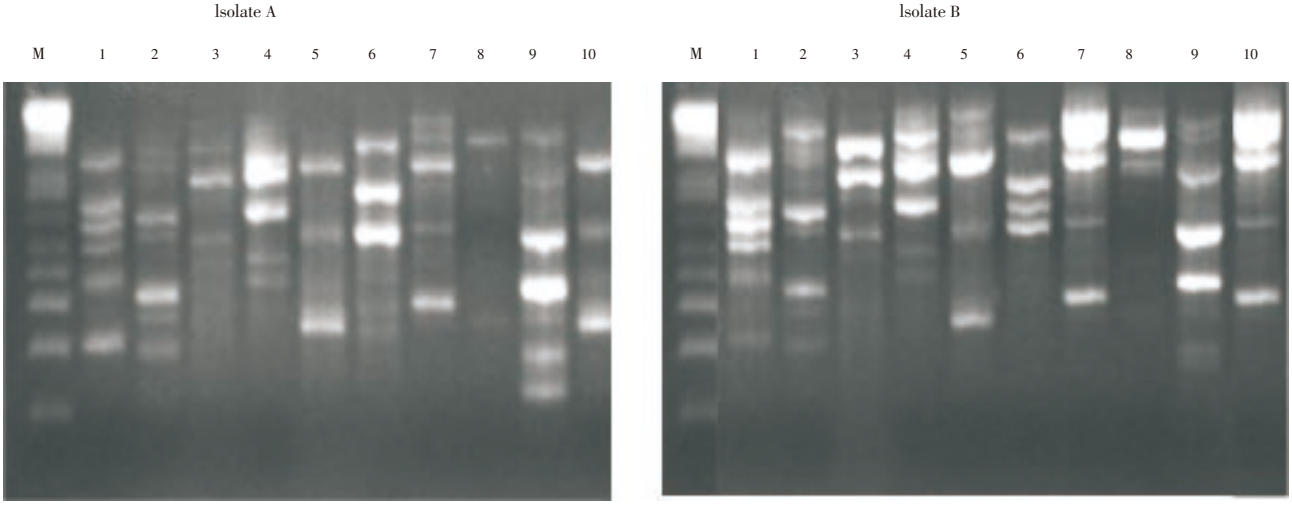

Figure 3. RAPD profiles showing polymorphism using 10 different primers on two isolates (A and B) of *M. tuberculosis* DNA.

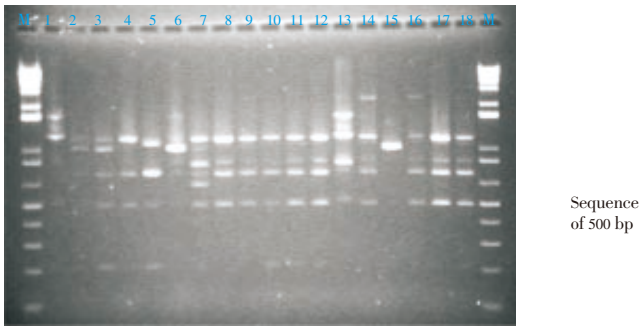

Figure 4. RAPD profiles showing polymorphism using primer JCT1

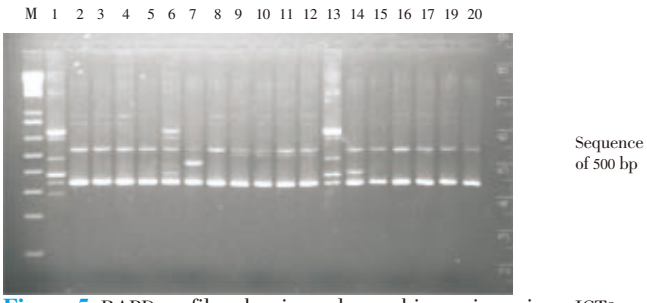

Figure 5. RAPD profiles showing polymorphism using primer JCT9

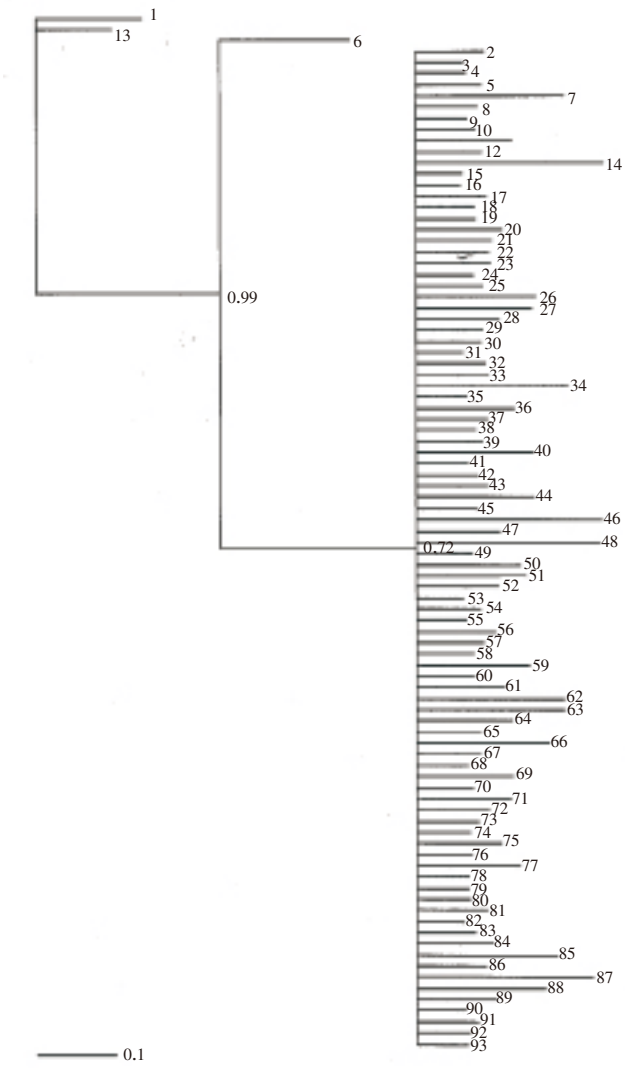

Figure 6. Dendogram derived from RAPD assays generated by using JCT1 Primer.

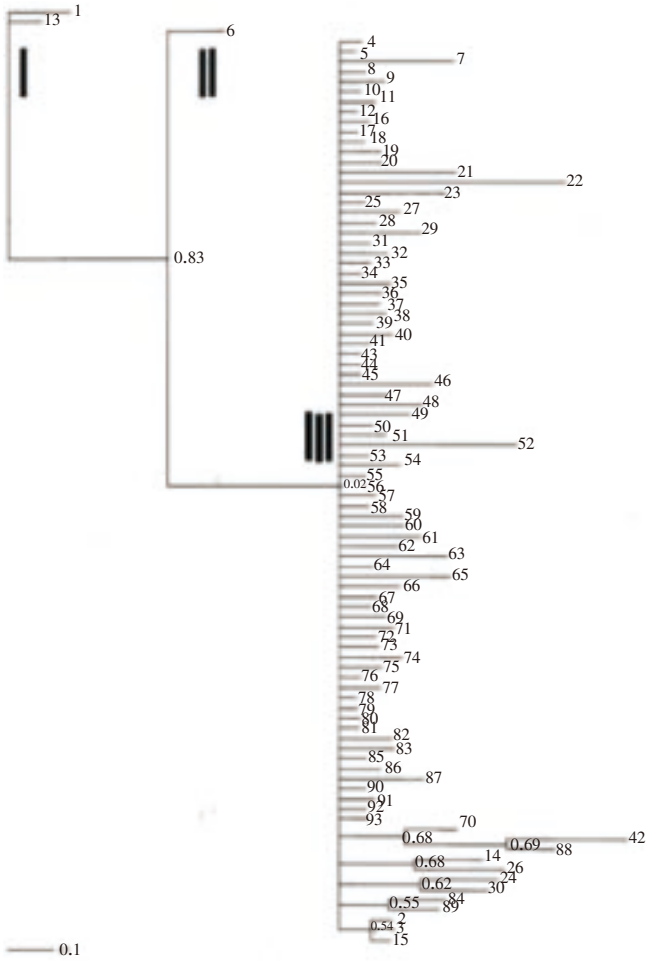

Figure 7. Dendogram derived from RAPD assays generated by using JCT1 and primer JCT9.

A dendogram was constructed firstly with primer JCT1 (Figure 6) and secondly with primers JCT9 (Figure 7) to compare the individuals. There are three groups (I to III) of *M tuberculosis* strains prevalent in Yaounde city, Cameroon. The major group III (96%) was present and transmitted continuously. Group I and II had been eradicated. We selected bands 200 and 500 bp that have a good potential for diagnostic analysis because they are specific only to complex tuberculosis. The 200 bp product not only has a good potential for diagnostic but this band also allowed for the discrimination of *M. tuberculosis* from *M. bovis* by a simple mutation on the atsa gene.

Table 2

Sequences of the 10 RAPD primers used in this study.

Notation	Primer sequence
JCT1	5′-TGCCGAGCTG-3′
JCT2	5′-TGCCGGCTTG-3′
JCT3	5′-CCCGTTGCCT-3′
JCT4	5′-GGACGACAAG-3′
JCT5	5′-ACCTCGGCAC-3′
JCT6	5′-GGCTGGTTCC-3′
JCT7	5′-CTGCGCTGGA-3′
JCT8	5′-ACCTGGGGAG-3′
JCT9	5′-GTCAGTGCGG-3′
JCT10	5′-ACCTCGGCAC-3′

4. Discussion

Drug-resistant tuberculosis is a serious problem throughout the world. Resistance to RIF (RIF) is mainly caused by the mutations in the *rpoB* gene coding the beta-subunit of RNA Polymerase[22,23]. Analysis of the conserved region within the *rpoB* gene of 5 RIF-resistant *M. tuberculosis* isolates showed that all have mutation within 69 bp segment of the gene, and two different types of mutations were detected within codon 516 (60%) and 531(40%). These findings are restricted to only two codon sites within the *rpoB* gene. The lack of diversity could be due to the small number of RIF-resistant strains that were studied and the limited population from which isolates were obtained. Adequate explanation of the molecular level must account for both the position of the affected amino acids and the particular substitution residue. The likelihood of pathogen survival may be enhanced by selection of mutations that balance a high level of antibiotic resistance with deleterious metabolic effects[24]. Without a detailed understanding of the structure-function relationships of the RNA polymerase B subunit, as determined by X-ray crystallographic and other types of analyses, it is impossible to offer adequate explanations of the effects of mutations at 516 and 531[25, 26]. Since all RIF-resistant *M. tuberculosis* isolates studied so far have missense mutations within a confined segment of the *rpoB* gene, molecular techniques could be used to analyze this segment of the gene for the detection of mutations associated with RIF resistance in tuberculosis[22, 23].

A comparison of the present results with other studies showed that the mutations at codon 531 also predominate in Poland[23]. In contrast with previously published data, the frequency of mutations at codon 516 was found to be high in Poland[27]. A high frequency of changes at this codon was also described recently for MDR *M. tuberculosis* isolates from East Hungary, Latvia[28], North India and East Asia[29]. The present study, demonstrates the feasibility of using TA cloning and sequencing to characterize *rpoB* mutations associated with RIF resistance. One of the main advantages of this technique is that it permits the identification of all mutations in the targeted sequence, and as demonstrated, successfully identifies uncharacterized mutant alleles. Because the TA cloning sequencing strategy is generally applicable to any gene, it is readily adaptable for the identification of point mutations associated with resistance to other anti- tuberculosis medications, such as isoniazid and streptomycin.

In the RAPD analysis, ten decamer primers were screened to obtain the two primers which revealed the most genetic polymorphisms among the isolate samples. Population genetics tests revealed a basically clonal structure for this population. Genetic analysis also showed a genetic polymorphism for the species *M. tuberculosis*, weak cluster individualization, and an unexpected genetic diversity for a population in such a high-incidence community.

The population genetics studies revealed a basically clonal structure for this population, without excluding rare genetic exchanges. Strains with high degree of changes in RAPD patterns correspond to the strains that diverged in their genomes. Strains with identical or small changes in RAPD patterns may have a common ancestor; they would correspond to recent transmission. For this study, we found that fingerprints generated by using two primers were necessary to render the method sufficiently differential. On the basis of these analyses, we consider that recent transmission of tuberculosis, and not only reactivation of latent disease, contributes substantially to the increase in tuberculosis in Yaounde-Cameroon. Our results show not only that the prevalence of *M. tuberculosis* in Cameroon city Yaounde is due to transmission rather than reactivation, but also that the lack of efficient diagnostic tool also may play a role in tuberculosis transmission. In low-resource countries, the best way to stop tuberculosis includes early diagnosis, followed by prompt treatment to minimize the transmission and also decrease spread of the resistant species.

Several studies have been done to detect *M. tuberculosis* in respiratory and other clinical samples by amplifying IS6110 sequences of *M. tuberculosis* by polymerase chain reaction (PCR) test with encouraging results. Most of these studies have mainly presented the DNA with small IS6110 sequence and others without IS6110 sequence of mycobacterial genome[29,30]. RAPD was also used to select the new site of diagnostic sequence by PCR. The 500 bp fragment selected herein is specific to *M. tuberculosis* and *M. bovis*. The specific band obtained (200 bp), has a good potential for diagnostic analysis because it is specific only to the complex tuberculosis and also it has a lower molecular weight. This band could easily be used as probe to discriminate *M. tuberculosis* to *M. bovis*. The global control of tuberculosis remains a challenge from the standpoint of diagnosis, detection of drug resistance, and treatment[31-34]. Identification of homogeneity by RAPD technique between strains of M. tuberculosis can help not noly in certifying the route of infection and the pattern of spread in a nationwide outbreak, but also in recognizing whether a particular infection represents reactivation or reinfection[35-38].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Tedom is grateful to Marine Biological Laboratory, Woods Hole, MA for a study fellowship. The financial support of IPICS/ISP (project CAM 01) is also gratefully acknowledged for financial assistance.

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