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Full Length Research Paper

Molecular characterization of Isoniazid and Rifampicin resistance strains of *Mycobacterium tuberculosis* isolated from new tuberculosis cases in Lagunes Region (Cote D'Ivoire)

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Tuberculosis remains in the world an important problem of public health. This study objective was to investigate the genetic basis of resistance to Isoniazid and Rifampicin of *Mycobacterium tuberculosis* complex strains collected in Lagunes region, an area of high burden of tuberculosis. During primary resistance study, 196 smears positive new cases of tuberculosis were recruited in Lagunes region. Out of a sample of 31 strains, 17 were Isoniazid mono-resistant strains, 7 were multi-drug resistant (MDR) strains and 7 were susceptible to both Isoniazid and Rifampicin. Genotype MTBC assays and sequencing of *mabA-inhA* regulatory region, *katG*, *inhA* and *rpoB* genes were performed on all the strains. At least one mutation in the genes studied was found in 20 (83%) of the 24 resistant strains. Ser315Thr mutation was prominent in Isoniazid mono-resistant strains (9/13) and in MDR strains (7/8). Three of 16 Isoniazid resistant strains with Ser315Thr mutation had an additional mutation in the regulatory region. No mutation were found in 6 of the 7 susceptible strains. Molecular methods in tuberculosis laboratory may improve detection of tuberculosis case with drug resistance case.

Key words: *Mycobacterium tuberculosis* complex, susceptibility testing, resistant genes.

INTRODUCTION

The Lagunes region is recognized to have a high burden of tuberculosis (N'guessan et al., 2008). Rifampicin (RMP) and Isoniazid (INH) remain the two most important first-line anti-tuberculosis drugs for the treatment of new cases of tuberculosis. Resistance to these major drugs represents a serious impediment to successful therapy.

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Because of the turnaround time for conventional susceptibility testing, patients infected with drug-resistant strains of *Mycobacterium tuberculosis* complex may be inadequately treated, thus facilitating the transmission of resistant strains (Yue et al., 2004). Indeed, laboratory diagnosis of tuberculosis and evaluation of drug resistance by routine phenotypic methods takes 6 to 9 weeks (Rastogi et al., 1989).

Rapid evaluation of drug resistance would improve choice of effective drug therapy and subsequent prevent drug-resistant strains propagation. The genes involved in

Table 1. Sequences of primers used in characterizating the Isoniazid and Rifampicin resistance strains of *Mycobacterium tuberculosis*.

Primer target (s)	Primer designation	Primer sequence (5' – 3')	PCR product size (bp)	Hybridization (°C)
rpoB	rifip1	GGT CGG CAT GTC GCG GAT GG	257	63
	ripip2	CGA CGT CGC GGA CCT CCA GC		
katG	katA	CCC GAT AAC ACC TCC TG		
	katCas	GTT TCG ACG TCG TTC ATG GC	817	59
	kat2	CTC GGC GAT GAG CGT TAC AG		
	kat4as	CCA GCG GTA AGC GCT TGT AG	1,318	59
	katC	CCG AGT ACA TGC TGC TCG AC		
	katEas	GGT GAT CGC ACA TCC AGC AC	609	59
Operon *inhA-mabA*	pro1	TCA ATA CAG CCG CAG CCA		
And its promoter	pro2	GTC ATC CGC ATG AGG AAT	493	53
	fabG1	TCA CGG CGG TAG AAG AGC		
	fabG2	CAT GTG CGT CCT TGT GTT	553	53
	INHA1	AGG ACG CAC ATG ACA AGC		
	INHA2	TCA TGA TCG GCA GCA GCG	412	53
	INHA3	CCA CAT CTC GGC GTA TTC		
	INHAD	CGA AAT GCA GGT AGT GCT C	601	53

resistance to RMP and INH and mutations leading to these resistances have been characterized by molecular method (Ramaswamy and Musser, 1998; Telenti et al., 1993). The objective of the study was to investigate the genetic basis of resistance to INH and RMP of strains of *M. tuberculosis* complex collected in Côte d'Ivoire.

MATERIAL AND METHODS

During the study on primary resistance conducted from 2004 to 2006, 196 strains of *M. tuberculosis* were isolated from new cases of tuberculosis in the Lagunes region. From this collection, a sample of 31 strains have the following characteristics: 17 strains were mono-resistant to Isoniazid (0.2 µg/ml), 7 strains were resistant to Isoniazid and Rifampicin (40 µg/ml) and from 172 strains susceptible to both Isoniazid and Rifampicin, 7 were selected at random, among them one was a dysgonic strain for which drug susceptibility tests were difficult to interpret. The DNA used for amplification was obtained by phenol-chloroform method (Chomczynski, 1993).

The Genotype MTBC assay (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer. The amplification mixture contained 35 µl of primer-nucleotide mix provided in the kit, 5 µl of 10x *Taq* polymerase incubation buffer containing 2 mM of $MgCl_2$, 2 units of thermostable *Taq* DNA polymerase, and 5 µl of extracted chromosomal DNA solution in final of 50 µl. The following amplification parameters were used: 5 min of denaturation, at 95°C, followed by 10 cycles of 30 s at 95°C and 2 min at 58°C, followed by 20 additional cycles of 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, ending with a final extension step of 8 min at 70°C. Hybridization and detection were performed with Twin Cubator semiautomated washing and shaking device according to the manufacturer's instructions and using the reagents provided with the kit. Briefly, 20 µl of amplified sample and 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 hybriddized amplicons was obtained by the addition of the strep-tavidin alkaline phosphatase conjugate.

DNA was submitted to PCR amplification using the oligo-nucleotides primers described in Table 1. The gene *katG* was amplified and sequenced using 3 pairs of primers (katA/katCas, kat2/kat4as, katC/katEas). The *mabA-inhA* regulatory region and the *inhA* gene were amplified using primers Pro1/Pro2, FabG1/FabG2, INH1/INH2, INH3/INHD. A 69-bp region of *rpoB* was amplified and sequenced using primers rifip1/rifip2. The ampli-fication protocol consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 63°C and 1 min at 72°C ending in a final extension step of 7 min at 72°C. After amplification, unincorporated nucleotides and primers were removed by filtration with Microcon 100 micro-concentrators (Amicon Inc., Beverly, Mass) and the gene target were sequenced in an ABI prism 310 DNA sequencer (Applied Biosystems Inc. Foster city, Calif). The sequences obtained were compared with the available sequences for the *katG* gene (GeneBank accession numbers X68081), the mabA-inhA operon (GeneBank accession numbers U66801 and U02492) and the *rpoB* gene (GeneBank accession numbers U12205).

RESULTS AND DISCUSSION

Genotype MTBC Assay confirmed the identification of the strains of *M. tuberculosis* complex. Overall, at least one mutation in the genes studied was found in 20 (83%) of the 24 resistant strains. Mutations were found in *katG*, *inhA*, and *mabA-inhA* regulatory region in 13 of 17 Isoniazid mono-resistant strains and in all the 7 multi-drug resistant strains (MDR, resistant to Isoniazid and

Table 2. Frequency of mutations founded in *Mycobacterium tuberculosis* resistant and susceptible strains.

DNA target	Amino-acid or nucleotide change	[a]INH n=17 (55%)	[b]MDR n=7 (22.5%)	[d]Susceptible n =7 (22.5%)	Total
*Kat*G	Ser315→ Thr	9 (53%)	6 (88%)	1 (14.3%)	16
mabA-inhA regulatory region	-8T → A	2[c]	0	0	2
	-8 T → C	0	1[c]	0	1
	-15C → T	2 (11.7%)	1	0	3
inhA	Met1→ Leu	1	0	0	1
	Val78→ Ala	1	0	0	1
No mutation		4	0	0	4

[a]INH = Isoniazid mono-resistant strains.
[b]MDR = Multi-drug resistant strains.
[c]double mutation in *katG* and in *mabA-inhA*.
[d]Susceptible = Susceptible strains including the dysgonic strain.

Table 3. Frequency of mutations described in *rpoB* gene of MDR and dysgonic strains.

Amino-acid change	Total
deletion 513 to 515	1(12.5%)
Asp516→Val	3 (37.5%)*
His522→Leu	1**
His526→Tyr	2 (25%)
His526→Arg	1**
Ser531→Leu	1 (12.5%)

* = Mutation in *rpoB* gene including dysgonic strain
**= These two mutations were associated in a single strain.

Rifampicin). No mutation was found in 6 of the 7 susceptible strains but *katG* mutation was found in the dysgonic strain, considered as susceptible but for which poor multiplication *in vitro* made the phenotypic test difficult to interpret (Table 2).

Ser315Thr mutation was prominent in Isoniazid mono-resistant strains (9/13) as well as in MDR strains (6/7). Mutation in *inhA* gene was observed in only 2 strains and mutation in the regulatory region of the gene *inhA* was found in 4 Isoniazid mono-resistant and 2 multi-drug resistant strains. Three of the 16 Isoniazid resistant strains with Ser315Thr mutation had an additional mutation in the regulatory region of *inhA* mutation (Table 2).

Mutation in *rpoB* was found in all the 7 Rifampicin-resistant strains and in the dysgonic strain which, in fact, was a MDR strain. These mutations were point mutation in amino acids 516, 521, 526 or 531 or deletion (Table 3).

Nucleic acid amplification technologies such as PCR are nowadays precious tools for identifying infectious pathogens such as *M. tuberculosis* and mutations responsible for acquired resistance to antituberculous agents. Sequencing of genes *katG*, *inhA* and promoter of *inhA* involved in resistance allows identifying a large part, around 70 - 80% of Isoniazid resistant strains (Brossier et al., 2006; Guo et al., 2006; Haas et al., 1997; Mokrousov et al., 2002). Sequencing *rpoB* allows identifying of almost all the Rifampicin-resistant strains (Brossier et al., 2006; Rossau et al., 1997; Telenti et al., 1997). It is what we found in the present study that focused on the strains of *M. tuberculosis* collected in Côte d'Ivoire during the study on primary resistance conducted in 2004 - 2006. Indeed, in 20/24 strains resistant to Isoniazid (83%) and in 7/7 strains resistant to Rifampicin mutations known to be responsible for resistance, were detected. The distribution of the mutations involved in INH-resistance was in the range of what is usually observed (Brossier et al., 2006; Guo et al., 2006; Haas et al., 1997; Mokrousov et al., 2002), i.e. Ser315Thr in *katG* in 16/25 (64%) of the strains. As in other studies, we found in some Isoniazid-resistance strains a Ser315Thr mutation associated with mutation in other genes such as *inhA* gene (Guo et al., 2006). Indeed, 6 of the 16 strains with Ser315Thr mutation had an additional mutation in the promoter region of *inhA* (Table 3).

Surprisingly, the mutation Ser531Leu, which is responsible for half of the cases of Rifampicin-resistance (Brossier et al., 2006; Rossau et al., 1997; Telenti et al., 1997) was found in only one of our 7 MDR strains.

We should raise the fact that one dysgonic strain, that was first classified as susceptible INH and RMP based on difficult to interpret phenotypic tests due to poor growing, was in fact resistant to both antibiotics based on genomic tests. This emphasizes the importance of proficiency testing and external quality controls as advocated by WHO as well as internal quality control that are essential components of quality assurance for laboratory services in tuberculosis control (WHO, 1998).

Molecular approach for drug susceptibility testing is particularly interesting for dysgonic strains of *M. tuberculosis* for 3 reasons: (a) phenotypic tests are difficult to interpret for these strains, (b) these strains are at risk to

be resistant to Isoniazid since some *katG* mutations are known to be associated with slow growth as described by Youatt (1969) and (c) many MDR strains are dysgonic (Gutiérrez MC et al., 1999).

Analysis of *rpoB* gene is particularly efficient in that aspect because Rifampicin-resistance is a good indicator of MDR and because sensitivity of *rpoB* test for detecting Rifampicin resistant is very good.

Conclusion

For tuberculosis laboratory, molecular methods may represent an opportunity to improve detection of patients infected by resistant strains of *M. tuberculosis*.

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