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Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Ontario, Canada

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Objectives: Ontario bears the greatest burden of tuberculosis in Canada, with 40% of all cases and 60% of multidrug-resistant cases. The purpose of this study was to genotypically characterize isoniazid- and rifampicin-resistant isolates and compare these results with phenotypic drug susceptibility testing data. This is the first Canadian study to examine gene mutations that contribute to multidrug-resistant tuberculosis.

Methods: A total of 751 tuberculosis isolates were tested for drug resistance using phenotypic antimicrobial susceptibility testing methods. Isolates were then characterized using molecular methods. Following DNA extraction, PCR amplification and sequence analysis were performed on the rifampicin resistance region of *rpoB*, as well as the region surrounding *katG*315 and the *inhA* promoter region associated with isoniazid resistance.

Results: Eighteen different mutation types were found in the *rpoB* region of rifampicin-resistant isolates. Isolates with mutations at residues *rpoB*531 (64.1%), *rpoB*526 (15.2%) and *rpoB*516 (8.7%) were the most common. In addition, an insertion was found at residue 514. Three phenotypically rifampicin-resistant isolates (3.3%) were genotypically wild-type. In isoniazid-resistant strains, mutations were found most commonly at *katG*315 (45.4%) as well as at the *inhA* promoter region (28.6%). Thirty-nine isolates (25.3%) were phenotypically isoniazid-resistant but genotypically wild-type. The *katG*315 mutation was statistically associated with multidrug-resistant isolates.

Conclusions: This study expands the knowledge of mutations that potentially contribute to drug resistance in tuberculosis and lays the foundation for developing molecular-based tests to determine drug resistance in clinical tuberculosis isolates.

Keywords: multidrug-resistant tuberculosis, isoniazid, rifampicin

Introduction

Tuberculosis (TB) remains a significant global public health concern. Treatment of TB infection relies primarily on the use of isoniazid and rifampicin with ethambutol and pyrazinamide as first-line drugs to treat TB.[1] The threat posed by TB is increasing with the emergence of drug-resistant TB isolates,[2] particularly multidrug-resistant (MDR) TB, defined as resistance to rifampicin and isoniazid.[3]

TB drug resistance is associated with mutations in several genes. In 95% of isolates, rifampicin resistance is mediated by mutations in an 81 bp region of the *rpoB* gene.[4] In contrast, isoniazid resistance is mediated by several genes, most commonly *katG*, especially at residue 315, and the promoter region of *inhA*.[4] Despite the characterization of the above genes, approximately one-quarter of isoniazid-resistant isolates are genotypically wild-type at the above sites, indicating the need for further investigation.

Molecular analysis of regional isolates is an essential first step to developing molecular-based detection methods for TB resistance. In this study the prevalence of mutations conferring rifampicin and isoniazid resistance was assessed using phenotypically drug-resistant and drug-susceptible isolates from the province of Ontario.

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Materials and methods

Mycobacterial isolates

Seven hundred and fifty-one TB isolates were selected for this study based on their phenotypic drug resistance characteristics. All isolates were obtained from specimens submitted for TB culture to the Ontario Agency for Health Protection and Promotion (OAHPP) between 1999 and 2007. Clinical specimens were processed and mycobacterial growth evaluated with the mycobacterial growth indicator tube in the BACTEC 960 instrument (Becton Dickinson, Sparks, MD, USA). Positive culture isolates were identified as TB using the AccuProbe test (Gen-Probe, San Diego, CA, USA).

Phenotypic drug susceptibility testing

Drug susceptibility was determined using the BACTEC 460 and 960 systems (Becton Dickinson), with an isoniazid critical concentration of 0.1 mg/L and a rifampicin critical concentration of 1.0 mg/L for the BACTEC 960 system and 2.0 mg/L for the BACTEC 460 system.

DNA extraction and PCR amplification of drug resistance genes

Bacterial DNA was extracted using a modified cetyl trimethylammonium bromide methodology.[5] Genotypic resistance was evaluated by PCR amplification followed by DNA sequencing of the *rpoB* rifampicin resistance region, the *katG*315 region and the *inhA* promoter region, using the Platinum *Pfx* DNA Polymerase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR primers for *rpoB* were *rpoB* F (5′-GTACGGTCGGCGAGCTGATCCAAA-3′) and *rpoB* R (5′-ATCAGACCGATGTTGGGCCCCT-3′). Primers for *katG* were *katG* F (5′- TCGACATTCGCGAGACGTTTCGG-3′) and *katG* R (5′- TTGACCTCCCACCCGACTTGTG-3′). Primers for *inhA* were *inhA* F (5′- CACCCGCAGCCAGGGCCTCG-3′) and *inhA* R (5′-CGATCCCCCGGTTTCCTCCGG-3′).[6] For each reaction, 5 ng of extracted genomic DNA was used. Amplification was performed using an iCycler thermocycler (Bio-Rad, Hercules, CA, USA).

DNA sequencing

Sequencing primers were *rpoB* F for *rpoB*, *katG* F for *katG* and *inhA* F for *inhA*. Sequencing was carried out using the ABI PRISM 3730xl and analysed using Vector NTI Contig Express and AlignX software (Invitrogen).

Statistical analysis

For all associations between different isolate groups χ^2 tests were used.

Results

Drug susceptibility patterns

Of 751 isolates tested, 219 were drug-resistant. Of these, 51.1% were isoniazid-monoresistant, 1.8% were rifampicin-monoresistant and 38.8% were MDR-TB, as defined by resistance to both isoniazid and rifampicin. Of the resistant isolates, 61.2% were also resistant to other first-line drugs. Eight percent of isolates were resistant to two first-line drugs but were not MDR.

Distribution of mutations associated with rifampicin resistance

Eighty-nine phenotypically rifampicin-resistant isolates and 182 phenotypically rifampicin-susceptible isolates were tested for mutations in the *rpoB* gene. Mutations were found in 96.6% of rifampicin-resistant isolates and 1.6% of the rifampicin-susceptible strains (possibly due to misinterpretation of phenotypic drug susceptibility testing), bringing the total number of isolates analysed to 92 (Table 1). Mutations at *rpoB*531, such as S531L, were the most common and were found in 64.1% of isolates. Mutations at *rpoB*526, such as H526Y, were found in 15.2% of isolates. Other mutations included L511P (2.2%), D516V (6.5%) and S522L (4.3%), among others. The insertion F514FF was found in 3.3% of isolates. Apart from single point mutations, three isolates had multiple mutations; however, most of the changes observed in these isolates were unique and are rarely found in the literature. Three isolates were phenotypically rifampicin-resistant, yet genotypically wild-type.

Distribution of mutations associated with isoniazid resistance

Due to the relative abundance of isoniazid-resistant isolates compared with isolates with other drug resistance patterns, a subset of study samples (isolates from 1999 to 2001) were chosen for analysis. One hundred and fifty-four phenotypically isoniazid-resistant isolates and 350 phenotypically isoniazid-susceptible isolates were tested for mutations in the *katG* gene and the *inhA* promoter region (Table 2). Of the phenotypically isoniazid-resistant isolates, 46.1% had mutations in *katG* only, most commonly a serine to threonine substitution at residue 315, and 25.3% had a mutation in the *inhA* promoter region only, most often at position −15. In addition, 3.2% of isolates had mutations in both *katG* and the *inhA* promoter region. The remaining 25.3% of isolates were wild-type at all loci. No mutations were found among the phenotypically isoniazid-susceptible strains.

Rifampicin resistance is an indicator of MDR-TB

Phenotypic data indicated that 95.5% of rifampicin-resistant strains were MDR-TB. In contrast, only 15.6% of the isoniazid-resistant isolates analysed were MDR-TB. This suggests that, in TB isolates from Ontario, rifampicin resistance is an indicator of MDR-TB whereas isoniazid resistance is not ($P < 0.0001$).

Mutations and MDR-TB

Genotypic data for both rifampicin and isoniazid resistance genes were available for a subset of 24 MDR-TB strains. Analysis of these isolates suggests that the distribution of mutations differs between MDR-TB and non-MDR strains. Seventy-five percent of MDR isolates exhibited a *katG*315 mutation, compared with 40% of isoniazid-monoresistant isolates. The *katG*315 mutation therefore strongly correlated with MDR strains when compared with isoniazid-resistant isolates ($P = 0.0018$). In contrast, 4.2% of MDR strains were genotypically wild-type at both *katG* and *inhA*, compared with 29.2% of isoniazid-resistant isolates, indicating that isoniazid-resistant isolates were much more likely than MDR isolates to have no

Table 1. Genotypic mutations associated with rifampicin-resistant isolates

rpoB codon[a]	M. tuberculosis mutation		Phenotypically rifampicin-resistant		Phenotypically rifampicin-susceptible (n=182)	Total (%)[b] (n=92)
	amino acid	nucleotide	MDR-TB (n=85)	not MDR (n=4)		
rpoB511	Leu430Pro	CTG→CCG	1	–	1	2 (2.2)
rpoB514	Phe433PhePhe	TTC→TTCTTC	3	–	–	3 (3.3)
rpoB516	Asp435Val	GAC→GTC	6	–	–	6 (6.5)
rpoB522	Ser441Leu	TCG→TTG	1	2	–	3 (3.3)
rpoB526	His445Tyr	CAC→TAC	5	–	–	5 (5.4)
	His445Asp	CAC→GAC	4	–	–	4 (4.3)
	His445Leu	CAC→CTC	1	–	1	2 (2.2)
	His445Cys	CAC→TGC	1	–	–	1 (1.1)
	His445Arg	CAC→CGC	1	–	–	1 (1.1)
rpoB531	Ser450Leu	TCG→TTG	56	–	–	56 (60.9)
	Ser450Met	TCG→ATG	–	1	–	1 (1.1)
	Ser450Trp	TCG→TGG	1	1	–	2 (2.2)
rpoB516+rpoB522[c]	Asp435Gly	GAC→GGC	–	–	1	1 (1.1)
	Ser441Leu	TCG→TTG				
rpoB516+rpoB529[c]	Asp435Ala	GAC→GCC	1	–	–	1 (1.1)
	Arg448Gln	CGA→CAA				
rpoB509+rpoB526+rpoB527[d]	Ser428Arg	AGC→AGG	1	–	–	1 (1.1)
	His445Pro	CAC→CCC				
	Lys446Gln	AAG→CAG				
Wild-type[e]	NA[f]	NA	3	–	179	3 (3.3)

[a]Codon numbering using standard *Escherichia coli* nomenclature.
[b]Includes rifampicin-resistant isolates and strains with *rpoB* mutations.
[c]Double *rpoB* mutant.
[d]Triple *rpoB* mutant.
[e]No mutations in sequenced region.
[f]Not applicable.

identified mutations conferring resistance (i.e. wild-type *katG* or *inhA* promoter sequences) ($P=0.0091$).

Discussion

The global rise of MDR-TB has emphasized the need for rapid, molecular-based testing to detect drug resistance in TB. In Canada, 11% of TB cases are resistant to at least one drug and 1%–2% are MDR-TB.[7] All isolates used in this study originated from the province of Ontario, which harbours the greatest burden of TB in Canada, with 40% of all cases and 60% of all MDR-TB cases. While the overall resistance rates in Canada are low, TB drug resistance in Ontario is of special concern, since nearly one-third of Ontario residents are foreign-born. Approximately 80% of TB in Ontario is in foreign-born patients and this population is six times more likely than Canadian-born patients to be infected with MDR-TB.[7]

All of the isolates used in this study were tested at the OAHPP in Toronto. The Mycobacteriology Laboratory processes >50000 patient specimens and 2000 referred isolates annually, making it one of the largest mycobacteria diagnostic and reference laboratories in North America.

Previous studies indicate that *rpoB*526 and *rpoB*531 mutations comprise the majority of rifampicin resistance mutations.[4] *rpoB*531 mutants are as fit as wild-type strains, and both *rpoB*526 and *rpoB*531 mutations can arise spontaneously *in vitro*.[8] In this study, 64.1% of rifampicin-resistant isolates analysed exhibited a mutation at *rpoB*531 and 15.2% of isolates had mutations at *rpoB*526. Mutations at other sites were found less frequently. Three phenotypically resistant isolates were genotypically wild-type. Rifampicin resistance was indicative of MDR-TB in 95.5% of isolates.

Although prevalence is variable, *katG* mutations in isoniazid-resistant isolates are found more commonly than *inhA* promoter region mutations, specifically at residue 315. Previous studies indicate that *katG*315 mutants are quite fit and retain some of their enzymatic activity, allowing them to function like a wild-type strain.[9] In addition, this mutation is associated with very high levels of isoniazid resistance compared with other

Table 2. Genotypic mutations associated with isoniazid-resistant isolates

Mutation	Amino acid change	Nucleotide change	Total (%) ($n=154$)
katG	Tyr304Ser	TAT→TCT	1 (0.6)
	Ser315Thr	AGC→ACC	61 (39.6)
	Ser315Asn	AGC→AAC	2 (1.3)
	Ser315Gly	AGC→GGC	1 (0.6)
	Ser315Ile	AGC→ATC	1 (0.6)
	Tyr336Cys	TAC→TGC	1 (0.6)
	Lys414Asn	AAG→AAC	1 (0.6)
	Ser457Ile	AGC→ATC	1 (0.6)
	not identified[c]	NA	2 (1.3)
mabA-inhA promoter	NA[d]	−8 T→A	1 (0.6)
	NA	−15 C→T	37 (24.0)
	NA	−34 C→T	1 (0.6)
katG+*mabA-inhA* promoter[a]	Ser315Thr, NA	AGC→ACC, −8 T→C	2 (1.3)
	Ser315Thr, NA	AGC→ACC, −15 C→T	3 (1.9)
Wild-type[b]	NA	NA	39 (25.3)

[a]Mutations identified at both loci.
[b]No mutations in sequenced regions of *katG* or the *mabA-inhA* promoter.
[c]Region could not be PCR amplified, potential *katG* deletion.
[d]Not applicable.

isoniazid-resistant isolates.[10] In this study, 45.4% of isoniazid-resistant isolates exhibited mutations in *katG*315. The mutation S315T was the most commonly found. Mutations in the promoter region of *inhA* were found in 28.6% of isolates tested, mainly at position −15. In Ontario, mutations at *katG*315 correlated strongly with MDR-TB isolates, but were less prevalent than in other regions of the world.

This study is a snapshot of TB drug resistance in Ontario. Although current MDR-TB rates are low, a rapid screening method would be a valuable tool for the Ontario provincial reference laboratory to rule out MDR-TB in high-risk cases or to perform surveillance due to the potential for expansion of the MDR-TB population.

Despite the many advantages of molecular testing, in this study genotypic analysis did not identify phenotypically resistant strains in 3.3% of rifampicin-resistant isolates and 25.3% of isoniazid-resistant isolates, highlighting the limitations of molecular testing and indicating that, currently, molecular testing could not replace phenotypic testing.

In summary, this study describes the frequency of mutations conferring drug resistance in TB isolates from Ontario. This analysis serves as a basis for future customized molecular applications for Ontario TB control.

Transparency declarations

None to declare.

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