Mutations in the rpoB gene of rifampin-resistant *Mycobacterium tuberculosis* isolates from Eastern Poland

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SUMMARY

This study investigated mutations in the *rpoB* gene of rifampin-resistant isolates obtained from patients living in Eastern Poland. A total of 37 phenotypically and/or genotypically confirmed *M. tuberculosis* rifampin-resistant clinical isolates were included in this study. The strains were selected from symptomatic patients with a diagnosis of pulmonary and extrapulmonary tuberculosis. A line probe assay kit (INNO-LiPA rif Tb) was used for any specific mutational pattern of *rpoB* gene.

Our data support the common notion that rifampin resistance genotypes with mutation at a critical codon, i.e. the one encoding Ser-531, is frequent in *M. tuberculosis* populations regardless of geographic origin. Our findings also suggest that in a geographic area such as Eastern Poland less common mutations of the *rpoB* gene occur more frequently. The frequency of substitution at codon 526 (His-Asp) was found to be high in Lublin. This study indicates that mutations associated with nucleotide replacements in codons 526 (His-Asp) and 531 (Ser-Leu) were associated with a high percentage of RMP resistance, whereas mutations in codons 516 (Asp-Val) and 526 (His-Tyr) were observed in a low percentage of RMP-resistance.

KEY WORDS: Mycobacterium tuberculosis, Rifampin resistance, rpoB gene, Mutations

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INTRODUCTION

Despite the recent progress in global control, tuberculosis remains a major public health problem in most developing countries. According to the World Health Organization 8 million cases of tuberculosis (TB) occur each year resulting in 3 million deaths (Hsing-Yu *et al.*, 2001, Perandin *et al.*, 2005). In response to the alarming statistics and trends the World Health Organization declared tuberculosis to be a global, public health emergency (Bártfai *et al.*, 2001, Morgan *et al.*, 2005, Prasad, 2005).

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The most alarming aspect of tuberculosis is the emergence of multidrug-resistant strains of *M. tuberculosis* (MDR-TB) defined as resistance to at least rifampin and isoniazid (I-Ching *et al.*, 2006, Morgan *et al.*, 2005, Prasad, 2005, Sharma *et al.*, 2003). This situation causes great concern worldwide because of the prolonged infectivity which increases the risk of transmission. The strains of *M. tuberculosis* (MTB) resistant to anti-TB drugs has been reported in most parts of the world but especially in Eastern Europe and in the countries where TB and HIV coinfection is endemic (Prasad, 2005, Shin, *et al.*, 2005). Recent data showed that about 13% of all new cases are resistant to at least one first line drug and 1.6% are resistant to both isoniazid (INH) and rifampin (RMP) (Prasad 2005).

Rifampin is one of the most important chemotherapeutic agents used to combat infections by *M. tuberculosis* and can be assumed to be a surrogate

marker for MDR-TB (I-Ching *et al.*, 2006, Morgan *et al.*, 2005, Sajduda *et al.*, 2004). The mechanism of action of the drug is to inhibit mycobacterial transcription by targeting DNA-dependent RNA polymerase. The development of resistance to rifampin is associated with mutations which occur within an 81-bp region of the *rpoB* gene corresponding to 507-533 codons that code for the subunit of RNA polymerase of *M. tuberculosis* (Bártfai *et al.*, 2001, I-Ching *et al.*, 2006, Sharma *et al.*, 2003). It is known that 90% of *M. tuberculosis* isolates resistant to RMP are also resistant to isoniazid. The confirmation of RMP resistance provides an effective screening tool for the identification of MDR strains.

There is very little information available on the specific mutation pattern and its detection in the *M. tuberculosis* strains isolated in Poland. The present study analyzed mutations in the *rpoB* gene of rifampin-resistant isolates obtained from patients living in Eastern Poland.

MATERIAL AND METHODS

Clinical samples

A total of 37 phenotypically and/or genotypically confirmed *M. tuberculosis* RMP-resistant clinical isolates collected over a period of 19 months (from January 2004 to July 2005) were included in this study. The strains were selected from symptomatic patients with a diagnosis of pulmonary (27 cases) and extrapulmonary (10 cases) tuberculosis who were hospitalized in a Public Hospital in Lublin, Eastern Poland (Table 1). Two out of three criteria were used to confirm the TB disease:

TABLE 1 - *Details of 37 clinical specimens included in the study.*

No.	*Clinical Isolates*	*Total (37)*
	Respiratory specimens	**27**
1	Sputum	23
	Bronchoalveolar lavage (BAL)	4
	Non-respiratory specimens	**10**
	Cerebrospinal fluid (CSF)	4
2	Urine	3
	Blood	1
	Biopsy material	2

1) sputum smears positive for AFB (acid-fast bacilli);
2) positive culture or PCR for *M. tuberculosis* and
3) clinical (signs/symptoms), the chest X-ray findings such as infiltrates or consolidations and/or cavitations (Morgan *et al.*, 2005, Sajduda *et al.*, 2004).

Drug susceptibility tests were performed by standard proportion method with resistance ratio as well as by INNO-LiPA Rif TB test (Innogenetics, Belgium). A line probe assay kit was used for any specific mutational pattern of *rpoB* gene. As a control *M. tuberculosis* H37Rv strain was used.

Strains identification

After microscopic examination clinical samples were simultaneously tested by two methods: conventional culture method on the Löwenstein-Jensen (L-J) slants for four to ten weeks and molecular method INNO-LiPA Rif Tb.

The cultures were subjected to phenotypic identification based on growth and colony characteristics, pigment production and biochemical tests.

Drug susceptibility testing

Conventional drug susceptibility was determined on LJ by the proportion method with resistance ratio. The susceptibility of mycobacteria was analyzed for 4 major antituberculosis agents including isoniazid (INH), rifampicin (RMP), streptomycin (STM), and etambutol (EB) in two concentrations as follow: INH 0.2/0.4 g/ml, RMP 40/80 g/ml, STM 2/4 g/ml and EMB 2/4 g/ml. Resistance is expressed as the percentage of colonies on drug-containing media in comparison with the growth on drug-free medium at the critical concentrations of the substances (inhibition of the growth of most cells in wild strains of *M. tuberculosis*).

When greater than or equal to 1% of bacillary population becomes resistant to the both critical concentrations of a drug the *M. tuberculosis* strain is considered resistant to the drug.

Molecular drug susceptibility to RMP was evaluated by using INNO-LiPa Rif TB kit according to the manufacturer's instructions.

Preparation of DNA extracts

To extract DNA from clinical samples they were liquefied and decontaminated to obtain the pellet. Human respiratory specimens were processed

with N-acetyl-L-cystein and NaOH method. Non-respiratory specimens were concentrated by centrifugation at 3000 g for 30 minutes. All preliminarily processed materials were kept at 80ºC for 10 minutes for disruption of the cell wall of possible mycobacteria. DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN, Germany).

Detection of mutations

The line probe assay kit (INNO-LiPA Rif. TB) was obtained from Innogenetics N.V., Zwijnaarde, Belgium and was used according to the manufacturer's instructions.

The rifampin resistance-determining region of the *rpoB* gene was amplified from *M. tuberculosis* isolates with specific biotinylated primers as indicated by the manufacturer using 1 unit AmpliTaq DNA polymerase per reaction mixture in a thermocycler.

The amplified biotinylated DNA material was hybridized with specific oligonucleotide probes immobilized as parallel lines on nitrocellulose paper strips provided with the kit. After hybridization alkaline phosphatase-labeled streptavidin was added and bound to any biotinylated hybrid previously formed. Incubation with 5-bromo-4-chloro 3-indolylphosphate-nitroblue tetrazolium chromogen resulted in purple precipitates on the paper strips. The presence of MTB in the sample was detected with the *M. tuberculosis* complex-specific probe. The reactivity of an amplified fragment with one or more of the 5 wild type probes (S1 to S5) was prevented by the presence of a mutation within the probe region. Four additional probes: R2 (D516V), R4a (H526Y), R4b (H526D) and R5 (S531L) are expected to hybridize the mutant sequences of the four most commonly observed mutations.

When all of the wild-type S probes gave a positive signal and none of the R probes reacted the *M. tuberculosis* isolate was considered susceptible to rifampin (INNO-LiPA S pattern). When at least one negative signal was obtained with the wild-type S probes the isolate was rifampin-resistant. When the resistance to rifampin was due to one of the four most frequently observed mutations described above a positive reaction was also obtained with one of the R probes and this was always accompanied by a negative reaction with the corresponding S probe (INNO-LiPA R pattern).

TABLE 2 - *Mutations of the rpoB gene found in RMPr M. tuberculosis isolates.*

No. of cases N=37 (%)	*LiPA pattern*		*Mutated codon*	*Specific mutation*
	R region (n=30 81%)	S region (n=6 16.2%)		
13 (35.1%)	R4b		H 526 D	His. -526 - Asp.
12 (32.4%)	R5		S 531 L	Ser. - 531 - Leu
3 (8.1%)	R2		D 516 V	Asp - 516 - Val
2 (5.4%)	R4a		H 526 Y	His - 526 - Tyr
3 (8.1%)		Δ S1	Q 513 K	
1 (2.7%)		Δ S2	Δ 518	
1 (2.7%)		Δ S5	L 533 P	
1 (2.7%)		Δ S1-Δ S2	Δ 514-516	
1 (2.7%)			wt*	

*wt = wild type

RESULTS

Out of the 37 RMP-resistant strains line probe assay confirmed the resistance in 36 (97.2%) cases. *In vitro* susceptibility testing of the isolates found 24 (64.8%) of them to be RMP-resistant.
Out of the 24 phenotypically RMP-resistant isolates, in one case precise mutation could not be localized.
The isolate hybridized with all S probes and did not give a positive result with the mutant (R) probes indicating a wild-type.
Four distinct nucleotide substitutions (Asp-516-Val, His-526-Tyr, His-526-Asp, Ser-531-Leu) accounting for resistance in 30 (81%) of the 37 RMP-resistant isolates were identified by the kit R probes specific for these mutations (LiPA patterns R4b, R5, R2, R4a respectively). Moreover, the genetic alteration within the 81-bp core region of *rpo B* gene was detected in 6 (16.2%) cases but the specific mutation was not identified by INNO-LiPA Rif. TB kit (patterns ΔS1-S5) (Table 1).
The most prevalent mutation sites were in codons 526 (35.1%), 531 (32.4%) and 516 (8.1%). In one case (2.7%) a double mutation was observed in codons 514-516 corresponding to LiPA patterns ΔS1-S2 (Table 2).

DISCUSSION

Genetic research suggests that multidrug-resistant tuberculosis may be an increasing threat in Poland (Sajduda *et al.*, 2004). Lublin, located in Eastern Poland, is the area where TB is most prevalent.
Therefore, we aimed to detect MDR-TB in clinical strains obtained from patients living in Eastern Poland who were diagnosed with tuberculosis as well as to characterize the mutations in the *rpo* gene.
Compared to traditional techniques for mycobacterial drug susceptibility testing, molecular methods are by far the most rapid.
The molecular techniques have largely been focused on RMP resistance because the genetic target is well described and the presence of RMP resistance is considered as an indicator of MDR-TB. Detection of mutations in the *rpoB* gene can reliably identify RMP-resistant strains (Kazue *et al.*, 1999, Morgan *et al.*, 2005, Sharma *et al.*, 2003, Shin *et al.*, 2005).
Several methods can identify RMP-resistant *M. tuberculosis* complex strains (Bártfai *et al.*, 2001, I-Ching *et al.*, 2006, Sajduda *et al.*, 2004, Shin *et al.*, 2005, Viveiros *et al.*, 2005). In this study we used the line probe assay INNO-LiPA Rif.TB (Innogenetics, Zwijndrecht, Belgium) for the early detection of RMP resistance in clinical isolates.
The LiPA is based on probes covering the RRDR (rifampin resistance determining region) sequence of *rpoB*.
The assay was performed in parallel with conventional isolation, identification and susceptibility testing procedures routinely used in mycobacteriology clinical laboratories.
In the present study the probe specific for MTB complex nucleotide sequence included in each strip of line probe generated an amplified product of approximately 256 bp for all *M. tuberculosis* strains studied (n=37) and H37Rv.
The LiPA assay revealed 36 RMP-resistant strains compared to 24 phenotypically RMP-resistant strains.
The molecular technique detected no mutation in one of the strains tested, although it was resistant to RMP as determined by the proportion method. A similar phenomenon has been observed with some phenotypically documented rifampin-resistant strains analysed in other studies (Bártfai *et al.*, 2001, Sajduda *et al.*, 2004, Viveiros *et al.*, 2005).
The mutation responsible for RMP-resistance in this strain could be localized outside the 81-bp hypervariable region (Sharma *et al.*, 2003). Other possibilities are that other rare *rpoB* mutations, heteroresistance (a mixture of susceptible and resistant subpopulations) or changes have occurred in genes whose products participate in antibiotic permeation or metabolism (Sajduda *et al.*, 2004).
All of the phenotypically RMP-susceptible strains yielded hybridization patterns with all 5 wild-type S probes and none of the resistant R probes (data not shown). Thus, excellent correlation was found between LiPA and the proportion method (100%).
This is in agreement with other studies which have shown concordance between the line probe

assay and phenotypic susceptibility tests (Hsing-Yu *et al.*, 2003, Perandin *et al.*, 2005).

The LiPA assay is able to identify the *M. tuberculosis* complex and simultaneously determines the presence of point mutations within the 81-bp of the *rpoB* gene.

The test can detect the type of mutation for only the four most common mutations in the region (Ser-531-Leu, His-526-Tyr, His-526-Asp and Asp-516-Val) while for isolates with other mutations it indicates only the presence of a genetic alteration (Bártfai Z, *et al.*, 2001, Kazue Hirano *et al.*, 1999).

Previous reports and this study suggest that the types and frequencies of particular mutations differ in different countries (Shin *et al.*, 2005, Viveiros *et al.*, 2005).

Worldwide the most frequent mutations are Ser-531-Leu followed by His-526-Tyr and His-526-Asp (Viveiros *et al.*, 2005). This study is the first to address the distribution of mutations in the *rpo* gene in the region of Eastern Poland. The most common substitutions occurred at codons 526 (His-526-Asp; 35.1%) and 531 (Ser-531-Leu; 32.4%) in the RRDR.

Less frequently Asp-516-Val mutations (8.1%) and His-526-Tyr mutations (5.4%) were observed.

Additionally, a genetic alteration within the 81-bp core region of *rpoB* gene was detected in 6 (16.2%) out of the 37 isolates but the specific mutation was not identified by line probe (LiPA patterns S1, S2, S5).

A double mutation was found in one strain. A comparison of these results with the results of Sajduda *et al.*, who characterized RMP-resistant MTB isolates in Poland, showed that the mutations at codon 531 also predominate in the Lublin area of Eastern Poland (Sajduda *et al.*, 2004).

In general, our data on *rpoB* mutation frequencies in isolates in Eastern Poland supported the common notion that rifampin resistance genotypes with mutation at a critical codon, i.e. the one encoding Ser-531, is frequent in *M. tuberculosis* populations regardless of geographic origin (Shin *et al.*, 2005, Viveiros *et al.*, 2005). Our findings also suggest that in a geographic area such as Eastern Poland less common mutations of the *rpoB* gene occur more frequently. In contrast with previously published data the frequency of substitution at codon 526 (His-Asp) was found to be high in Lublin (Sajduda *et al.*, 2004). This study indicates that mutations associated with nucleotide replacements in codons 526 (His-Asp) and 531 (Ser-Leu) were associated with a high percentage of RMP resistance whereas mutations in codons 516 (Asp-Val) and 526 (His-Tyr) were observed in a low percentage of RMP resistance.

These differences reflect the complex and crucial interaction between the drug and its target at the molecular level where the position of the affected allele seems to be critical.

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