

Pyrazinamide-Monoresistant *Mycobacterium tuberculosis* in the United States

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Mycobacterium bovis is naturally resistant to the antituberculosis drug pyrazinamide (PZA). To determine whether all *Mycobacterium tuberculosis* complex isolates demonstrating PZA monoresistance were truly *M. bovis*, we examined the phenotype and genotype of isolates reported as PZA monoresistant in five counties in California from January 1996 through June 1999. Isolates reported by local laboratories to be PZA monoresistant were sent to the state reference laboratory for repeat susceptibility testing using the BACTEC radiometric method and to the Centers for Disease Control and Prevention for *pncA* sequencing and PCR-restriction fragment length polymorphism (RFLP) analysis of the *oxyR* gene. Of 1,916 isolates, 14 were reported as PZA monoresistant and 11 were available for retesting. On repeat testing, 6 of the 11 isolates were identified as PZA-susceptible *M. tuberculosis*, 1 was identified as PZA-monoresistant *M. bovis*, and 1 was identified as *M. bovis* BCG. The three remaining isolates were identified as PZA-monoresistant *M. tuberculosis*. Sequencing of the *pncA* and *oxyR* genes genotypically confirmed the two *M. bovis* and the six susceptible *M. tuberculosis* species. Each of the three PZA-monoresistant *M. tuberculosis* isolates had different, previously unreported, *pncA* gene mutations: a 24-bp deletion in frame after codon 88, a base substitution at codon 104 (Ser₁₀₄Cys), and a base substitution at codon 90 (Ile₉₀Ser). This study demonstrates that PZA monoresistance is not an absolute marker of *M. bovis* species but may also occur in *M. tuberculosis*, associated with a number of different mutational events in the *pncA* gene. It is the first report of PZA-monoresistant *M. tuberculosis* in the United States.

The members of *Mycobacterium tuberculosis* complex (*Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, and *Mycobacterium tuberculosis*) are closely related genetically but differ in their epidemiology and in their clinical presentation as tuberculosis (TB) in humans (3, 15, 19, 25). Differentiation of *M. bovis* from *M. tuberculosis* species is important from the public health perspective since control measures, treatment, and the natural history of the disease due to *M. bovis* infection are significantly different from those of *M. tuberculosis* (5).

In the clinical laboratory, phenotypic tests such as colony morphology, nitrate, and niacin tests can be used to distinguish *M. bovis* from *M. tuberculosis* species. The two species can also be distinguished by their susceptibility to thiophen-2-carboxylic acid (TCH) and pyrazinamide (PZA) (16). Although PZA-susceptible *M. bovis* species have been described, they are quite rare, and the finding of PZA monoresistance is generally considered a hallmark of *M. bovis* species (18).

Some laboratories in the United States use PZA monoresistance to distinguish *M. bovis* from other species in the *M. tuberculosis* complex; however, the proportion of laboratories using this criterion is unknown. Due to technical difficulty in

obtaining growth in the very acidic medium required for PZA activity, the results of PZA susceptibility testing are often unreliable (6, 12). As a result, PZA susceptibility tests are not performed routinely in all laboratories or else PZA monoresistance is often not detected and so the true prevalence of *M. bovis* infection is unknown.

Genotypic identification of *M. bovis* species can be performed by identifying characteristic mutations in the *pncA* and *oxyR* genes of *M. bovis* species, distinguishing it from other members of the *M. tuberculosis* complex, but this is not generally available in the clinical laboratory (27, 29). PZA resistance in *M. bovis* species is associated with a single point mutation from C to G at position 169 in the *pncA* gene, causing the replacement of histidine (CAC) with aspartic acid (GAC) at amino acid position 57. Mutation in the *pncA* gene, which encodes the enzyme pyrazinamidase (PZAase), which converts the inactive pro-drug PZA to the active moiety pyrazinoic acid, is associated with defective PZAase activity, resulting in PZA resistance (13, 26). A second genotypic marker used to identify *M. bovis* species is a polymorphism of G to A at position 285 in the *oxyR* gene (29). Another molecular method of identification relies on the fact that most strains of *M. bovis* have 1 to 6 copies of IS6110, whereas most *M. tuberculosis* strains have 10 to 16 copies (31). However, some *M. tuberculosis* strains also have only a few copies of IS6110 and overlap with *M. bovis*, and certain *M. bovis* strains have multiple copies of IS6110; thus, this method is unreliable (4, 20, 31).

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In this study we investigated the reliability of routine PZA susceptibility testing in some U.S. laboratories as a parameter for the differentiation of *M. bovis* from other members of the *M. tuberculosis* complex. During the course of this investigation we identified the first reported cases of PZA-monoresistant TB in the United States.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA isolation. To investigate the use of PZA monoresistance as a parameter for the speciation of *M. tuberculosis* complex, we carried out a search of all isolates reported as PZA resistant in the National Tuberculosis Genotyping and Surveillance Network (NTGSN) sponsored by the Centers for Disease Control and Prevention (CDC). As part of the NTGSN, from January 1996 through June 1999 a single isolate from each culture-positive TB case from five counties (Alameda, Solano, Santa Clara, Contra Costa, and San Mateo) in the San Francisco Bay area of California was sent for IS6110 restriction fragment length polymorphism (RFLP) DNA fingerprinting to the Department of Health, Berkeley, Calif. DNA extraction for PCR experiments was performed using mechanical lysis with a bead-beating procedure (21).

BACTEC drug susceptibility testing. Initial drug susceptibility testing was performed in local laboratories and repeated in the California Department of Health laboratory using the BACTEC radiometric susceptibility method (24). All isolates were retested for susceptibility to isoniazid (INH), rifampin (RIF), ethambutol (EMB), and streptomycin (STR) (24). PZA susceptibility testing was carried out in Middlebrook 7H12B broth at pH 6.0, and resistance was defined as no growth in medium containing 100 µg of PZA per ml, the recommended critical concentration for PZA for the determination of resistance (S. H. Siddiqui, BACTEC Manual). Isolates were reported as PZA monoresistant if they were resistant only to PZA and susceptible to the other four drugs.

Phenotypic characterization. Phenotypic identification of *M. bovis* species was based on the following four criteria: (i) the presence of buff dysgonic colonies, (ii) a negative niacin test, (iii) a negative nitrate test, and (iv) susceptibility to TCH (18). *M. tuberculosis* complex organisms were differentiated from other *Mycobacterium* species using high-performance liquid chromatography (HPLC) of mycolic acids. HPLC cannot differentiate *M. bovis* species from other members of the *M. tuberculosis* complex, but it can differentiate *M. bovis* BCG from other members of *M. tuberculosis* complex (1).

Genotypic characterization. (i) Amplification and sequencing of *pncA* gene. The entire *pncA* open reading frame, as well as 124 bp of upstream and 59 bp of downstream sequence, was PCR amplified. A 744-bp PCR product was generated using the primers *pncA*-8 (5'-GGTTGGGTGGCCGCCGTGTCAG-3') and *pncA*-11 (5'-GCTTTCGCGGAGCGCTCCA-3'). The *pncA* open reading frame (561 bp) begins at nucleotide 125 of the 744-bp PCR product and ends at nucleotide 685. Each 50-µl PCR mixture contained 1.0 µl of template DNA, 2.5 U of *Taq* DNA polymerase (Boehringer-Mannheim, Indianapolis, Ind.), deoxynucleotide triphosphates (dNTPs; 200 µM, each), and a 0.5 µM concentration of each primer in 1× PCR buffer. Amplification was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Inc., Foster City, Calif.) using a "touchdown" amplification approach in which the primer annealing temperature was decreased 0.5°C per cycle for the first 20 cycles, from 68°C for the first cycle to 58°C for cycles 20 to 35. The amplification profile consisted of an initial 5 min of denaturation at 94°C; 35 cycles of 94°C for 30 s, annealing for 30 s, and elongation at 72°C for 30 s; and a final 8-min elongation. Unincorporated primers and dNTPs were removed from the reaction mixtures using QIAquick PCR purification columns (Qiagen, Inc., Santa Clarita, Calif.).

Automated DNA sequencing was performed using rhodamine DyeDeoxy Terminator chemistry according to the protocol supplied by the manufacturer (Perkin-Elmer, Inc.). The fluorescent products were electrophoresed on an ABI model 373A instrument (Perkin-Elmer, Inc.). The *pncA* amplicons were sequenced using four internal primers. Primers *pncA*-10 (5'-GCTGGTCATGTT CGGATCG-3') and *pncA*-2R (5'-GAACACCGCCTCGATTGCCG-3') were used to sequence nucleotide residues 20 to 406 of the 744-bp amplicon. Primers *pncA*-6 (5'-CCTCGTGGCCACCGC-3') and *pncA*-9 (5'-CGCCAACAAG TTCAATCCCG-3') were used to sequence the region from residues 318 to 720. All post-run analysis was performed using Sequence Navigator version 1.0.1 software (Perkin-Elmer, Inc.). Each sequencing run included the PZA-susceptible strain *M. tuberculosis* H₃₇Rv (ATCC 27294) as a wild-type control. Each sequence was compared to both the control strain and the published *pncA* sequence (GenBank accession no. U59967).

(ii) PCR-RFLP analysis of allelic variation at *oxyR* nucleotide position 285. A 548-bp fragment of *oxyR* containing nucleotide 285 was amplified by PCR as

previously described (29). The PCR product (10 µl) was digested with 4 U of *AluI* (New England Biolabs, Beverly, Mass.), a restriction enzyme that cleaves at an AGCT sequence. The *oxyR* gene of *M. bovis* has an AGCT sequence beginning at the adenine residue located at nucleotide 285. Digestion was carried out at 37°C for 90 min, and the resulting DNA fragments were electrophoretically separated with a 1.8% agarose gel containing 0.03% ethidium bromide at a constant voltage of 90 V for approximately 2 h. Digested and undigested samples from each strain were electrophoresed in adjacent lanes. The DNA bands were visualized with a UV transilluminator and were documented by photography. There are three *AluI* restriction sites in the 548-bp sequence of *oxyR* in *M. tuberculosis* complex organisms with a guanine at nucleotide 285. Digestion with *AluI* should yield four DNA fragments of 236, 227, 55, and 30 bp. *M. bovis* isolates (with an adenine at position 285) have four restriction sites for *AluI* in the amplified *oxyR* gene segment and, therefore, five DNA fragments (236, 148, 79, 55, and 30 bp) should be produced.

(iii) IS6110 RFLP DNA fingerprinting. IS6110 RFLP DNA fingerprinting was performed on all available isolates using methods previously described (30).

RESULTS

BACTEC drug susceptibility testing. During the study period, 2,395 cases of TB were reported to the CDC from this site, and 1,916 of these were culture positive. IS6110 RFLP DNA fingerprinting had been performed on isolates from 1,436 cases (ca. 80% of all culture-positive isolates). Using the NTGSN database, we reviewed susceptibility data on all 1,436 isolates and identified 24 reported as PZA resistant. Of the 24 cases, 10 had isolates reported as resistant to at least two drugs, including PZA, and 14 had isolates reported as PZA monoresistant, of which 11 were available for repeat susceptibility testing. On repeat susceptibility testing six isolates were identified as PZA susceptible and five were reconfirmed as PZA monoresistant.

Phenotypic characteristics. The six isolates found to be PZA susceptible on repeat drug susceptibility testing were identified as *M. tuberculosis* isolates by colony morphology and biochemical testing. Of the remaining isolates, five were again found to be PZA monoresistant; two were identified as *M. bovis* and three were reported as *M. tuberculosis* based on colony morphology and biochemical characteristics (Table 1). Of note, three of the nine isolates identified as *M. tuberculosis* were found to be nitrate negative; one had a mutation in the *pncA* gene, and all three had phenotypic and genotypic criteria for identification as *M. tuberculosis* species (Table 1).

IS6110 RFLP DNA fingerprinting. IS6110 RFLP DNA fingerprinting revealed a single band in both the *M. bovis* and *M. bovis* BCG isolates. The three isolates identified as *M. tuberculosis* and confirmed as PZA monoresistant had IS6110 RFLP DNA fingerprint patterns that varied from 13 to 17 bands and were considered unrelated strains. The remaining six *M. tuberculosis* isolates had from 1 to 14 bands on IS6110 RFLP DNA fingerprinting, with three having only one or two bands.

Genotypic characteristics. Genotypic speciation by PCR-RFLP of the *oxyR* gene and sequencing the *pncA* gene further confirmed the biochemical identification of the six isolates identified as PZA-susceptible *M. tuberculosis* and the two PZA-monoresistant *M. bovis* isolates. All six PZA-susceptible *M. tuberculosis* isolates lacked the C-to-G mutation at position 169 in the *pncA* gene and the G-to-A polymorphism at position 285 in the *oxyR* gene, while each *M. bovis* isolate contained both the mutation and the polymorphism of the *pncA* and the *oxyR* genes, respectively (25) (Table 1). Mutations in the *pncA* genes

TABLE 1. Phenotypic and genotypic characterization of 11 *M. tuberculosis* isolates reported as PZA mono-resistant on initial susceptibility testing in local laboratories in the San Francisco area of California

Isolate	Species identification	Repeat sensitivity test ^a	Colony morphology	Niacin test	Nitrate test	IS6110 (no. of copies)	<i>pncA</i> type	<i>oxyR</i> (residue at position 285)
1	<i>M. tuberculosis</i>	R	Eugonic	+	–	14	Deletion ^b	G
2	<i>M. tuberculosis</i>	R	Eugonic	+	+	17	Ser ₁₀₄ Cys	G
3	<i>M. tuberculosis</i>	S	Eugonic	+	–	14	Wild type	G
4	<i>M. tuberculosis</i>	S	Eugonic	+	+	13	Wild type	G
5	<i>M. tuberculosis</i>	R	Eugonic	+	+	13	Ile ₉₀ Ser	G
6	<i>M. tuberculosis</i>	S	Eugonic	+	–	2	Wild type	G
7	<i>M. tuberculosis</i>	S	Eugonic	+	+	1	Wild type	G
8	<i>M. tuberculosis</i>	S	Eugonic	+	+	11	Wild type	G
9	<i>M. bovis</i>	R	Dysgonic	–	–	1	His ₅₇ Asp	A
10	<i>M. bovis</i> BCG	R	Dysgonic	–	–	1	His ₅₇ Asp	A
11	<i>M. tuberculosis</i>	S	Dysgonic	+	+	1	Wild type	G

^a R, resistant; S, sensitive.

^b Deletion, a 24-bp deletion after codon 88.

were found in each of the three PZA-mono-resistant *M. tuberculosis* isolates: a 24-bp deletion in frame after codon 88, a base substitution at codon 104 (Ser₁₀₄Cys), and a base pair substitution at codon 90 (Ile₉₀Ser). These three isolates did not contain the characteristic polymorphism in the *oxyR* gene associated with *M. bovis* species.

DISCUSSION

The finding of three TB cases with positive cultures for PZA-mono-resistant *M. tuberculosis* and high-copy-band numbers of IS6110 on RFLP DNA fingerprinting has not been previously reported in the U.S. Cheng et al. (2) recently described PZA-mono-resistant *M. tuberculosis* in a cohort of Canadian patients. They did not state how *M. tuberculosis* was spaciated; however, the mutation they demonstrated in these 21 isolates was not the characteristic *M. bovis* mutation. The 21 isolates with the same mutation in the *pncA* gene (20 PZA mono-resistant and 1 multidrug resistant) also had the same RFLP DNA fingerprint pattern, suggesting that a single strain was readily transmitted among individuals in this community.

TB disease in humans can be caused by *M. tuberculosis* or *M. bovis* species, and although they clinically cause very similar diseases, there are important differences in the epidemiology, treatment, and public health control measures for each (3, 19, 28). *M. tuberculosis* is primarily transmitted by direct exposure to aerosolized infectious particles and is the major cause of human TB (8, 22, 23). *M. bovis* is essentially a zoonosis that affects cattle and many domestic and wild animals; it can be transmitted by direct exposure or indirectly by ingestion of contaminated milk or milk products (19). In areas where bovine and human disease coexist and are endemic, the distinction of *M. bovis* from *M. tuberculosis* is important in monitoring the spread of *M. bovis* to humans and the need for improved veterinary public health measures (5).

With the introduction of genetic probes and HPLC analysis of mycolic acids, the use of classical biochemical tests for the identification of *M. tuberculosis* complex isolates has declined. However, neither of these rapid methods distinguishes *M. tuberculosis* from *M. bovis*, and many clinical laboratories in the United States use PZA mono-resistance as an indicator of *M. bovis* species in the laboratory. The results of this study highlight two difficulties with this approach: poor reproducibility

and lack of reliability. The poor reproducibility of susceptibility tests is illustrated by the finding that, when repeat susceptibility testing was carried out, 6 of the 11 isolates reported as PZA mono-resistant in peripheral laboratories were in fact PZA susceptible. National data on the reproducibility of PZA susceptibility testing were not yet available at the time of this study, although a preliminary assessment, using the BACTEC radiometric method, suggests that errors occur in <5% of PZA susceptibility tests repeated in the other laboratories (Beverly Metchock, CDC, personal communication). PZA mono-resistance in this study was not a reliable marker of *M. bovis* species, as three of the five isolates with PZA mono-resistance were identified as *M. tuberculosis*. Analysis of the polymorphism in the *oxyR* gene is currently the easiest, most rapid, and most reliable method for identifying *M. bovis* (7).

Although some studies have suggested that detection of one or few copies of IS6110 is characteristic of *M. bovis* species, we found that three of the *M. tuberculosis* isolates had only one or two copies of this insertion sequence, which is more suggestive of *M. bovis*, demonstrating that IS6110 RFLP is also not a reliable laboratory tool in the identification of *M. bovis* (31). Further, many strains of *M. tuberculosis* from Vietnam and other countries have been reported to have few or no copies of IS6110 (20). More recently, researchers conducting a large study in Australia found strains of *M. bovis* showing four or five IS6110 bands on DNA fingerprinting, demonstrating that finding multiple bands of IS6110 does not exclude *M. bovis* (4).

PZA is an important first-line anti-TB drug. Along with INH, RIF, STR, and EMB, PZA is an important component of the WHO Directly Observed Treatment Short Course Chemotherapy strategy (31). The introduction of PZA in combination therapy with RIF and INH had a significant impact on anti-TB regimens by reducing the duration of treatment from 9 to 6 months (9, 10). PZA has an important sterilizing effect, as measured by negative cultures at 2 months, and significantly reduces relapse rates in 6-month regimens (10, 11). On the basis of these data we can infer that infection with PZA-mono-resistant *M. tuberculosis* might be associated with a delayed response, delayed sterilization, higher relapse rates, and treatment failures. The finding of clinical TB cases due to PZA-mono-resistant *M. tuberculosis* is of particular concern in resource-poor countries where drug regimens using PZA are the standard practice for the treatment of TB (14).

In the move toward TB elimination, early and accurate identification of drug-resistant TB is an important first step in improved TB control. Careful evaluation in the clinical laboratory of low-cost molecular methods to reliably identify PZA resistance and to identify *M. bovis* species would be of benefit in some areas. Further work is needed to examine the clinical impact of PZA-monoresistant *M. tuberculosis*.

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