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Original Research Article

Genetic basis of Resistance to Rifampin and Isoniazid in Cases of Failure and Relapse from first- line Drugs In Côte d'Ivoire

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A B S T R A C T

Multidrug-resistant Tuberculosis undermines our fight against TB. Abetter understanding of rifampin and isoniazid-resistance mechanisms can help to effectively fight against this disease. For failure and relapse cases with positive smear, two sputum were collected in centers of care and were transported at +4°C in National Reference TB laboratory at Institute Pasteur. After decontamination by NALC/NaOH method, DNA was extracted by Genolyse® kit. Genotype MTBDR*plus*® assay was performed according to the manufacturer recommendations. A total of 451 sputum were analyzed, 31 results were not interpretable. For the TB diagnosis, results of microscopic examination were consistent with those obtained with MTBDR plus assay. A total of 242 results of Genotype MTBDR*plus*® assay were analyzed. In the *rpoB* gene, the *D516V* mutation was the most frequent in failure: 33, 68% (32/95, 95% CI:24, 31-44, 11) against 22, 45% (33/147, 95% CI: 15, 98-30, 05) in relapse with no statistically significant difference(p=0,054). In the *katG* gene, the *S315T* mutation was the most observed with or without a mutation in their *inhA* promoter -8 positions. Punctual mutation is the main cause of TB-drug resistance in relapse and failure cases.

K e y w o r d s

Mutations, Failure, Relapse, Rifampin, isoniazid

Introduction

Patients in therapeutic failure or relapse cases of pulmonary tuberculosis pose a real public health problem in Côte d'Ivoire (Kouassi, 2004). Among these patients, the possibility of multidrug-resistant tuberculosis (MDR-TB) is high (Horo et *al*., 2010). Thus, with a prevalence estimated at 53.2% in 2013 (N'guessan et *al*., 2013), MDR-TB is likely to compromise the TB control if it is not diagnosed quickly and properly treated. The usually resistance mechanisms described are in connection with well-known gene mutations. Over 95% of rifampin-resistant strains have mutations in a well-defined, 81 base pair (pb) (27 codons) central region of the gene that encodes the

β-subunit of RNA polymerase (*rpoB*) (Ramaswamy et *al*., 1998). The most frequent mutations in the *rpoB*gene are *D516V, H526Y, H526D,* and *S531L*.

Isoniazid (INH) resistance mechanisms are particularly complex because they involve several genes. The *S315T* mutation in *katG* gene is the most current mutation in strains INH-resistant, and occurs in 50%-95% of INH resistant strains (Zhang et *al*., 2009).Approximately 8-35% of INH resistant strains have mutations in the promoter of *inhA* gene.

These mutations *C15T, A16G, T8C*, and *T8A* reflecting a low level of INH resistance (MIC=0.2-1 µg/ml) (Telenti, 1993; Musser 1998).The data in the literature describe that for rifampin (RMP) resistance, the most common mutation (65-95%) deteriorate the codon *526* or codon *531* and result in a high degree of resistance (MIC > 32 µg/ml). Alteration of others codons as codon *516* causes the appearance of a low resistance level (MIC< 32 µg/ml) (Ramaswamy et *al*., 1998).In South Africa, Barnard et *al*., 2008 showed that mutation *S531L* was observed in the more RMP-resistant strains (Barnard et *al.*, 2008).Concerning INH, Tessema et *al*., in Ethiopia showed that mutation *S315T* was the most observed in MDR-TB isolates of *M. tuberculosis* (Tessema et *al*., 2012). TB with local and regional specificities (Brossier et *al*., 2006), what is the situation among patients eligible for retreatment in our country.

Materials and Methods

Study sites

This study proceeded from March to June 2013. Patients experiencing failure and relapse were enrolled in the Regional Reference Centers of TB in Côte d'Ivoire. These patients were selected on the basis of result of the smears for the BAAR detection after Ziehl-Neelsen stain. Genotype MTBDR*plus*® assay wasperformed at the National Reference Laboratory for TB (TB-NRL) at Pasteur Institute Abidjan (IPCI).

Samples collection and transport

Among suspect patients of failure and relapse, two samples were collected and were used to carry out the microscopic analysis to confirm the cases. After confirmation, the samples were individually wrapped and transported to TB-NRC refrigerated at +4 °C in coolers for Genotype MTBDR*plus*® assay.

Mutations detection

DNA extraction

Sputum samples were processed using the US Centers for Disease Control and Prevention (US CDC) recommended method of N-acetyl-L-Cysteine 4% NaOH-2.9% Citrate. Five milliliters of sputum were transferred in 50 ml of plastic centrifuge tube. Five milliliters of NALC solution were added to the sputum. The preparation was mixed by vortexing for 1 min and incubated at room temperature for 15 min. In the centrifuge tube, 35 ml of sterile phosphate buffer pH 6.8 were added and centrifuged at 3.000g for 20 min. Supernatant was carefully eliminated. Pellet was re-suspended with 2 ml of sterile phosphate buffer; 200 µl of re-suspended pellet were used to perform a smear which was stained using the Ziehl-Neelsen method. The Genolyse kit was used for bacterial DNA extraction; 500 microliters of sediment were transferred in an eppendorf tube of 1500 µl. The suspension was centrifuged at 10.000g in an aerosol-tight rotor for 15min. The supernatant was discarded; 100 µl of lysis

buffer were added to the sediments. The bacterial suspension was homogenized by vortexing. The bacterial suspension was inactivated at 95°C for 5 min; 100 µl of neutralization buffer were added to the preparation. The DNA contained in the supernatant was transferred to a fresh tube. A negative control was included in each run of sputum sample decontaminated for DNA extraction.

Amplification of DNA extracted from sputum samples

With sputum containing AFB, Genotype MTBDR*plus*® assay version 2.0 (Hain Life science, Nehren, Gmbh, Germany) was performed as recommended by the manufacturer. The amplification mixture contained 35 µl of primer-nucleotide Mix B, 10 µl of Mix A (5µl 10xPCR buffer, 2 µl of Mgcl2, 3 µl of molecular water, 1 unit of thermostableTaq DNA polymerase) and 5 µl of extracted chromosomal DNA solution. Amplification parameters used were: 15 min of denaturation at 95°C, followed by 20 cycles of 30 s at 95°C and 2 min at 65°C, followed by 30 additional cycles of 25 s at 95°C, 40 s at 53°C, and 40 at 70°C (1cycle).

Hybridization

Hybridization and detection were performed with a TwinCubator semi-automated washing and shaking device according to the manufacturer's instructions and using the reagents provided with the kit. Twenty microliters of denaturation solution was mixed with 20 µl of amplified sample. The mixed solution was incubated at room temperature for 5 min. One milliliter of pre-warmed hybridization buffer was added before the membrane strips were placed and shaken in the hybridization solution for 30 min at 45°c. After two washing steps, a colorimetric detection of the hybridized amplicons was obtained by the addition of the streptavidin alkaline phosphatase conjugate.

An internal quality control program with positive and negative controls was implemented during the study. An interpretable Genotype MTBDR*plus*® assay was defined as a test strip with all control markers positive, including results of the markers for positive control (H37Rv strain), negative control for DNA extraction and for mix preparation.

Statistical tests

Data collected were analyzed using Epi-info software. The rate of mutations in *rpoB*, *katG*, and *inhA* gene in category of patients experiencing failure and relapse with its confidence interval (CI) 95% were estimated. Chi 2 test was used to compare proportions with a significance level of 5%.

Results and Discussion

A total of 451 patients were included in this study. These patients were all previously treated for smear positive TB. Microscopic examination of sputum concentrated showed that all the samples analyzed were interpreted at least 1+ (Table 1). Among these patients, there were 58.09% (262/451) relapse and 41.91% (189/451) failure. The distribution of patients according to the sex showed that 70.07% (316/451) were male and 29.93% (135/541) were female. The majority of these patients were in the age group of 25-40 years (Table 2).From sputum samples received, 39.4% (178; 95% CI: 34.96-44.16) were reported susceptible to RMP and INH. RMP mono resistance was detected in 2.66% (12; 95% CI: 1.45-4.73) and INH mono resistance at 7.76% (35; 95% CI: 5.54-10.73). RMP and INH resistance (MDR-TB) was detected in 43.24% (195;

95% CI: 38.63-47.96). At 6.87 % (31; 95% CI:4.79-9.72) samples, wild type probes *WT3/WT4, WT7,* and *WT8,* with the majority *WT8* probe, were absent but the modified protein (mutation) was not detected by the method used Genotype MTBDR*plus*® (Table 3). A total of 242 patients who had at least one known and interpretable mutation for each antibiotic were analyzed to estimate the rate of mutation which dominates. Among them, 147 (60.74%) were relapse and 95 (39.26%) were failure. Among the RMP-resistant strains, three (3) cases in relapse had simultaneously *D516V* and *H526D* mutations. For RMP resistance, *D516V* mutation of *rpoB* gene was most observed in failure with a rate of 33.68% (32/95, 95% CI: 24.31-44.11) against 22.45% (33/147, 95% CI: 15.98-30.06) in relapse and no statistically significant difference(p=0,054). Then comes the *H526D* mutation with a rate of 23.81% (33/147, 95% CI: 17.18-31.5) in relapse against 11.58% (11/95, 95% CI: 5.92-19.77) at the failures and a statistically significant difference (p=0, 01) (Table 4). Concerning INH resistance, the *katG S315T* mutation is also more prevalent among both failures: 95.79% (91/95, 95% CI: 89.59-98.84) as in relapse: 86.39% (127/147, 95% CI: 79.77-91.49) with a statistically significant difference (p=0, 01) with or without a mutation in their*inhA* promoter -8 position (Table 5). Multidrug-resistant tuberculosis (MDR-TB) is a major threat to programs against tuberculosis worldwide(Sharma et *al*., 2006).

Rapid identification of drug, particularly MDR-TB is most important to help reduce the spread of disease (WHO, 2008). New diagnostic tools for TB have been developed and validated, in particular molecular methods (Garcia, 2003). Some on these methods are based on the knowledge that resistance to RMP and INH in *M. tuberculosis*most often attributed to mutations in *rpoB, katG* and *inhA* genes. Genotype MTBDR*plus*® assay is an excellent tool for early detection of resistance mutations to anti-TB drugs. This method has been used and validated by several authors worldwide (Brossier et *al*., 2006;Hillemann et *al*., 2006).We used it in this study. Our results go up that the molecular basis of resistance remain unknown to 42.32% (81/189) of patients with treatment failure and 37.02% (97/262) of patients with a relapse profile, which are detected RMP and INH sensitive (table 3). In relapse it seems understandable because relapse TB is defined as subject who has been treated for TB, was declared "cured" and returned with TB confirmed by bacteriological examination (WHO, 2001; PNLT, 2005).

It may therefore be an endogenous reactivation by the same body as that caused the first episode of TB and exogenous reinfection with a new different sensitive agent responsible for the previous episode. But in failures, this rate of 42.32% is excessive. Mutations not detected in these patients with suspected treatment failure would fall outside the 81 bp region of *rpoB*gene called region determining RMP resistance (RRDR).

However, literature data report that more than 95% of mutations of resistance to RMP are the most common place in this central region of 81 base pair (pb) of the *rpob* gene (Ramaswamy et *al*., 1998).Our results are valid because Genotype MTBDR*plus*® directly on sputum smear-positive was validated in Côte d'Ivoire. The sensitivity and specificity for the detection of RMP resistance were 100% and 73.2% and for the detection of INH resistance 95% and 95.1% respectively (N'guessan et *al*., 2014).It may therefore be biases in patient selection. That

is why it is important to popularize these molecular methods for diagnosis of MDR-TB in our developing countries. Genotype MTBDR*plus*® technology has allowed us to detect in 242 patients with suspected MDR-TB at least one resistance mutation known and interpretable. When comparing rates of resistance mutations in the *rpoB* gene, we found that the *D516V* mutation resulting in an amino acid exchange was more frequent among patients in therapeutic failure situation: 33, 68% (32/95, 95% CI: 24, 31-44, 11) against 22, 45% (33/147, 95% CI: 15, 98-30, 06) in relapse. The comparison showed no statistically significant difference (p=0,054). The *H526D* mutation is the second mechanism of RMP resistance observed in this study with a frequency of 23, 81% (33/147, 95%CI: 17, 18-31, 5) in relapse against 11, 58% (11/95, 95% CI: 5, 92-19, 77) in failures and a statistically significant difference (p=0, 01) (Table 4).

Literature data describe that the most common mutations in *rpoB* gene deteriorate*codon526* or *codon531* and result in a high degree of RMP resistance(MIC > 32 µg/ml)(Kapur et *al*., 1994).Viewed broadly on target, *codon 526* of *rpoB* gene, with two mutated alleles (*H526Y* and *H526D*) is the target of mutation.

This seems corroborated with Ivorian study of N'guessan et *al*., which reported that among relapse situation patients, 31,3% (40/128) had a point mutation at *codon 526* of *rpoB* gene (N'guessan et *al*., 2014). It has been shown that the relative frequencies with mutations in codons 531, 526, and 516 varies and differs according to different geographic locations (Isfahani et *al*., 2006; Mohammad et *al*., 2006), but Miotto et *al*., study in Burkina Faso, an adjoining country at Côte d'Ivoire also showed a predominance of codon 526 (31, 2%) in RMP-resistant strains (Miotto et *al*., 2009).

It is important to understand the relationship of clinical condition of patients with TB mutations and high levels of RMP resistance to determine if patients are initially infected with multi resistant strains or the emergence of MDR-TB is due to inadequate or improper treatment of antibiotics has resulted in the acquisition of mutations and resistance to antibiotics. In this study, we found that *D516V* mutation was the most frequent. Indeed, it is about a low degree of RMP resistance (MIC < 32 µg/ml), key antibiotic of TB treatment. The comparison showed no statistically significant difference (p=0,054). This RMP resistance is one of the main reasons for the first-line treatment failure. RMP resistance of *M. tuberculosis*is the amplification by the man action of a natural phenomenon (Davis, 1971).Several factors influence the success of TB treatment.

Human errors include when a person does not correctly following the treatment to completion provides selective pressure. This favors resistant bacilli which then multiply and become predominant. With repeated political crises in Côte d'Ivoire and theirs storm which eroded good practice such as direct supervision of treatment, it's clear that if the RMP resistance were to amplify and generalize, TB would become incurable in this country with limited resources. Additionally, some HIV-positive patients may have a high risk to develop TB resistant to RMP because of molecular absorption problems (WHO, 1996; Ramaswamy et *al*., 1998).

The method does not identify all mutations. Evidence in some patients 6, 87% (31, 95% CI: 4, 79-9, 72), wild-type probes *WT3/WT4*, *WT7*, and *WT8* with the majority *WT8* probe, were absent but modified protein (mutation) was not detected.

Table.1 Results of microscopic examination according to the scale of WHO and the UICTMR (Rieder et *al*., 2007)

	FAILURE (N=189)			RELAPSE (N=262)		
MICROSCOPY	**n**	**%**	**CI 95%**	**n**	**%**	**CI 95%**
1+	51	26,98	20,80-33,91	42	16,03	11,80-21,04
2+	49	25,93	19,84-32,79	74	28,24	22,88-34,11
3+	89	47,09	39,80-54,47	146	55,73	49,48-61,84

Table.2 Description of patients according to the age group and the sex

	FEMALE (N=135)				MALE (N=316)			
	FAILURE (n=66)		RELAPSE (n=69)		FAILURE (n=123)		RELAPSE (n=193)	
AGE GROUP	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
[10-25]	15	22,73	20	16,26	10	14,49	30	15,54
[25-40]	34	51,52	72	58,54	38	55,07	117	60,62
[40-55]	15	22,73	26	21,14	17	24,64	38	19,69
[55-70]	1	1,52	4	3,25	3	4,35	6	3,11
[70-85]	1	1,52	1	0,82	0	0	1	0,52
[85-90]	0	0	0	0,00	0	0,00	1	0,52

Table.3 Sensitivity of rifampin (RMP) and isoniazid (INH) according to the categories of patients

	FAILURE		RELAPSE	
Profile of sensitivity	**n**	%	**n**	%
RMP and INH resistance (MDR-TB)	80	42,32	115	4,89
RMP mono resistance	2	1,05	10	3,81
INH mono resistance	13	6,87	22	8,39
RMP and INH sensitive	81	42,85	97	37,02
Uninterpretable	13	6,87	18	6,87
Total	**189**	100	**262**	100

Table.4 Frequency of *rpoB*mutations observed in the two populations with at least resistance

		FAILURE (N=95)			RELAPSE (N=147)			
GENE	**MUTATIONS**	**n**	**%**	**CI 95%**	**n**	**%**	**CI 95%**	**p-value**
GENE *rpoB*	*D516V*	32	33,68	24,31-44,11	33	22,45	15,98-30,06	0,054
	H526Y	20	21,05	13,36-30,62	30	20,41	14,21-27,83	0,9
	H526D	11	11,58	5,92-19,77	35	23,81	17,18-31,5	**0,01**
	S531L	16	16,84	9,94-25,90	19	12,93	7,96-19,45	0,39

Table.5 Frequency of *katG, inhA* mutations observed in the two populations with at least resistance

			FAILURE (N=95)			RELAPSE (N=147)		
GENE	MUTATIONS	n	%	CI 95 %	n	%	CI 95%	p-value
GENE*katG*	*S315T*	91	95,79	89,57-98,84	127	86,39	79,77-91,49	**0,01**
GENE*inhA*	*C15T*	1	1,05	0,03-5,73	7	4,76	1,94-9,57	0,11
	T8C	28	29,47	20,56-39,71	34	23,13	16,58-30,79	0,27
	T8A	13	13,68	7,49-22,26	20	13,61	8,51-20,23	0,98
GENE*katG/inhA*	*S315T+ C15T*	0	0	0,00-3,81	1	0,68	0,02-3,73	0,42
	S315T+ T8C	28	29,47	20,56-39,71	34	23,13	16,58-30,79	0,27
	S315T+ T8A	17	17,89	10,78-27,10	27	18,37	12,47-25,59	0,92

These patients were excluded because the mutated profile could not be confirmed in the *rpoB* gene. If the method is well wherever it was used, but in this study case, there was a weak point in this molecular method.

There is a real polymorphism in the wild-type probe *WT8* of*rpoB* gene that explores simultaneously *codons 531* and *533*(Hillemann et *al*.,2007). RMP resistance can induce mutation detected or not (Ma xh et *al*., 2006; Ohno et *al*., 1996). This is why the interpretation of the wild type probe absence without detection of the modified protein is to be confirmed. Sequencing or antibiotic susceptibility tests (DST) justify the profile of patients. In this study, the molecular method used has detected three (3) cases of infections with two strains of *M. tuberculosis*. Indeed, it was noted the simultaneous presence of *D516V* and *H526D* mutations in the *rpoB* gene in three patients experiencing relapse. It is not clear that this observation is of dual infection by two different agents of *M. tuberculosis*. This is observed in patients who have been treated for a previous episode of TB.

There may cases of exogenous reinfection by a new agent different from the agent responsible for the first TB episode. The genotypes of M. tuberculosis strains circulating in Côte d'Ivoire does not appear to be stable. Indeed, in a context of endemic TB, a mixed infection is not an exception. But this must be confirmed with genetic fingerprints of the initial strains and those of the recovery of TB (Harries et *al*., 2003).However, this technology is usually not available in resource-poor settings. Contrary to what was observed in the *rpoB* gene, we noticed that it had a high frequency of mutation *S315T* in *katG* gene.In failure: 95, 79 % (91/95, 95% CI: 89, 57-98, 84) and at relapse: 86, 39% (127/147, 95% CI: 79, 77-91, 49) with a statistically significant difference (p=0, 01)(Table 5). Indeed, INH is the most TB-drug widely used (Mitchison, 1998).This high frequency observed in this study as in Russia (Buyankhishiget et *al*., 2012) explains that INH resistance occurs more frequently than those which concern most TB-drugs.

The More TB-drugs are used, the more resistance develops and amplifies. It is therefore reasonable to assume that knowledge of the molecular basis of the

phenomenon of antibiotic resistance will develop more new molecules active against *M. tuberculosis*. We also observed mutations associated with *S315T* positions *T8C* and *T8A* of nucleic acid *inhA* promoter (Table 5).

In all cases, *S315T* mutationin *katG* gene is the main mechanism of INH resistance. Our results are consistent with those of N'guessan et *al.,* who identified that the *D516V* mutation in *rpoB* gene and *S315T* mutation in *katG* gene were more common in MDR strains in an Ivorian study in 2008 (N'guessan et *al.*, 2008).Genotype MTBDR*plus*®assay is predictive only because all the mutations responsible for resistance to RMP and INH are not yet known or tested (Vernet et *al*., 2004; Rigouts et *al*., 2011). It would be interesting later in this work using the sequencing of different target (*inhA* promoter, *rpoB* and *katG* gene) as the best gold standard (Telenti et *al*., 1993) to reveal all the mutations for resistance to both major TB-drugs.

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