

## Susceptibility of *Mycobacterium tuberculosis* to sulfamethoxazole, trimethoprim and their combination over a 12 year period in Taiwan

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Received 9 September 2011; returned 13 October 2011; revised 31 October 2011; accepted 31 October 2011

**Objectives:** This study was designed to determine the susceptibility of clinical isolates of multidrug-resistant (MDR) and non-MDR *Mycobacterium tuberculosis* to sulfamethoxazole, trimethoprim and trimethoprim/sulfamethoxazole over a 12 year period in Taiwan.

**Patients and methods:** We examined a total of 117 clinical isolates of *M. tuberculosis* collected from Southern Taiwan, 116 from 1995 to 2006 and an extensively drug-resistant (XDR) isolate in 2009. These included 28 isolates susceptible to all four first-line agents, 52 MDR isolates and 36 isolates with a mixed combination of drug resistance patterns other than MDR and 1 XDR isolate.

**Results:** Sulfamethoxazole inhibited 80% growth of all 117 isolates regardless of their susceptibility to the first-line agents at an MIC<sub>90</sub> of 9.5 mg/L. The concentration required to inhibit 99% growth was 38 mg/L. There were no significant changes in the MIC<sub>50</sub> or MIC<sub>90</sub> of sulfamethoxazole over a 12 year period. All 117 isolates were resistant to trimethoprim at >8 mg/L. The combination of trimethoprim/sulfamethoxazole at a ratio of 1:19 had no additive or synergistic effects.

**Conclusions:** Sulfamethoxazole inhibited the growth of clinical isolates of *M. tuberculosis* at achievable concentrations in plasma after oral administration. Susceptibility to sulfamethoxazole remained constant over a 12 year period. Trimethoprim was inactive against *M. tuberculosis* and trimethoprim/sulfamethoxazole provided no additional activity. Although the current and prior studies demonstrate that sulfamethoxazole is active against *M. tuberculosis* the search needs to continue for more active, lipid-soluble sulphonamides that are better absorbed into tissues and have improved therapeutic efficacy.

**Keywords:** sulphones, sulphonamides, treatment

### Introduction

Tuberculosis (TB) is one of the major causes of death worldwide. Treatment is complicated by the need for prolonged therapy, multidrug regimens and the need for good compliance. The global emergence of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB has made treatment even more difficult. Therefore, there is a continuing need for novel, minimally toxic drugs that are effective against tubercle bacteria.<sup>1,2</sup>

In addition to developing new compounds, expanding the indications of established drugs would lead to a fast avenue for the treatment of tuberculosis. Previously licensed drugs

have the advantage of known pharmacological properties, safety and potential for drug interactions. For example, the combination of meropenem and clavulanate has been shown to be effective against drug-susceptible and drug-resistant XDR clinical isolates, and persistent bacteria.<sup>3</sup> However, its use is limited to severe cases of tuberculosis because of the need for parenteral administration.

Sulphones and sulphonamides were used for the treatment of tuberculosis in the early 1950s. Extensive data are available for the old sulphonamide and sulphone compounds in the treatment of *Mycobacterium tuberculosis* (MTB) *in vitro* and *in vivo*, including data that were generated from experiments

in animals and humans.<sup>4</sup> These early-phase drugs were abandoned for the treatment of tuberculosis mostly because of their toxicity<sup>5</sup> and the availability of potent streptomycin, isoniazid and rifampicin at that time.

The synergistic activity of a combination of sulfamethoxazole and trimethoprim against a wide variety of Gram-positive and Gram-negative bacteria has been well established.<sup>6</sup> Recently, there have been two examinations of the susceptibility of *M. tuberculosis* to trimethoprim/sulfamethoxazole and to sulfamethoxazole alone. Forgacs *et al.*<sup>5</sup> found that 98% of their 44 isolates were susceptible to the trimethoprim/sulfamethoxazole combination at an MIC of  $\leq 19$  mg/L. These included six MDR-TB isolates. Ong *et al.*,<sup>7</sup> using the Mycobacterium Growth Indicator Tube [MGIT; Becton Dickinson (BD)] broth dilution drug susceptibility test system, found that 12 isolates susceptible to the first-line agents were susceptible to sulfamethoxazole at a concentration  $\leq 38$  mg/L. They also showed that sulfamethoxazole was bacteriostatic against *M. tuberculosis*. This prompted us to examine the activity of sulfamethoxazole, trimethoprim and their combination against isolates of MTB collected over a 12 year period in Southern Taiwan.

## Materials and methods

### *M. tuberculosis* isolates

The collection consisted of a total of 117 clinical strains: 116 isolated at the Kaohsiung Veterans General Hospital in Southern Taiwan over a 12 year period (1995–2006) and one XDR-TB strain isolated in 2009. *M. tuberculosis* complex strains isolated before 2000 were cultured in a BACTEC 460 instrument (BD Diagnostic Instrument Systems, Towson, MD, USA). All inoculated Bactec 12B vials were tested twice a week for the first 3 weeks and then once a week for the remaining 3 weeks. Positive vials were subjected to smear microscopy. Final identification of an MTB complex was done by the BACTEC NAP (*p*-nitro- $\alpha$ -acetylamino- $\beta$ -hydroxy propiophenone) differentiation test (BD Biosciences, Sparks, MD, USA). A decrease or unchanging growth index (GI) compared with the control vial indicates an *M. tuberculosis* complex, whereas an increase in GI indicates probable non-tuberculous mycobacteria. The strains isolated after 2001 were cultured by a BACTEC MGIT 960 (BD Biosciences). The instrument automatically monitors fluorescence every 60 min. A series of algorithms programmed in the instrument enables determination of a presumptive positive. Any sample that was identified as positive was removed from the instrument and subjected to smear microscopy. The BD ProTectET CTB assay (BD Diagnostic Systems, Baltimore, MD, USA) was used to identify the *M. tuberculosis* complex isolates. The above-mentioned methods used in current clinical settings, including ours, simply identify bacteria causing TB. Most of these TB-causing bacteria are *M. tuberculosis*, but the possible involvement of *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canettii* and *Mycobacterium microti* could not be completely excluded. Therefore, we use '*M. tuberculosis* complexes' or '*M. tuberculosis* isolates' in this report for clarity. The definition of drug-resistant *M. tuberculosis* is as follows: MDR, resistant to at least isoniazid and rifampicin; and XDR, resistant to at least rifampicin, isoniazid, any fluoroquinolone and to at least one of three injectable drugs used in anti-TB treatment (capreomycin, kanamycin and amikacin).

### Antimicrobial agents

Sulfamethoxazole and trimethoprim were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). Sulfamethoxazole was dissolved in a few drops of 1 N NaOH. The stock solutions were stored as aliquots at  $-20^{\circ}\text{C}$ . Working solutions were prepared by dilution with distilled water.

An appropriate amount of working solution was introduced into 7H11 medium (BD Biosciences) or agar. Sulfamethoxazole was tested at the following final concentrations in agar medium: 1.2, 2.4, 4.75, 9.5, 19, 38, 76 and 152 mg/L. Concentrations of easily dissolved trimethoprim ranged from 0.06 to 8 mg/L. The ratio of trimethoprim to sulfamethoxazole in the combination mixture was 1:19.

### Susceptibility testing

MICs were determined using the standard agar dilution method.<sup>8</sup> Briefly, 7H11 agar-containing medium was prepared from a dehydrated base as recommended by the manufacturer. After autoclaving and cooling to  $50$ – $56^{\circ}\text{C}$ , oleic acid/albumin/dextrose/catalase (OADC) supplement (BD Biosciences) and appropriate drug working solutions were aseptically added to the medium mixtures in 2-fold dilutions. The final medium mixture (4 mL each) was pipetted into individual plastic quadrant Petri plates. One quadrant in each plate receiving no drug in the medium mixture was used to serve as a control for the bacterial growth while the other quadrants had a series of 2-fold-diluted antibiotics in the same medium. To prepare the bacteria, *M. tuberculosis* colonies were suspended in 7H9 broth (BD Biosciences). The mixtures were vortexed and allowed to settle for 20 min to remove any debris. The supernatant fluid was then transferred to a second tube. This process was repeated twice. Then, the cells were diluted in 10 mL of 50 mM sodium phosphate buffer (pH 7.0) and adjusted with a colorimeter (Vitek Systems, Inc., Hazelwood, MO, USA) to yield 1 McFarland unit. The suspension contained approximately  $10^8$  cfu/mL. The broth was diluted 1:1000 to provide inocula of  $10^4$  cfu to each of the four quadrants. The plates were incubated for 3 weeks at  $35^{\circ}\text{C}$ . MICs were defined as the lowest concentrations that inhibited a defined percentage (80% or 99%) of the bacterial growth relative to the drug-free quadrant,<sup>9</sup> which contained between 100 and 500 colonies.<sup>10</sup> The MICs acting on 50% and 90% of the total bacterial population were termed MIC<sub>50</sub> and MIC<sub>90</sub>, respectively. Since there are no standardized guidelines for susceptibility testing of *M. tuberculosis* against sulfamethoxazole and trimethoprim, we adopted the CLSI (formerly NCCLS) criteria for susceptibility of *Mycobacterium kansasii* and *Mycobacterium marinum* to these drugs.<sup>11</sup> Resistance to sulfamethoxazole and trimethoprim were defined as MICs of  $\geq 38.2$  mg/L and  $\geq 2.0$  mg/L, respectively. These cut-offs also correspond to the achievable concentration of sulfamethoxazole following a single oral dose of 800 mg (160 mg for trimethoprim).<sup>12</sup> Quality control for trimethoprim was performed by broth microdilution testing with *Staphylococcus aureus* ATCC 29213 according to the CLSI M7-A7 methodology.<sup>13</sup> The endpoints were read as 80% or greater reduction in growth as compared with the control. The expected MIC range for the quality control strain was 1–4 mg/L according to the CLSI tables.<sup>14</sup>

## Results

### Clinical isolates of *M. tuberculosis*

The distribution of the 117 isolates of *M. tuberculosis* recovered in Southern Taiwan (116 isolates from 1995–2006 and an XDR strain isolated in 2009) are shown in Table 1; they are grouped according to the years of isolation and susceptibility patterns to anti-TB drugs.

### Susceptibility of *M. tuberculosis* to sulfamethoxazole and trimethoprim

The MICs of sulfamethoxazole and trimethoprim/sulfamethoxazole that inhibited 80% growth of *M. tuberculosis* are shown in

**Table 1.** Summary of 117 isolates of *M. tuberculosis* according to year of recovery and patterns of susceptibility to anti-TB drugs

Year	Number of isolates			Subtotal
	susceptible <sup>a</sup>	MDR <sup>b</sup>	mixed patterns <sup>c</sup>	
1995–97	7	15	9	31
1998–2000	7	12	6	25
2001–03	8	10	14	32
2004–06	6	15	7	28
2009	0	1 (XDR)	0	1
Total	28	53	36	117

<sup>a</sup>Susceptible to isoniazid, rifampicin, streptomycin and ethambutol.

<sup>b</sup>Resistant to isoniazid and rifampicin.

<sup>c</sup>Resistance patterns other than those defined by MDR and XDR.

**Table 2.** Activities of sulfamethoxazole alone and in combination with trimethoprim against 117 clinical isolates of *M. tuberculosis*, grouped according to susceptibility to the first-line agents

MIC (mg/L)	Number of isolates with 80% inhibition of growth					
	Susceptible <sup>a</sup>		MDR <sup>b</sup>		Mixed patterns <sup>c</sup>	
	SMX	SXT	SMX	SXT	SMX	SXT
≤1.19	1	1	4	3	0	0
2.38	1	1	2	3	1	2
4.75	6	6	11	11	14	13
9.5	18	18	32	33	19	19
19	2	2	4	3	2	2
Total	28	28	53	53	36	36

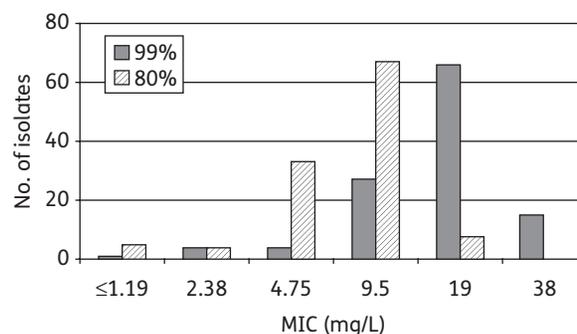
SMX, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole.

<sup>a</sup>Susceptible to isoniazid, rifampicin, streptomycin and ethambutol.

<sup>b</sup>Resistant to isoniazid and rifampicin; includes one XDR-TB isolate (MICs of SMX and SXT were both 9.5 mg/L).

<sup>c</sup>Other combinations of resistance patterns.

Table 2. Sulfamethoxazole was highly active (MIC ≤4.75 mg/L) against 42/117 (35.9%) of the isolates, but 19 mg/L was required for 8/117 (6.8%) of the isolates. Collectively, sulfamethoxazole inhibited growth of all 117 isolates at concentrations not greater than 19 mg/L—levels achievable in plasma after oral administration, regardless of their susceptibility to the first-line agents. The MIC<sub>50</sub> and MIC<sub>90</sub> of sulfamethoxazole to inhibit 80% growth were both 9.5 mg/L. The MICs of sulfamethoxazole alone were nearly identical to those of the combined trimethoprim/sulfamethoxazole. All 117 isolates were resistant to trimethoprim at >8 mg/L when trimethoprim alone was tested. The activity of sulfamethoxazole against 117 clinical isolates of *M. tuberculosis* according its ability to inhibit 80% and 99% of bacterial growth is summarized in Figure 1. Among them, only 15/117 (12.8%) required 38 mg/L sulfamethoxazole to inhibit 99% of the bacterial growth.

**Figure 1.** Summary of sulfamethoxazole activity against 117 clinical isolates of *M. tuberculosis* collected from Southern Taiwan according to 80% and 99% inhibition of growth.**Table 3.** MIC<sub>50</sub> and MIC<sub>90</sub> of sulfamethoxazole at 80% inhibition of growth for 116 isolates of *M. tuberculosis* isolated in Southern Taiwan and grouped according to the years recovered

Year	No.	Sulfamethoxazole (mg/L)		
		MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>
1995–97	31	1.19–19	9.5	9.5
1998–2000	25	≤1.19–9.5	9.5	9.5
2001–03	32	2.38–19	9.5	19 <sup>a</sup>
2004–06	28	≤1.19–19	9.5	9.5
Total	116	≤1.19–19	9.5	9.5

<sup>a</sup>Four strains susceptible to 19 mg/L sulfamethoxazole in this period.

## 12 year trend in susceptibility of *M. tuberculosis* to sulfamethoxazole

The MIC<sub>50</sub> and MIC<sub>90</sub> of sulfamethoxazole that inhibited 80% growth of *M. tuberculosis* by year of isolation are shown in Table 3. Susceptibility to sulfamethoxazole remained about the same during the study period except for 2001–03. The MIC<sub>90</sub> for this period increased from 9.5 to 19 mg/L; this was due to a set of four strains isolated during this 3 year period having an MIC<sub>90</sub> of 19 mg/L. However, this occurrence did not notably affect the overall trend.

## Effect of the medium on susceptibility of *M. tuberculosis* to sulfamethoxazole and trimethoprim/sulfamethoxazole

Media 7H10 and 7H11 differ by the absence or presence of digested casein, respectively. To assure that the lack of synergism with the combination of trimethoprim/sulfamethoxazole was not due to medium difference,<sup>5</sup> MICs of sulfamethoxazole and trimethoprim/sulfamethoxazole were re-assessed with 20 representative MDR-TB isolates. The MICs of sulfamethoxazole and trimethoprim/sulfamethoxazole in 7H10 were 2-fold less than those seen for 7H11 with the isolates tested. Otherwise, the patterns were quite similar (Table 4).

**Table 4.** Effect of medium (7H10 and 7H11) on the MICs of sulfamethoxazole and trimethoprim/sulfamethoxazole for a subset of 20 representative isolates of *M. tuberculosis*

antibiotic	80% inhibition MIC (mg/L)						99% inhibition MIC (mg/L)					
	7H11			7H10			7H11			7H10		
	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>
SMX	≤4.75–19	9.5	19	≤4.75–9.5	≤4.75	9.5	9.5–38	19	38	≤4.75–19	9.5	19
SXT	≤4.75–19	9.5	19	≤4.75–9.5	≤4.75	9.5	9.5–38	19	38	≤4.75–19	9.5	19

SMX, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole.

## Discussion

The current study confirms earlier reports that sulfamethoxazole has activity against *M. tuberculosis* at concentrations achievable in plasma, which are 30–60 mg/L after oral administration of 800 mg of sulfamethoxazole and 160 mg of trimethoprim.<sup>12</sup> Our findings for sulfamethoxazole alone are similar to those of Forgacs *et al.*,<sup>5</sup> who also used the agar dilution method but with 7H10 medium, instead of our 7H11. They reported that 98% of 44 isolates had MICs of trimethoprim/sulfamethoxazole of ≤19 mg/L. We found that at 80% inhibition of bacterial growth, all our 117 isolates had MICs of sulfamethoxazole of ≤19 mg/L. They reported that 11/44 (25%) of their isolates were susceptible at 1- to 2-fold lower concentrations of sulfamethoxazole when tested in combination with trimethoprim.<sup>5</sup> We found that in our isolates the trimethoprim/sulfamethoxazole combination was not more active than sulfamethoxazole alone. However, we did find that in 7H10 all isolates seemed to be more susceptible to sulfamethoxazole (or trimethoprim/sulfamethoxazole) than in 7H11, since the MICs were 2-fold reduced. Therefore, the enzyme-digested casein-containing 7H11 agar may reduce the drug susceptibility although it enhances the growth of some recalcitrant isolates of drug-resistant *M. tuberculosis*.<sup>15</sup>

Ong *et al.*,<sup>7</sup> using the MGIT 960 system, have found that the MIC of sulfamethoxazole against a collection of 12 *M. tuberculosis* isolates was ≤38 mg/L. Wallace *et al.*,<sup>16</sup> using the 7H9 microdilution method, found that ≥90% of 10 isolates of MTB were susceptible to 8 mg/L sulfamethoxazole. We found that 109/117 (93.2%) of our strains were susceptible to 9.5 mg/L sulfamethoxazole and that the susceptibility to sulfamethoxazole was independent of the resistance to the first-line agents (Table 2). Taking these results together, we could generally conclude that many clinical isolates, if not all, remain susceptible to sulfamethoxazole.

All of the tested isolates of *M. tuberculosis* from Southern Taiwan in our 12 year collection were resistant to trimethoprim at >8 mg/L. This concentration cannot be achieved in plasma after oral administration of the trimethoprim/sulfamethoxazole standard dose. Our results are in accord with those of Suling *et al.*<sup>17</sup> and Jacobs,<sup>18</sup> who also found that *M. tuberculosis* is resistant to trimethoprim. It is not known so far whether the mechanism of trimethoprim resistance in *M. tuberculosis* is due to overexpression of *dfrA* as suggested by studies with *M. smegmatis*.<sup>19</sup>

Wiktor *et al.*<sup>20</sup> reported that trimethoprim/sulfamethoxazole reduced mortality by 41% in African HIV-1-infected patients

with tuberculosis. They attributed this phenomenon to better control of secondary infections. We agree with Köser *et al.*<sup>21</sup> that 'A clear understanding of the resistance mechanisms of trimethoprim/sulfamethoxazole in *M. tuberculosis* is urgently needed since the WHO has renewed its call for widespread use of this drug combination for the prophylactic treatment of patients with HIV.' Excessive use of antimicrobial drugs, including trimethoprim/sulfamethoxazole, is known to be widespread in developing countries.<sup>22,23</sup> Our finding that susceptibility to sulfamethoxazole of *M. tuberculosis* did not notably change over a 12 year period in Taiwan is somewhat reassuring in that strain resistance of *M. tuberculosis* to sulfamethoxazole has not been evident. However, we cannot predict whether it may emerge in the future.

In summary, we have confirmed that sulfamethoxazole exhibits *in vitro* activity against clinical isolates of *M. tuberculosis* and that inhibiting 80% of the growth of all 117 isolates could be achieved at 19 mg/L. To achieve 99% inhibition of growth for all isolates, 38 mg/L sulfamethoxazole is needed and this concentration is still achievable in plasma if orally administered at 800 mg. However, sulfamethoxazole has several unfavourable pharmacological properties; these include poor lipid solubility and a restricted distribution pattern, typically confined to the intravascular space. On the other hand, trimethoprim alone was not active against our isolates of *M. tuberculosis* and it did not augment the activity of sulfamethoxazole. Intriguingly, there were no significant changes in susceptibility to sulfamethoxazole over a 12 year period. Therefore, it is suggested that a search for more active, lipid-soluble compounds with improved tissue distribution might enhance the therapeutic value of sulphonamides/sulphones toward treating tuberculosis.

## Acknowledgements

We are grateful to Chia-Chien Lee for his excellent technical assistance.

## Funding

This work was financially supported by Kaohsiung Veterans General Hospital (VGHKS 97-67 to T. S. H.), the National Science Council of Taiwan (NSC99-2320-B-075B-002 to T. S. H.), the National Health Research Institutes (Taiwan) (NHRI-98A1-CLCO-0707091 to Y.-S. C.) and a grant from the Ministry of Education, Aim for the Top University Plan (to W.-J. S.).

## Transparency declarations

None to declare.

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