

The Use of the PCR-Based Dot-Blot Hybridization Assay to Detect Resistance Markers to Rifampicin and Streptomycin in *Mycobacterium tuberculosis* Isolates from the SW Region of Cameroon

Irene Ane-Anyangwe^{1*}, Wilfred Fon Mbacham², Henry Dilonga Meriki³, Teyim Pride^{1,2}, Theresa Nkuo-Akenji¹, Veronique Mbeng Penlap², Leopold Djomkam Tietcheu², Damian Nota Anong¹, Akindeh Mbuh Nji², Vincent P. K. Titanji¹

¹Department of Biochemistry and Microbiology, Faculty of Science, University of Buea, Buea, Cameroon

²Laboratory for Public Health Research Biotechnologies, Biotechnology Centre, University of Yaoundé, Yaoundé, Cameroon

³Mezam Polyclinic, Bamenda, Cameroon

Email: ianyanyangwe@yahoo.com

Received 16 March 2016; accepted 4 June 2016; published 7 June 2016

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Abstract

Drug sensitivity testing to establish resistance to TB drugs takes many months to arrive at. Public health physicians have difficulties with such an approach due to long wait periods and cannot use it to establish community wide prevalence as a way to understand where resistance may be emerging faster and to limit its spread. The objective of this study was to use the dot-blot hybridization technique in the detection of resistance to rifampicin (RIF) and streptomycin (SM) in South-Western Cameroon and to compare the technique with the routine culture and drug susceptibility testing for detecting resistance in a resource poor country, Cameroon. A hospital-based study was conducted at the Regional hospitals of Buea and Limbe and Tiko Central Clinic. Tuberculosis (TB) patients aged 15 to 50 (mean age: 30.50 ± 8.33 standard deviation) were recruited for the study between December 2006 and April 2007. Cultures from 59 patients were tested for rifampicin and streptomycin sensitivity by the modified proportion method and mutational analysis for *rpoB* codon 516 and *rrs* codon 513 was performed by the dot-blot hybridization technique. Of the 59 sputum samples collected (36 were males and 23 were females) came from Buea 19 (32.2%), Limbe

*Corresponding author.

How to cite this paper: Ane-Anyangwe, I., Mbacham, W.F., Meriki, H.D., Pride, T., Nkuo-Akenji, T., Penlap, V.M., Tietcheu, L.D., Anong, D.N., Nji, A.M. and Titanji, V.P.K. (2016) The Use of the PCR-Based Dot-Blot Hybridization Assay to Detect Resistance Markers to Rifampicin and Streptomycin in *Mycobacterium tuberculosis* Isolates from the SW Region of Cameroon. *Journal of Tuberculosis Research*, 4, 72-79. <http://dx.doi.org/10.4236/jtr.2016.42009>

20 (33.9%) and Tiko 20 (33.9%) towns respectively. Amplification for the gene showed that there was (59) 100% amplification with primers used for *rpoB* genes and 43 (72.9%) amplification with primers used for the *rrs* gene. Mutational analysis demonstrated that resistance to RIF was common in females (52.1%) than males (41.7%) while 6% of the samples were indeterminate. 12 (20.3%) samples showed phenotypic and genotypic resistance to RIF compared to 34 samples (58.1%) for SM. Phenotypic resistance and genotypic susceptibility were found in 5 (8.5%) RIF and 3 (4.7%) SM compared to phenotypic susceptibility and genotypic resistance that were found in 2 (3.5%) RIF and 3(4.7%) SM. Double mutation on *rpoB* and *rrs* genes occurred in 8 (13.6%) DNA samples. Resistance to RIF and SM due to mutations on the *rpoB* and *rrs* genes respectively in the SW region was found to be high and comparable to the drug susceptibility testing by 92%, (95% CI: 75.7 - 99.1). The Dot-blot technique will be useful in rapidly assessing the effectiveness of national TB control programs in limiting the spread of resistance strains in Cameroon.

Keywords

PCR-Based DOT-Blot Analysis, Rifamycin, Streptomycin, SW Region

1. Introduction

Tuberculosis (TB) remains one of the world's most serious problems, causing about 3 million deaths per year. It accounts for about one third of all preventable adult deaths globally [1] [2]. TB will undoubtedly increase in prevalence in most countries due to increasing number of multidrug resistant (MDR) *Mycobacterium tuberculosis* strains [3] [4] and the Human Immunodeficiency Virus (HIV) pandemic [5]. As such the World Health Organization declared TB to be a global public health emergency [5].

The emergence of multi-drug resistant strains of *Mycobacterium tuberculosis*, with resistance to at least rifampin (RIF) and isoniazid (INH) is due to particular genomic mutations of *Mycobacterium tuberculosis*. Ten (10) genes are known to be linked to resistance to the first line antituberculosis drugs. These drugs include *KatG*, *inhA*, *aphC*, *kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance; *rpsL* and *rrs* for Streptomycin (SM); *embB* for Ethambutol (EMB) *apncA* for Pyrazinamid (PZA) resistance. Rifamycin is a key component of the World Health Organization Directly Observed Therapy Scheme ([DOTS], short course) regimen and, since RIF mono-resistance is extremely rare, INH resistance is usually preceded by that of RIF. Resistance to RIF is considered to be the MDR marker [6] and has been shown in studies that RIF resistance of between 95% - 98% is caused by mutation in the *rpoB* gene encoding the RNA Polymerase B-subunit [7] [8]. Sputum smear microscopy has been the most cost effective diagnostic technique used for TB in most developing countries, where culturing is usually impossible. Early diagnosis, effective treatment and successful cessation of transmission are major strategies in the control of TB.

A PCR-based dot-blot hybridization strategy using labeled specific probes has been used to screen for mutation in genes responsible for resistance to drugs [9] [10]. Molecular methods can be used to determine the genetic changes that lead to antibiotics resistance. However, the use of such methods is restricted to well-equipped laboratories. For countries which do routine culture of specimens for *Mycobacterium tuberculosis*, the ability to type isolates using small amounts of DNA may be of additional valuable. This study aimed at identifying the presence of Drug Resistance (DR) by comparing phenotypic (DST) and genotypic (Dot-blot) results, targeting *rpoB* and *rrs* genes.

2. Methods

2.1. Admission Criteria and Informed Consent

Ethical clearance for this work was obtained from the South West Regional Delegation of Public Health. Following the patient's or guardian's consent, early morning sputa (0.5 - 1 mL) were collected. Only participants diagnosed as smear positive pulmonary tuberculosis were enrolled in the study.

2.2. Sample Collection

Sputum samples were collected between December 2006 and April 2007 from 59 TB patients coming from different localities within the South West Region for pulmonary Tuberculosis diagnosis at the Regional hospitals of Buea and Limbe and Tiko Central Clinic. Data on medical history, age, gender, residence, bacterial colony counts were collected. Smears were prepared, stained with Ziehl-Neelsen stain [11] and viewed under a light microscope at $\times 1000$ magnification. The 59 sputum samples for culture were put into 50 ml screw-capped falcon tubes and sent to the Mezam Polyclinic within 24 hours, where culture and phenotypic drug sensitivity testing were carried out.

2.3. Cultured Sample for DNA Analysis

Culture and antibiotic susceptibility testing was done as described previously [12]. Specimens were liquefied and decontaminated using N-acetyl-L-cysteine-sodium hydroxide (BD MycoPrepTM, Becton Dickinson Diagnostic System, Maryland, and USA) following manufacturer's instructions [13] and concentrated by centrifugation at 4000 rpm for 15 - 20 minutes at room temperature [14]. Samples were inoculated in triplicate on three Lowenstein-Jensen media slants (one supplemented with 0.4% pyruvate). The cultures were incubated at 37°C and examined weekly for a maximum duration of 8 weeks. Identification of isolates as *Mycobacterium tuberculosis* was by microscopy of Ziehl-Neelsen stained smears, colony morphology, nitrate reduction, niacin accumulation, catalase activity at 25°C and 68°C [15]. Drug susceptibility testing was performed on Lowenstein-Jensen medium slants by the standard indirect modified proportion method of Canetti *et al.* [16]. A loop full of the cultured cells was put into a 1.5 mL micro centrifuge tube for DNA extraction. About 1.0 mL of sterile distilled water was added and placed in a heating block at 100°C for 30 min. The lysate was centrifuged for 2 minutes and the supernatant used for PCR assay according to Nolte *et al.* [17]. Positive control was achieved with the reference strain H37Rv a well characterized molecular clinical isolate of *M. tuberculosis* [12]. Distilled water served as negative control.

2.4. PCR Amplification

Some 2 μ L of genomic DNA was used as template for amplification in a 23 μ L reaction mixture consisting each at final concentrations of magnesium chloride 2.5 mM; dNTPs 200 μ M each, 5' primer and 3' Primer, 0.2 μ M and 1.5 U of Taq polymerase (Promega). The reaction was conducted as follows: 93°C for 3 minutes followed by 35 cycles at 93°C for 1 minute, annealing at T_m for 1 minute and extension step at 72°C for 2 minutes. Final extension was done at 72°C for 10 minutes. The optimal annealing temperature was 58°C for *rpoB* gene and 64°C for *rrs* gene.

2.5. Amplification and Dot-Blot Hybridization

Efficient amplification of PCR product was confirmed by gel electrophoresis on 12% polyacrylamide gel. To verify the possibility of an omission in a gene sequence, a mix-in experiment was performed with 1 μ L of each DNA sample that did not amplify in the presence of another gene that did amplify. Oligonucleotides were labeled at the 5' end by phosphorylation with [³²P] ATP (Amersham) as described previously.¹⁸ PCR products were heat-denatured at 95°C for 10 minutes and applied under vacuum to a Hybond-N+ nylon filter (Amersham) in a dot-blot apparatus (Bio-Rad). The DNA was fixed unto the membrane by baking at 80°C for 1 hour. For radio isotopic detection, each filter was hybridized in 5X SSPE buffer and finally washed in 1.5 \times SSPE buffer for 10 minutes at 74°C as described previously [18]. Autoradiography was done at room temperature for 2 - 3 hours [19]. To re-probe, the membrane was stripped by incubation at 46 °C in 1 M NaOH and neutralization was carried out according to the method of Victor, *et al.* [18].

2.6. Statistical Analysis

Data analysis was done using Microsoft Excel 2003 and SPSS for Windows version 11.0 (Somers, NY). The Pearson's Chi square test was used to compare qualitative variables. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Study Population Characteristics

A total of 59 patients (36 males and 23 females) were enrolled in the study. Participants were aged ranging from of 15 to 50 years, mean age: 30.50 ± 8.33 standard deviation (SD). Majority of the participants (42.4%) were between the ages 21 and 30 years. Nineteen patients (32.2%) came from Buea, 20 (33.9%) from Limbe and 20 (33.9%) from Tiko, which are the three major health areas in Fako Division of South-Western Cameroon (**Table 1**).

3.2. Drug Resistance Pattern by Age and Sex

There was no significant difference in the drug resistance to both drugs with sex ($p = 0.774$ for RIF) and ($p = 0.778$ for SM). However, more females 12/23 (52.1%) had RIF resistant TB than males 15/36 (41.7%), while more males 9/36 (25%) had SM resistant TB than females 5/23 (21.7%). No resistance to RIF was found in subjects ≤ 20 years, while the highest resistance to SM was seen in the age group 21 - 30 (28%), closely followed by those above 40 years (25%). The highest resistance to SM was recorded in the age group 21 - 30 years (56%), closely followed by age group 31 - 40 years (43.8%), and the age group greater than 40 years (41.7%) and that less than 20 years (25%).

3.3. Dot-Blot Mutational Analysis

Figure 1 shows the mix-in experiment used to verify the possibility of an omission in a gene sequence. All 59 (100%) amplified with the *rpoB* primer. Mutations for *rpoB* genes occurred in 14 (23.7%) of the samples (**Table 1a**). Forty three (72.9%) samples amplified with primers specific for the *rrs* genes (**Table 1**). Mutation resulting in resistance to streptomycin occurred in 27 (62.8%) of these (**Table 1**). Mutational analysis for drug resistance could not be performed on the remaining 16 (27.1%) samples, since they could not amplify with *rrs* primer.

3.4. Comparison between Phenotypic and Genotypic Resistances

Twelve of the 59 samples (20.3%) showed both resistance to drugs in vitro and carried the mutations that confer resistances to rifamycin (**Table 1**), while 25 (58.1%) of the 43 amplified were resistant both by drug susceptibility testing (DST) and by dot-blot to streptomycin (**Table 3**). Five out of 59 (8.5%) were resistant to rifamycin yet did not bear the mutations that confer resistance to rifamycin (**Table 2**). Similarly, 2 out of 43 (4.7%) were resistant to phenotypic DST but would not show resistant conferring mutations to streptomycin (**Table 3**). On the other hand 2 of the 59 (3.4%) bacterial DNA samples showed susceptibility in vitro yet and bore the gene

Table 1. Study participants characteristics.

Characteristics	n (%)	
	Categories	
Age group (years)	≤ 20	4 (6.8)
	21 - 30	25 (42.4)
	31 - 40	18 (30.5)
	>40	12 (20.3)
Sex	Male	36 (61.0)
	Female	23 (39.0)
Site of Collection	Tiko	20 (33.9)
	Buea	19 (32.2)
	Limbe	20 (33.9)

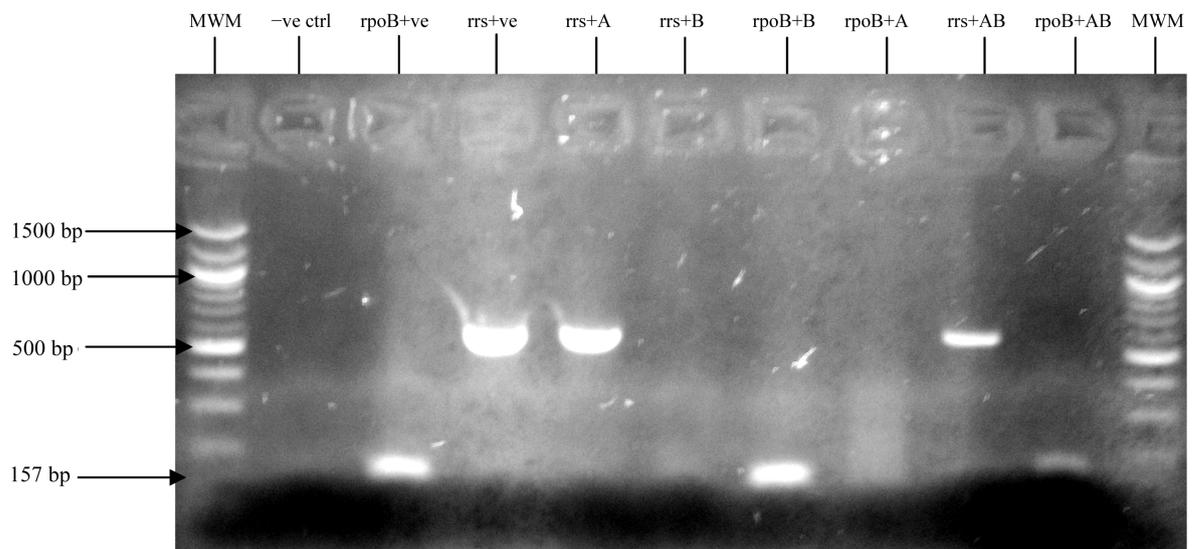


Figure 1. Mix-experiment gel electrophoresis. Mix-in experiment showing the amplified and non-amplified results. Amplification was identified by the presence of band in the wells on the gels. Bands were seen for molecular weight markers, positive controls for the genes *rpoB* and *rrs*, sample A was amplified with both *rpoB* and *rrs* genes, sample B was amplified only with *rpoB* gene as primer, and a mixture of A and B were amplified with both *rpoB* and *rrs* genes. No amplification identified by the absence of bands was seen for negative control and for sample B with *rrs* as primer.

Table 2. Comparability between *rpoB* gene dot-blot and phenotypic drug susceptibility testing.

	RIFAMYCIN (<i>rpoB</i> gene)	Phenotypic DST, n (%)			% Sensitivity	% specificity	% PPV	% NPV
		R	S	Total	(95% CI)	(95% CI)	(95% CI)	(95% CI)
DOT-BLOT	R	12	2	14	70.6	95.2	85.7	88.9
	S	5	40	45	(44.0 - 89.7)	(83.8 - 99.4)	(57.2 - 98.2)	(76.0 - 96.3)
	Total	17	42	59				

Table 3. Comparability between *rrs* gene dot-blot and phenotypic drug susceptibility testing.

	STREPTOMYCIN (<i>rrs</i> gene)	Phenotypic DST, n (%)			% Sensitivity	% specificity	% PPV	% NPV
		R	S	Total	(95% CI)	(95% CI)	(95% CI)	(95% CI)
DOT-BLOT	R	25	2	27	92.6	87.5	92.6	87.5
	S	2	14	16	(75.7 - 99.1)	(61.7 - 98.5)	(75.7 - 99.1)	(61.6 - 98.5)
	UA	16	0	16				
	Total	32	27	59				

Legend: R—Resistance; S—Sensitive; UA—Unamplified; DST—Drug Susceptibility Testing; In (b) above there were 16 which did not amplify with primers to *rrs* gene. Interestingly these come from the group that was expected to show mutations conferring resistance. Mix-in positive DNA experiments showed there were no inhibitions nor nuclease activity since the added DNA did amplify.

mutation for resistance to rifamycin (**Table 2**). For Streptomycin it was 2 out of the 43 (4.7%) bacterial DNA samples that amplified (**Table 3**). Forty (67.8%) of the samples showed both phenotypic and genotypic susceptibility to rifamycin and 14 (32.6%) were both phenotypically and genotypically susceptible to streptomycin (**Table 2** and **Table 3**). Double mutation on both the *rpoB* and *rrs* genes resulting in multiple (double) resistance was found in 8 (13.6%) of the samples.

Discrepancies: Some discrepancies were identified between the phenotypic and genotypic drug pattern. False negative resistance, that is, resistance by phenotype and susceptible by genotype was found to be 8.5% in rifamycin and 4.7% in streptomycin. False positive resistance, that is, susceptible by phenotype and resistant by genotype was 3.4% in rifamycin and 4.7% in streptomycin. There was a significant difference in this discrepancies $p < 0.001$ to both drugs. False negative results were predominant in rifamycin while false positives were predominant in streptomycin. Mix-in experiment results demonstrate that amplification was possible in previously unamplified samples of the SM gene *rrs*.

4. Discussions

A high prevalence of drug resistant strains of *Mycobacterium tuberculosis* both phenotypically (28.8% for RIF and 54.2% for SM) and genotypic resistance (23.7% for RIF and 45.8% for SM) (Table 2 & Table 3) from the South Western Cameroon is reported. We showed that the Dot-blot compared to DST had a high positive predictive value (92.6%, 95% CI: 75.7 - 99.1) suggesting that the Dot-blot could be used in public health settings to establish the prevalence of mutations to TB drugs and the potential of a population to fail therapy.

Amplification was complete 59 (100%) with *rpoB* (Table 2) but in only 43 (72.9%) samples with *rrs* gene (Table 3). A mix-in experiment was performed with the 16 samples which did not amplify. It turns out that all 16 samples are complementary to the quadrant expected to show resistant mutations (Table 3). The absence of signal could not have been due to the presence of an inhibitor or nuclease as the added positive DNA did amplified with other primers. It is possible that one mechanism for Streptomycin resistance could be gene segment omissions and warrants further investigations. Double mutation on both the *rpoB* and *rrs* genes occurred in 8 (13.6%) DNA samples, is indicative of multiple resistances. Mono-resistance to rifamycin is rare and its presence is indicative of multiple drug resistance (MDR) [6]. Monotherapy for streptomycin was used 50 years ago to treat tuberculosis and this monotherapy resulted to high resistance [20]. Resistance in this study was common in streptomycin than in rifamycin. This explains why the principle behind modern chemotherapy for tuberculosis lies in the association of several drugs to which the bacilli are sensitive to [21]. Females were more resistant (52.1%) than males (41.2%) to rifamycin while males more resistant to streptomycin (25%) than females (21.7%). Sex, previous treatment, age, and work type were some of the risk factors that have been reported for drug resistant TB [22] [23].

In comparing phenotypic and genotypic results, some discrepancies were noted. False positive phenotypic resistance in rifamycin (3.4%) and streptomycin (4.7%) occurred. False negative phenotypic resistance was predominant in rifamycin (8.5%) than streptomycin (4.7%). It had been found out that the routine drug resistance testing in *M. tuberculosis* is difficult and can sometimes give inconsistent results [6] and it is one of the most difficult technique to standardize for a diagnostic mycobacteriology laboratory due to alterations in the anti-mycobacterial activity of various drugs when incorporated into media [24]. There is therefore an urgent need to develop and make use of a high thorough put tool for detecting MDR. Considering that most drug resistant cases occur in resource poor countries [5]. Rapid detection of drug resistance could optimize treatment and improve the outcome of patients with drug resistant TB, but especially important in the prevention of transmission of drug resistant TB. It can also be used in drug surveillance studies, and will be cost-effective in resource poor countries where most MDR-TB patients reside. Drug susceptibility test is usually performed in order to provide information for the treatment of individual patients. These molecular methods cannot be used for individual patient management but can serve public health approach surveys in establishing how wide spread resistance to various drugs might be. Sensitivity testing by culture requires 3 - 8 weeks and is not usually reliable. Molecular methods are designed to exploit the observation that specific mutation found in resistant strain are absent in susceptible organisms.

5. Conclusion

We conclude that the PCR-based dot-blot hybridization technique is comparable to the DST in establishing the resistance profile of a population to RIF. The dot blot is rapid, reproducible, not technically demanding and shortens the time of diagnosis from several weeks to two days.

Limitations of the Study

The difficulty to isolate DNA directly from sputum was a limitation. Other limitations included the fact that all

the samples did not amplify for *rrs* genes, and due to the lack of MTB culture facility in the South West Region in Cameroon, sputum had to be carried all the way to the North West Region for culture and DST.

Acknowledgements

IAA was supported by the University of Buea staff development grant; VPKT was supported by the IPICS/CAM01 Project; TMP received financial supported from the Epidemiology Research Laboratory and IAEA Grant Program RAF6040. WFM was supported by the Yaoundé University 1 grant program UYI/FS/04567Z4/wfm/13, the RAF6040 IAEA grant and the GABRIEL Network through the AFRICARAMI EU Project and the Mezam Polyclinic tuberculosis unit.

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