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Full Length Research Paper

Detection of resistance-associated mutations in *Mycobacterium tuberculosis* isolates in Cameroon using a dot-blot hybridisation technique

Wilfred Fon Mbacham[1,2]*, Leopold Djomkam Tientcheu[1,2], Veronique Beng Penlap[1,2], Christopher Kuaban[3], Sara Eyangoh[4], Hubert Wang[5], Jean Bickii[6], Palmer Masumbe Netongo[1,2], William Titi Lembe[5], Olama Abega[7], Njikam Njifutie[8], Teyim Pride[1] and Baldip Khan[9]

[1]Biotechnology Centre of Nkolbisson, University of Yaounde I, Cameroon.
[2]Department of Biochemistry, Faculty of Science, University of Yaoundé I, Cameroon.
[3]The Faculty of Medicine and Biomedical Sciences, University of Yaounde I, Cameroon.
[4]Centre Pasteur du Cameroun (CPC), Yaounde, Cameroon.
[5]National Committee for the Fight against Tuberculosis, MINSANTE, Cameroon.
[6]The Institute for Medical and Medicinal Plant Research, MINRESI, Cameroon.
[7]Department of Physics, Faculty of Science, University of Yaounde I, Cameroon.
[8]Department of Animal Biology and Physiology, University of Yaoundé I, Cameroon.
[9]International Atomic Energy Agency, Vienna, Austria.

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***Mycobacterium tuberculosis* strains collected from patients with pulmonary tuberculosis in the West and Centre regions of Cameroon were culture-tested for the major anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol and streptomycin). Of the 112 predetermined samples included in the study, 21 (18.7%) were sensitive to all the drugs, while 91 (81.3%) were resistant to at least one drug. Resistance to isoniazid was the most common (79.1%), followed by rifampicin (65.9%), streptomycin (62.6%) and ethambutol (38.5%), 50% of the samples were qualified as '(resistant to at least isoniazid and rifampicin). A PCR-based dot-blot hybridization strategy was used to detect mutations at different loci in five genes associated with resistance to the drugs tested. For rifampicin resistance, the mutation on codon 526 of the *rpoB* gene was the most common (66.3%), followed by the codon 516 (60.5%) and the codon 531 (31.4%). The mutation on codon 513 of the *rrs* gene was the most encountered in streptomycin resistant strains (77.8%); while the mutation on codon 43 of *rpsL* gene was always associated to that of *rrs* gene. The mutation on codon 531 of the *rpoB* gene for rifampicin resistance (95.6%) was most prevalent in the samples from the Centre region compared to the West region (P = 0.0003). Generally, no significant differences were obtained on the prevalences of the other mutations analysed based on the regions, gender or the age of patients ($P > 0.05$). The dot-blot analysis, gave no result for the codon 306 of *embB* gene associated with ethambutol resistance under experimental conditions used in this study. The PCR-based dot-blot hybridization strategy was tested to validate the procedure on stored samples and could be a good surveillance method for rapid detection of the evolution of drug resistant *M. tuberculosis* in Cameroon.**

Key words: Drug resistance, gene mutations; dot-blot hybridization, tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a disease of major public health concern worldwide. It remains one of the world's most serious afflictions accounting for approximately 3 million deaths per year (Mathema et al., 2006). In Cameroon, TB remains a common disease with an estimated incidence of 100 cases per 100,000 populations/year (Kuaban et

*Corresponding author. E-mail: wfmbacham@yahoo.com.

Table 1. Probes used for PCR amplification.

Gene	Probe	Sequence	Tm (°C)
katG	katG315wt	GATCACC**AGC**GGCATCGAGG	66
	katG315mu	GATCACC**ACC**GGCATCGAGG	66
	katG315dmu	GCGATCACC**ACA**GGCATCGA	64
rpoB	rpoB531wt	AGCGCCGACTG**TCG**GCGCTG	70
	rpoB531mu	AGCGCCGACTG**TTG**GCGCTG	68
	rpoB526wt	GGGTTGACC**CAC**AAGCGC	60
	rpoB526mu	GGGTTGACC**GAC**AAGCGC	60
	rpoB516wt	TTCATG**GAC**CAGAACAACCG	60
	rpoB516mu	TTCATG**GTC**CAGAACAACCG	60
rpsL	rpsL43wt	ACCACTCCG**AAG**AAGCCGAA	62
	rpsL43wt	ACCACTCCG**AGG**AAGCCGAA	64
rrs	rrs512/3wt	ACGTGCCAG**CAG**CCGCG	60
	rrs512/3mu	ACGTGCCAG**CTG**CCGCG	60
embB	embB306wt	CCTGGGCATGGCCCGAGTCG	70

al., 2000a, b). This epidemic is further complicated by the emergence of drug resistant strains of *Mycobacterium tuberculosis* which pose a threat to national tuberculosis control programme and often, result in high-level mortality (Victor et al., 1999).

The level of anti-tuberculosis drug resistance in a community is usually considered as a measure of the success of its tuberculosis control programme and gives indication of suitable drug regimens for future use. Traditional drug susceptibility testing depends on a positive culture for diagnosis and subcultures for susceptibility testing. Usually 4 to 12 weeks are required for a positive culture to be concluded' and a further 4 to 8 weeks susceptibility testing on solid media using the indirect proportion method as described by Canetti et al. (1963). Drug susceptibility testing is not only time-consuming, but there are significant technical problems in the standardization of these tests, such as difficulties to establish an appropriate inoculum volume, the stability of the drugs in different culture media and the reliability of results when testing the drug (Victor et al., 1999; Mitchison, 2005). Furthermore, this laboratory delay can constitute a future obstacle for the treatment of patients infected by drug resistant *M. tuberculosis* strains. Rapid diagnosis of drug resistance may help to stop the chain of transmission of multidrug-resistant tuberculosis. In *M. tuberculosis,* the molecular basis of drug resistance to all the first-line drugs is due to mutations in target genes.

In Cameroon, information on the prevalence of specific mutations associated with drug resistance in *rpoB* for rifampicin, *katG* for isoniazid, *embB* for ethambutol, and *rrs* and *rpsL* genes for streptomycin in *M. tuberculosis* strains is scarce. This study aimed to implement the PCR-based dot-blot hybridization strategy using radioactively labelled DNA probes as tools to rapidly identify specific mutations within the drug resistant genes of *M. tuberculosis* strains recovered from TB patients.

MATERIALS AND METHODS

Clinical isolates

Clinical isolates included in this study were collected from patients tested for tuberculosis in the West and Centre province of Cameroon. Ethical clearance was obtained from the Cameroon National Ethics Committee and consent was sought from patients recruited between 2002 and 2006 from the Western (76 patients) and Center (36 patients) regions of Cameroon noted for high rates of tuberculosis (Kuaban et al., 2000a, b). The isolates were all identified as *M. tuberculosis* after culture on Löwenstein-Jensen (LJ) and Löwenstein-Jensen containing Pyruvate (LJ + P) media. Antibiotic susceptibility testing was performed using the indirect proportion method on LJ as described by Canetti et al. (1963). The following anti-tuberculosis drugs were tested: rifampicin (RMP) 40 mg/l, isoniazid (INH) 0.2 mg/l, streptomycin (SM) 4 mg/l, and ethambutol (EMB) 2 mg/l. Drug resistance was defined as growth on a drug-containing medium greater than or equal to 1% of that on the control medium.

PCR amplification

Well characterised *M. tuberculosis* reference H37Rv and drug resistant reference strains *of M. tuberculosis* were used as controls in PCR and dot-blot hybridization assays. The probes used in this study as shown in Table 1 were directed toward mutations most frequently described in the literature. All samples were tested for mutations at the following codons: *katG315, rpoB531, rpoB526, rpoB516, rpsL 43, rpsL88, rrs-513, rrs-491*, and *embB306*. Primers to amplify regions of the *katG*, *rpoB*, *embB*, *rrs* and *rpsL* genes

Table 2. Distribution of study TB patients with culture-adapted *M. tuberculosis.*

Age group	Centre (n = 36)				West (n = 76)				Total	
	Male		Female		Male		Female			
	n	%	n	%	n	%	n	%	n	%
15-24	1	14.3	6	85.7	7	36.8	12	63.2	26	23.2
25-34	**11**	**57.9**	**8**	**42.1**	**16**	**50**	**16**	**50**	**51**	**45.5**
35-44	6	75	2	25	6	54.5	5	45.5	19	17
45-54	1	50	1	50	8	80	2	20	12	10.7
55-	0	0	0	0	2	50	2	50	4	3.6
Total	19	52.8	17	47.2	39	51.3	37	48.7	112	100

were used together with genomic DNA contained in 2.5 µl of the lysate of each sample as template for amplification in a 50 µl reaction mixture consisting of 50 µM magnesium chloride, 200 µM of each dNTP (Promega, UK), 0.25 µM of each forward and reverse primer and 0.15U of *Taq* polymerase (Hotstar Quiagen). Reaction mixtures were amplified in a Biometra® 2000 thermal cycler as follows; 95°C for 15 min followed by 45 cycles at 94°C for 1 min, annealing at corresponding temperatures set for 1 min and extensions at 72°C for 10 min. Amplifications were confirmed by 1.5% agarose gel electrophoresis

Dot-blot hybridization

Wild-type and mutant-specific oligonucleotides to screen for the presence or absence of specific mutations in five genes associated with resistance to anti-tuberculosis drugs were obtained from the Medical Research Council, Centre for Molecular and Cellular Biology, University of Stellenbosch, South Africa. Oligonucleotides were 5' end-labelled with [γ-^{32}P]-ATP (Amersham) in a 50 µl reaction mixture consisting of oligonucleotides, 10 pM; T4 polynucleotide kinase (PNK) [Amersham], 10 U/µl; PNK buffer, 1X and [γ-^{32}P]-ATP, 30 µCi. The reaction mixture was incubated at 37°C for 30 min and then inactivated at 72°C for 30 min. An aliquot of 10 µl of each PCR product was buffered in 0.4 M NaOH and 25 mM EDTA final concentration in 200 µl and heat-denatured at 95°C for 10 min. This was then applied into a Hybon-N$^+$ nylon filter (Amersham) under vacuum in a dot-blot apparatus (Biorad). The DNA was fixed onto the membrane by baking at 80°C for 2 h. Each membrane (one membrane per gene) was hybridized with the corresponding probes in a hybridization mixture as described by Victor et al. (1999). The membranes were washed in 50 ml of 2X SSPE containing 0.1% SDS for 30 min at room temperature (25 to 27°C) followed by a second wash in 50 ml of 1X SSPE + 0.1% SDS for 30 min at the annealing temperature of each probe. Autoradiography was done at room temperature using ultra-sensitive Kodak films after 24 to 48 h of exposure. To reprove, the membranes were stripped by incubation at 50°C for 30 min in 200 ml of 0.4 M NaOH with constant shaking and neutralized for 30 min at 42°C in 200 ml of neutralizing buffer containing 0.2 M Tris-HCl (pH 7.5), 0.1% SDS, 0.1X SSC.

Statistical analysis

Data were analysed using the SPSS version 12 software. Proportions were compared by the chi squared (X^2) test to establish differences between age, gender or geographical regions. A difference was considered significant if $p < 0.05$.

RESULTS

First-line drug resistance patterns

The patient profile of the study is contained in Table 2. Therein we observed that of the 112 patients from whom clinical *M. tuberculosis* isolates were obtained, 58 (51.8%) were males and 54 (48.2%) females. The ages of patients ranged from 15 to 70 years. The drug resistance distribution according to the patient's gender and age showed no significant differences ($p > 0.05$). The susceptibility to first-line anti-TB drugs rifampicin, isoniazid, streptomycin and ethambutol was determined by the indirect proportions method on LJ medium as described by Cannetti et al. (1963). Of the 112 isolates, 21 (18.7%) were susceptible to all the antibiotics tested and 91 (81.3%) were resistant to at least one of the first-line anti-tuberculosis drugs. Resistance to rifampicin and isoniazid (classified as multiple drugs resistance) were found in 56 (50%) of the 112 strains isolates (Table 3). Resistance to any one of the drugs was observed in 26 (23.2%) isolates, 21 isolates were resistant to two drugs (18.8%), 20 to three drugs (17.9%) and 24 to all four drugs (21.1%). Of the 91 resistant isolates, the resistance to isoniazid was most common (79.1%), followed by rifampicin (65.9%) streptomycin (62.6%) and ethambutol (38.5%).

Detection and analysis of gene mutations

Amplification by PCR was successful for 90 TB isolates, and these were included for molecular characterization. Of these, 10 were cultured and tested as susceptible to all four drugs while 80 were resistant to at least one drug. Fifty four (54) were isolates of patients from the Western region and 36 isolates were from Centre region of

Table 3. Pattern of resistance to antituberculosis drugs, resistance among isolates from 112 patients with pulmonary tuberculosis in the West and Centre province of Cameroon.

Resistance to	Drug	Number of resistant	% of all patient (n = 112)	% of all resistant (n = 91)
One drug	H	9	8	9.9
	R	3	2.7	3.3
	S	12	10.7	13.2
	E	2	1.8	2.2
	Total	26	23.2	28.6
Two drugs	HR	12	10.7	13.2
	HS	7	6.3	7.7
	RS	1	0.9	1.1
	SE	1	0.9	1.1
	Total	21	18.8	23
Three drugs	HRS	12	10.7	13.2
	HRE	8	7.1	8.8
	Total	20	17.9	22
Four drugs	HRSE	24	21.1	26.4
Total resistant		91	81.3	100
Total susceptible		21	18.7	
*MDR	(HR & X)	56	50	61.5

H, isoniazide; R, rifampicin; S, streptomycin; E, ethambutol; X, 0 or S or E or SE, *MDR ,multidrug resistant.

Cameroon. Of the 90 isolates analysed for *rpoB* gene mutation, 4 (4.4%) had no mutation while 86 (95.6%) presented a mutation in at least one of the three codons analysed. Mutation in codon 526 was most common in 66.3% of the isolates, followed by that of codon 516 (60.5%) and 531 (31.4%). Single mutations within one codon only were most encountered at position 526 (31.4%), followed by 516 (11.6%) and 531 (1.2%). Mutation within two codons were the most prevalent in the clinical *M. tuberculosis* isolates studied (54.7%), the most common was *rpoB*516-526 pair (25.6%) followed by *rpoB*516-531 (20.9%) and *rpoB*526-531 (7%). Mutations involving all three codons were rare (2.3%) (Table 4).

However, the analysis of the prevalence of codon mutations in *rpoB* gene with respect to the two regions showed that there seem to be an equal distribution of the mutation of codon 526 and 516 in the Centre and Western regions of Cameroon (p = 1 and 0,896 respectively) whereas the mutation of codon 531 was most prevalent in isolates from the Centre region than the isolate from the West region of Cameroon ($p = 0.0003$). No significant difference was observed in the prevalence of the mutations on codons 516, 526, and 531 of *rpoB* gene when the gender and the age of patients were considered ($p > 0.05$).

The 570 bp region of *rrs* gene containing the codon 513 mutation is associated with streptomycin (SM) resistance. Autoradiography after hybridization of *rrs* gene with rrs513mu probe demonstrated that 77.8% or 70 of the isolates were *rrs* 513 mutant alleles. Of these 70 isolates each time, 35 of them had in addition the 43 *rpsL* gene mutation. However, the analysis of the prevalence of *rrs* and *rpsL* genes mutations with respect to the 2 regions demonstrated no significant difference in the distribution of these mutant allelles in the Centre and Western regions of Cameroon ($P > 0.05$). Equally no significant difference was observed on the prevalence of *rrs* and *rpsL* genes when the gender and the ages of patients were considered ($P > 0.05$).

Analysis of the *katG* gene mutation associated with the resistance to isoniazid, demonstrated that 24 (26.7%) were free of mutation at codon 315, while 66 (73.3%) had mutation at codon 315. No significant differences were noted on the prevalence's of these mutations in *M. tuberculosis* isolates with respect to region, gender and age of patients ($p > 0.05$). The analysis of codons 306 (*embB* gene) and 315 dmu (*katG* gene) with *embB*306 and *katG*315dmu probes respectively did not result in any hybridization experiments under experimental conditions described earlier in our laboratory.

DISCUSSION

Studies conducted so far to map anti-tuberculosis drug resistance in Cameroon (Kuaban et al., 2000a, b) were

Table 4. Pattern of codon mutations in *rpoB* gene from 90 clinical *M. tuberculosis* resistant isolates.

Mutation in:	Codon	Number of mutant	% of all isolate (n = 90)	% of all mutant (n = 86)
One codon	*rpoB526*	27	30	31.4
	rpoB516	10	11.1	11.6
	rpoB531	1	1.1	1.2
	Total	37	41.1	43
Two codons	*rpoB526-516*	22	24.4	25.6
	rpoB526-531	6	6.7	7
	rpoB516-531	18	20	20.9
	Total	47	52.2	54.7
Three codons	*rpoB526-516-531*	2	2.2	2.3
Total of mutant		86	95.6	100
Total of wild-type		4	4.4	

restricted only to phenotypic observations. Although, these studies gave an indication on the drug resistance profile of the country, molecular resistance markers could give a clearer. Previous studies have shown that the PCR-based dot-blot hybridization technique with probes for the wild-type and mutant alleles can be used to detect mutations in different loci of genes associated with resistance to isoniazid, rifampicin, streptomycin and ethambutol (Victor et al., 1999). In this study, high mutation rates revealed in the literature for codons 315 (*katG*), 531, 526, 516 (*rpoB*), 43 (*rpsL*), 513 (rrs) and 306 (*embB*), were the initial focus of this study to evaluate the distribution of these mutants in samples collected from two regions (Western and Centre) with high prevalences of TB in Cameroon (Kuaban et al., 2000) (Figure 1).

Resistance mutations observed in a majority of rifampicin resistant strains (>95%) are due to small deletions or insertions within an 81-base pair rifampicin resistance-determining region (RRDR) of the *rpoB* gene, between codons 507 and 533 (Ramaswany and Musser, 1998). These mutations include those at codon 516, 526 or 531 (Barfai et al., 2001; Kapur et al., 1994; Ahmad et al., 2002; Ramaswany and Musser, 1998). The analysis of *rpoB* gene by the dot-blot hybridization strategy is a clear indication of the versatility of the approach and could discriminate between sensitive and mutant alleles of the stored samples.

The *embB* gene associated with ethambutol resistance was successfully amplified by PCR, but the dot-blot hybridization analysis did not give any result. Similarly, non-hybridizations were encountered in other African countries where dot-blot hybridization was used to detect mutation in *embB* gene resulting in the change of approach to using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) (Johnson et al., 2006). This technique uses a triplet of primers set to efficiently detect in one step the genotypes with resistance to ethambutol. No result was also obtained when the *katG*315dmu probe was used to analyse mutation in *katG* gene associated with resistance to isoniazid. This result may be the result of the absence of the double mutation in the *katG* gene of the isolates analysed since this mutation was rarely detected in other regions of the world (Victor et al., 2002). However, the hybridization of *katG* gene with *katG*315wt probe presented a higher prevalence of codon 315 mutation within the *katG* gene (73.3%) comparable to that demonstrated by Victor et al., 2002) wherein the mutation of codon 315 were present in 60% of clinical *M. tuberculosis* isolates resistant to isoniazid worldwide.

The dot-blot hybridization pattern of clinical isolate from the Western and Centre regions of Cameroon showed that mutations of codon 526 (66.3%) and 516 (60.5%) were predominant in the rifampicin resistance-determining region (RRDR) of *rpoB* gene while codon 531 mutation was less frequent (31.4%). Our findings were different from that obtained in Egypt, where they reported a frequency of 40% mutation at codon 526 and 20% mutation at codon 516 (Nagwa et al., 2004). Our study however, showed similar results with that obtained in India, were they reported a 47% mutation occurring at codon 531 (Aparna et al., 2010). However a worldwide study of the mutation of the codon in the RRDR of *rpoB* showed that the mutation at codon 531 was the most predominant (45%) followed by that of codon 526 (21%) and mutations in codon 516 were rarely encountered (8%), (Rossau et al., 1997). This study however, showed a high prevalence of mutation at codon 516, similar to results published in Iran (Farahnoosh et al., 2008) where they reported 66% mutation in codon 516 of Central Asia family isolates. Such diversity of the prevalence of mutations within codons of *rpoB* gene does confirm the variability of these mutations within geographical regions worldwide and may also suggest movements of MDR

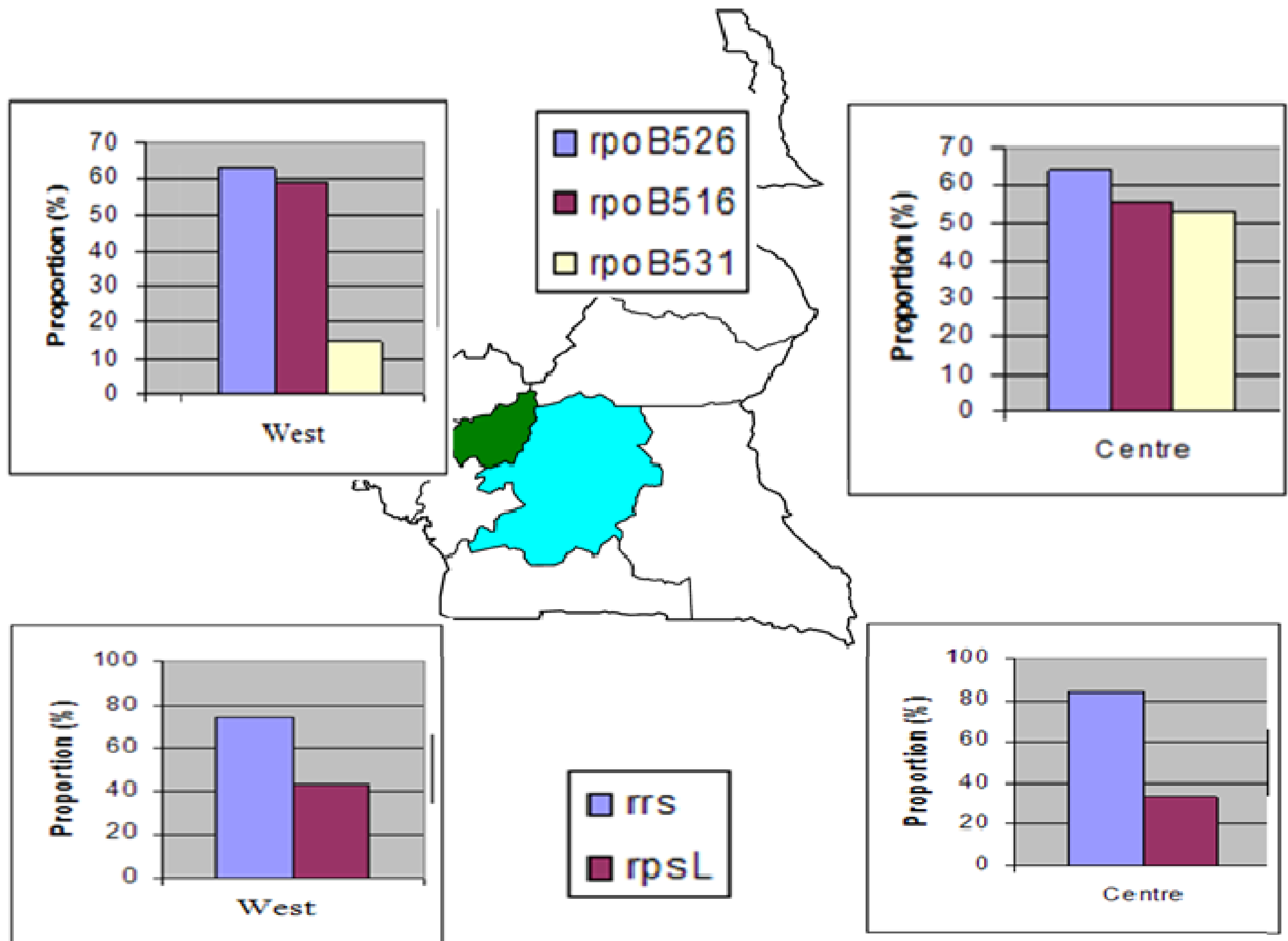

Figure 1. Prevalence of mutation in *rrs, rpsl* and *rpoB* genes in *M. tuberculosis* resistant isolates in Cameroon. Isolates originated from the Centre (n = 36) and West (n = 54) regions of Cameroon. rpoB526, rpoB516 and rpoB531 represent the codons with mutations in rpoB gene associated with Rifampicin resistance. Proportion represent the percentage of isolates containing mutation in the two regions (For rpoB526 and rpoB516 P > 0.05 and for rpoB531 P = 0.0003).

M. tuberculosis strains between regions of the world.

Mutations within *rpoB* gene suggest that codon 526 and 516 mutations were more or less equally distributed in the Centre and Western regions of Cameroon ($p > 0.05$). The high prevalence of codon 531 mutation in the Centre region (52.8%) compared to the Western region (14.8% - $p = 0.0003$), may demonstrate the region-specific genetic variability of drug resistant MTB. The analysis of the prevalence of mutations in *rrs* and *rpsL* genes however, suggested that there was no variation on the prevalence of these mutations within these two geographical regions studied. The 43 *rpsL* mutant gene seemed to occur in tandem with the 513 *rrs* mutant gene making it an early selectable marker for a rapid screening of streptomycin resistance in clinical isolates.

This rapid testing molecular technology needs to be developed further to proceed from sputum samples to detection thereby shorten the time for decision to treat. For the first time in Cameroon a dot-blot hybridization strategy was used to successfully establish drug resistance profile in clinical *M. tuberculosis* isolates. The method is reproducible and not technically demanding and can be adapted to amplify and detect mutations conferring resistance to anti-tuberculosis drugs directly from sputum samples (Victor et al., 1999) in a public health operational sense for quick surveillance (Mathema et al., 2006) and monitoring the evolution of anti-tuberculosis drug resistance in Cameroon.

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