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In Vitro Susceptibility of *Mycobacterium tuberculosis* to Trimethoprim and Sulfonamides in France

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Five type strains and 55 *Mycobacterium tuberculosis* complex clinical isolates were found resistant to trimethoprim with a MIC of >200 mg/liter and susceptible to both sulfadiazine and cotrimoxazole with a MIC_{90} of 10 mg/liter. Two *M. canettii* isolates uniquely yielded silent mutations $C_{156} \rightarrow T$ and $G_{238} \rightarrow C$ in the *folP1* gene. Combined with scarce published data, these data indicate that sulfamides could be considered alternative antituberculous antibiotics.

Human tuberculosis caused by *Mycobacterium tuberculosis* complex (MTC) mycobacteria is responsible for more than 1.8 million deaths yearly (1, 2). The emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains is limiting the choice of antituberculous antibiotics to toxic, injectable second-line antibiotics (3, 4). Therefore, there is an interest in reevaluating alternative older antibiotics used as antituberculous agents in past decades. Among such antibiotics, sulfonamides are structural analogues of para-aminobenzoic acid (pABA), inhibiting the dihydropteroate synthase (DHPS) and blocking the folic acid synthesis pathway of *M. tuberculosis* (5). Few data have been issued regarding the reevaluation of sulfonamides as antituberculous antibiotics, in the area of XDR tuberculosis. Here, we determined the *in vitro* susceptibility of a collection of modern isolates, including XDR isolates, to sulfadiazine (SDZ), trimethoprim (TMP), and cotrimoxazole (sulfamethoxazole [SMX]-TMP).

A stock solution of cotrimoxazole (SMX administered at 400 mg combined with TMP at 80 mg in a 5-ml volume) (Roche, Neuilly, France) was stocked at +4°C. A 2 mg/ml stock solution of sulfadiazine (Sigma-Aldrich, Saint-Quentin Fallavier, France) was freshly prepared by dissolution into 10% NH_4OH (Sigma-Aldrich) and was filtered using a 0.22-μm-pore-size filter (Millipore, Molsheim, France). Likewise, a 12 mg/ml trimethoprim stock solution was freshly prepared in dimethyl sulfoxide (Euromedex, Souffelweyersheim, France) and filter sterilized.

A total of 55 *M. tuberculosis* clinical isolates, including 3 XDR isolates routinely made in the Institut Hospitalier Universitaire Méditerranée Infection Reference Laboratory for Mycobacteria and *M. tuberculosis* H37Rv CIP 103471, *Mycobacterium canettii* CIP 140010059, *Mycobacterium bovis* CIP 671203, *M. bovis* BCG Pasteur, and *M. bovis* BCG Tokyo, were examined in this study. All strains were cultured onto Middlebrook 7H10 agar at 37°C for 4 weeks, colonies were transferred into a sterile tube containing 5 to 10 sterile glass beads (Sigma-Aldrich) and 3 ml of Middlebrook 7H9 broth (Sigma-Aldrich) and processed with a vortex device, and the turbidity of the suspension was adjusted to 0.5 McFarland (Densimat; bioMérieux, La Balme-les-Grottes, France).

As for the susceptibility tests, 0.8 ml OADC (oleic acid, bovine serum albumin, dextrose, and catalase) (Becton, Dickinson, Sparks, MD) was added per MGIT tube (Becton, Dickinson). For each tested isolate, one drug-free tube was used as a positive-growth control and four to six additional tubes were inoculated with 5 mg/liter, 10 mg/liter, 20 mg/liter, or 40 mg/liter cotrimoxazole, 5 mg/liter, 10 mg/liter, 20 mg/liter, or 40 mg/liter sulfadiazine, or 10 mg/liter, 20 mg/liter, 40 μg/ml, 50 mg/liter, 100 mg/liter, or 200 mg/liter trimethoprim. Tubes were then inoculated with 0.5 ml of the mycobacterial suspension and incubated into the Bactec MGIT 960 instrument (Becton, Dickinson) for 7 days. Acid-fast staining (Ziehl-Neelsen) was performed on smears made from each signal-positive tube to confirm the presence of mycobacteria and eliminate any contaminant.

In the presence of positive controls, susceptibility testing yielded a MIC_{90} of 10 mg/liter for cotrimoxazole and sulfadiazine. All MTC mycobacteria were inhibited by 20 mg/liter cotrimoxazole and sulfadiazine (Table 1), whereas they were all resistant to trimethoprim, with a MIC of >200 mg/liter.

We then sequenced the PCR-amplified *folP1* gene encoding DHPS in tested strains, by incorporating mycobacterial DNA into a PCR using primer pair *folp1*-F (5′-AGTCATAGGTGTCGGCCAAG-3′) and *folp1*-R (5′-GCGGACTGTTCAAAACCAAT-3′) and a program consisting in an initial denaturation at 95°C for 15 min followed by 45 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 5 min. A negative control was used in each PCR run. Purified PCR products were sequenced using a BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) and primer pair *folp1-1* (5′-ATTCCGGTCGCGACCAGTT-3′) and *folp1-2* (5′-CTGGCCAGCGTCGCCGAC-3′). Comparison of the nucleotide sequence with a *M. tuberculosis* reference sequence (GenBank accession no. NC_000962.3) using the CLUSTAL W program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) indicated that 58 MTC strains were wild type whereas the *M. canettii* clinical isolate and type strain had silent mutations at codon 156 (GAC → GAT [aspartic acid]) and codon 238 (GCG → GCC [alanine]).

Here, the *in vitro* susceptibility and sequencing data were authenticated, as negative controls used in both culture-based and

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TABLE 1 Result of testing of MTC susceptibility to cotrimoxazole, trimethoprim, and sulfadiazine

Drug(s)	No. of resistant strains at indicated drug concn/no. of tested strains						
	5 mg/liter	10 mg/liter	20 mg/liter	40 mg/liter	50 mg/liter	100 mg/liter	200 mg/liter
SMX-TMP	60/60	2/60	0/60	0/60			
SDZ	60/60	7/60	0/60	0/60			
TMP		60/60	60/60	60/60	60/60	60/60	60/60

PCR-based experiments remained negative. The MGIT system here used has been previously reported (6). Our results showed good agreement with a previous study conducted in Massachusetts where 98% of 44 isolates, which included 6 MDR-TB isolates, were susceptible to cotrimoxazole, with a MIC of ≤19 mg/liter (7). Likewise, another study in Australia showed that 12 *M. tuberculosis* clinical isolates were susceptible to SMX, with a MIC of ≤38 mg/liter (6). Subsequently, in Taiwan, 117 *M. tuberculosis* clinical isolates, including 28 susceptible, 36 resistant, and 52 MDR isolates and 1 XDR isolate, were found to be susceptible to SMX, with a MIC_{90} of 9.5 mg/liter (8). Here, we found that the MIC for cotrimoxazole equaled the MIC for sulfadiazine, in agreement with a previous report that the MIC of cotrimoxazole was identical to that of SMX alone (6). These data suggest a lack of synergistic effect between SMX and TMP against MTC organisms (7), and, accordingly, we found all MTC mycobacteria to be resistant to TMP. However, the MICs determined here for sulfonamides are far below the 98 to 120 mg/liter serum concentration of sulfamethoxazole in individuals receiving either intravenous or high-dosage oral trimethoprim and sulfamethoxazole (9). These *in vitro* susceptibility data confirmed that 96.6% of the cotrimoxazole- and sulfadiazine-susceptible MTC had identical wild-type *folP1* sequences whereas the remaining two susceptible *M. canettii* isolates harbored two silent *folP1* mutations, representing the natural polymorphism of this gene.

In conclusion, we have demonstrated that MTC mycobacteria are susceptible *in vitro* to sulfonamides at concentrations easily achieved in patients. Sulfonamides could be considered second-line antibiotics in combination with other efficient antituberculous antibiotics for the treatment of drug-resistant tuberculosis.

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