Title:

Is Streptococcus pyogenes resistant or susceptible to trimethoprim-sulphamethoxazole?

Running Title:

S. pyogenes and trimethoprim-sulphamethoxazole

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ABSTRACT

Streptococcus pyogenes is commonly believed resistant to trimethoprim-sulphamethoxazole (SXT), resulting in reservations about using SXT for skin and soft tissue infections (SSTI) where S. pyogenes is involved. S. pyogenes in vitro susceptibility to SXT depends on the medium’s thymidine content. Thymidine allows S. pyogenes to bypass the sulphur mediated inhibition of folate metabolism and historically, resulted in apparently reduced susceptibility of S. pyogenes to sulphur antibacterials. Low thymidine concentration in Mueller Hinton Agar (MHA) is now regulated. We explored S. pyogenes susceptibility to SXT on various media. Using two sets of 100 clinical S. pyogenes isolates, we tested for susceptibility using SXT Etests® on: MHA containing defibrinated horse blood and 20mg/L β-NAD (MHF), MHA with sheep blood (MHS), MHA, MHA with horse blood (MHBA), and MHA with lysed horse blood (MHLHBA). EUCAST breakpoints defined susceptibility (MIC ≤ 1 mg/L) and resistance (MIC > 2 mg/L). In study 1, 99% of S. pyogenes isolates were susceptible to SXT on MHA, MHBA and MHLHBA with geometric mean MIC 0.04, 0.04 and 0.05 mg/L respectively. In Study 2, all 100 S. pyogenes isolates were susceptible to SXT on MHF, MHS, MHA, and MHLHBA with geometric mean MIC 0.07, 0.16, 0.07 and 0.09 mg/L respectively. This study confirms in vitro susceptibility of S. pyogenes to SXT, providing support for the use of SXT for SSTIs. A clinical trial using SXT for impetigo is ongoing.
Introduction

Streptococcus pyogenes was one of the first bacterial infections to be treated with sulphur antibacterials in the 1930s (16) and proved to be clinically effective in the treatment and prophylaxis of S. pyogenes infections (10, 16, 23, 29). However, when sulfadiazine, an early short-acting sulphur antibacterial, was used in mass prophylaxis programs to prevent S. pyogenes tonsillitis and acute rheumatic fever (ARF) in military recruits in the 1940s, the clinical efficacy of this antibacterial was limited due to the presumed development of resistance (13, 15, 27, 31, 42) amongst some strains. Initial antibacterial susceptibility testing (AST) of S. pyogenes to sulphur antibacterials using a broth dilution method demonstrated some strains were resistant (25, 53); however, AST was in its infancy and no standardised reference methods existed at that time. This early experience resulted in the belief that trimethoprim-sulphamethoxazole (SXT) is ineffective against S. pyogenes and its use has been discouraged in clinical practice for decades (35).

Subsequent antibacterial susceptibility experiments showed apparently reduced susceptibility of S. pyogenes (and other bacteria) (6, 40) to the sulphur antibacterials due to antagonism of the inhibition of folate metabolism. Harper and Cawston discovered an inhibitory substance in 1945, eventually identified as thymidine, which was interfering with the ability of sulphur antibacterials to kill the organism (25). Because the activity of SXT is determined by the antibacterial’s ability to deprive an organism of folate coenzymes (7), there is a direct relationship between the thymidine levels in culture media and SXT resistance (11). High thymidine content in agar provides an exogenous substrate which can be used by an organism to maintain...
S. pyogenes and SXT Page 4

folate metabolism and hence appear resistant to SXT. In early studies, most culture media contained sufficient thymidine to antagonise the inhibitory effects of sulphur drugs and hence produced resistant results when this class of antibacterials was tested (6).

Notably, lysed horse blood was found to contain the enzyme thymidine phosphorylase which neutralised thymidine (46) and overcame this effect (21, 25). No other mammalian blood contains thymidine phosphorylase (21). However, the addition of lysed horse blood was not recommended for AST, despite several authors (5, 6, 21, 54) advising supplementation with lysed horse blood for any medium used to test sulphur antibacterial susceptibility, if the thymidine concentration was above 0.03µg/mL (5) (below which inhibition does not occur). In this context, the notion that S. pyogenes was resistant to sulphur antibacterials perpetuated.

SXT was introduced in 1968 (3, 28, 43), and has since become one of the most widely used antibacterials in the world. However, recommendations against the use of sulphur antibacterials, including SXT, for S. pyogenes infections continue in the belief that the organism is intrinsically resistant (33, 47, 51). Two studies (33, 51) have reported S. pyogenes uniformly resistant to SXT, but this was prior to thymidine content standardisation in MHA and was on agar containing sheep blood. There have also been reported clinical failures in the use of this agent in eradicating S. pyogenes from nasopharyngeal carriage (30). However, other centres have demonstrated full in vitro susceptibility of S. pyogenes to SXT (14, 22, 36, 54). Since 2006, when thymidine content of MHA became strictly regulated by the Clinical and Laboratory Standards Institute (CLSI) to maintain a low level of thymidine and hence avoid
inhibition (M6-A2 protocol) (9), it has no longer been necessary to add lysed horse
blood to the medium for AST. However, current methods do use agar supplemented
with mammalian blood.

Given the prevailing view that caution should be exercised in using SXT for
infections involving *S. pyogenes* and the paucity of clinical data of SXT efficacy
against *S. pyogenes*, we sought to confirm or disprove the notion that *S. pyogenes* is
resistant to SXT *in vitro* on various antibacterial susceptibility testing media. Co-
infection of *S. pyogenes* with *Staphylococcus aureus* in skin and soft tissue infections
and the rising prevalence of methicillin resistant *S. aureus* (MRSA) provide added
stimulus to explore the utility of SXT in the treatment of these infections.

**Materials and Methods**

**Swab collection and identification of *S. pyogenes***

Skin, throat or nose swabs were collected using a rayon tipped cotton swab (Copan,
Interpath Services, Melbourne Australia). In study 1, swabs were plated on horse
blood agar (HBA, Oxoid Basingstoke UK) and HBA containing colistin and nalidixic
acid (HBA + CNA, Oxoid) within 48 hours of collection. In study 2, swabs were
stored in skim milk tryptone glucose glycerol broth (STGGB) at −70°C prior to
plating on the above media. Incubation was at 37°C for 16 hours in 5% CO₂. Beta
haemolytic colonies were identified morphologically and confirmation of *S. pyogenes*
was with the Lancefield streptococcal grouping test for group A (Oxoid). Isolates
were stored in glycerol at −70°C until subsequent replating for AST.
Selection of isolates:

Study 1
We began by exploring the issue with a preliminary study of 100 skin and throat isolates of *S. pyogenes* collected from 3 remote Australian Aboriginal communities between 2003 and 2005 during surveillance studies (39). We performed a SXT E test® strip (bioMérieux, France) on 3 different agars - MHA, MHA supplemented with horse blood (MHBA) and MHA supplemented with lysed horse blood (MHLHBA) (Oxoid). Interpretation was based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (20) released in 2010.

Study 2
The Skin Sore Trial is a randomised, controlled trial comparing benzathine penicillin G (BPG) treatment of impetigo (the standard of care), with oral SXT. The first 100 *S. pyogenes* isolates from skin and nasal swabs from participants in the Skin Sore Trial were used in Study 2. The participants were aged 3 months – 13 years and were from 4 remote Aboriginal communities of the Top End of the Northern Territory, Australia, recruited in 2010. Swabs were collected from the anterior nares and at least 2 purulent or crusted sores from all children. Swabs were collected from skin sores on days 0 (pre treatment), day 2 (mid treatment) and day 7 (completion of treatment). Consent for participation and collection of specimens was obtained from the guardian or parent of each participant. The study was approved by the Northern Territory Top End Human Research Ethics Committee (HREC 09/08) and has been registered with the Australian and New Zealand Clinical Trials Registry (ACTRN 12609000858291).
Antibacterial Susceptibility Testing Methods and agar used

The two internationally accredited, standardised methods for AST (Table 1), are the CLSI (8), and EUCAST (19) methods. CLSI does not reference breakpoints for *S. pyogenes* susceptibility to SXT and recommends the use of Mueller Hinton agar (MHA) supplemented with 5% sheep blood for testing susceptibility of *S. pyogenes* to other antibacterials. In contrast, EUCAST has released breakpoints for disc diffusion method and Minimum Inhibitory Concentration (MIC) on appropriate media. The medium recommended for *Streptococcus* groups A, B, C and G is MHA supplemented with 5% defibrinated horse blood + 20mg/L B-Nicotinamide Adenine Dinucleotide. This agar is commonly referred to as MHF (www.eucast.org, accessed 25 July 2012).

Two experimental agars were also used for susceptibility testing. MHA is routinely used in a number of other antibacterial susceptibility tests not involving beta haemolytic streptococci. MHLHBA was used to explore the hypothesis based on historical literature that the lysing of horse blood releases thymidine phosphorylase which breaks thymidine down to thymine.

Using the EUCAST breakpoints (20) with MIC $\leq$ 1 mg/L as sensitive and MIC > 2 mg/L as resistant and a SXT Etest® to perform antibacterial susceptibility, these studies compared the various media that have been recommended for AST. The Etest® interpretation is based on the trimethoprim component of the trimethoprim-sulphamethoxazole combination, in a ratio of 1:19. The EUCAST methodology using an Etest® was chosen due to the availability of published breakpoints, however due to the widespread use of CLSI, the medium upon which this organism is tested was also used and results obtained referenced to the EUCAST breakpoints.
Antibacterial Susceptibility Testing

Single colonies were isolated from frozen stocks following overnight incubation on HBA in 5% CO₂ at 37°C. Susceptibility testing for SXT was performed using a 0.5 McFarland suspension to create a confluent lawn inoculum and then applying a SXT Etest® as per the manufacturer’s instructions. Plates were read following incubation in 5% CO₂ at 35°C for 16 – 20 hours by 2 readers. The MIC was recorded where the inhibition ellipse intersected the scale. Where a difference in result of more than 2 gradations was noted between the 2 readers, a repeat test was performed with a fresh subculture of S. pyogenes. As SXT is a bacteriostatic antibacterial, this mode of action can alter the appearance of an MIC endpoint, resulting in hazy zones. Where haze was present, both the 80% and 100% points of ellipse intersection were recorded. A penicillin, erythromycin and clindamycin disc diffusion according to CLSI guidelines (8) was conducted concurrently on all 100 strains in Study 2.

Quality Control

In Study 2, for every 20 S. pyogenes clinical isolates, a control strain of S. pyogenes (ATCC19615) was also tested on the 4 agars and found to be susceptible. Quality control of the SXT Etests® was performed throughout the study using Escherichia coli (ATCC 25922) on MHA and Streptococcus pneumoniae (ATCC 49619) on MHS. The reference ranges for each organism were achieved, namely 0.064 – 0.25 µg/mL for E. coli and 0.125 - 1 µg/mL for S. pneumoniae.

STATA version 12.0 (STATAcorp, College Station, TX) was used to determine the geometric means. The data was logarithmically transformed to a normal distribution and paired t-tests used to determine the difference between agars for Studies 1 and 2.
**Results**

*Study 1*

On MHBA and MHLHBA, *S. pyogenes* isolated from the skin (n=36) and throat (n=64) were uniformly susceptible to SXT (Table 2). Ninety-nine isolates tested on MHA were susceptible to SXT (Table 2). The single isolate which appeared resistant on MHA was susceptible on both MHBA and MHLHBA.

There was no statistically significant difference between the geometric mean MIC measures on MHA and MHBA. However, isolates tested on both MHA and MHBA had a lower MIC than isolates tested on MHLHBA (Table 3).

*Study 2:*

One hundred isolates of *S. pyogenes* from 43 children were included in this analysis. *S. pyogenes* isolates utilised were from sores (n=98) and the anterior nares (n=2). The majority of isolates (76%) were from day 0 (before antibacterial treatment), 19% from day 2 and 5% from day 7. Sixty-four of the swabs from which *S. pyogenes* was identified also cultured *S. aureus*. Of these, 14% were methicillin-resistant *S. aureus* (MRSA) and 86% methicillin-susceptible *S. aureus* (MSSA).

All 100 isolates of *S. pyogenes* were susceptible to SXT on all agars by both readers (Table 4). Inter-rater reliability was excellent with 96% of all MIC readings within +/- 1 MIC gradation. In view of this, all analyses were done on results from reader 1. All 100 *S. pyogenes* isolates were also susceptible to penicillin, erythromycin and clindamycin.
The geometric means were similar for MHA, MHBA and MHLHBA (Table 4). MHS had a higher geometric mean MIC than the other media. This was statistically significant, with isolates tested on MHS having a higher geometric mean MIC than the same isolates tested on all other agars (Table 5). Despite the higher MIC, all isolates tested on MHS were still susceptible to SXT. There was no difference in MIC between isolates tested on MHA and MHF. As in study 1, isolates tested on MHA had a lower MIC than the same isolates tested on MHLHBA. This was also found for isolates tested on MHF (Table 5).

Ongoing surveillance with *in vitro* susceptibility testing is needed to monitor for changes in rates of SXT resistance with increased use of SXT. To date, we have tested 910 *S. pyogenes* isolates cultured from impetigo and anterior nares of children randomised in the Skin Sore Trial on MHF using a SXT Etest® according to EUCAST guidelines. Only 8 (0.9%) have been found to be resistant with MIC > 2mg/L (unpublished data). These results are consistent with those reported from the EUCAST group.

**Discussion**

Although SXT is no longer commonly recommended for treatment of respiratory tract infections, it remains one of the most widely used and cheapest antibacterials in the world, and is an important option for treatment of SSTI, where *S. pyogenes* and *S. aureus* are often co-pathogens (4, 12, 24, 34). In the era of rising MRSA prevalence, antibacterials that are active against both bacteria are highly valued.
Impetigo is a significant therapeutic problem in remote communities in the Northern Territory of Australia (37, 49, 50) with community-associated MRSA having become highly prevalent in this region (50). Impetigo is also an endemic problem in many less developed countries (41, 45) and MRSA is likely to be on the rise in these contexts also (50). In patients with MRSA and \textit{S. pyogenes} co-infection, finding a single oral agent that is effective, affordable and easy to use would be a significant advance. Penicillins and cephalosporins are no longer an option for MRSA. In the Northern Territory context, clindamycin is not an option with up to 22% of MRSA isolates resistant (49), aside from its poor palatability in young children and the difficulties in maintaining adherence to a thrice-daily regimen. Tetracyclines are not recommended in children under 8 years of age (17) and linezolid is currently too expensive for the empiric treatment of such a common childhood condition. SXT, which is cheap, widely available, well tolerated and requires only twice-daily dosing, is a potential single agent for treatment of both MRSA and \textit{S. pyogenes}. Several studies have confirmed the ongoing susceptibility of \textit{S. aureus} to SXT in this region of Australia (38, 49).

The breakpoints utilised for this study were defined by EUCAST using data collated from a wide range of sources on more than 2500 isolates of \textit{S. pyogenes} tested for susceptibility to SXT using a variety of methods (32). Of the 2596 tests reported from multiple sources, geographical areas and time periods, 2559 were susceptible to SXT \( \leq 1 \text{mg/L} \) and 23 isolates were resistant (0.9%). (European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC distribution website \texttt{http://mic.eucast.org}, last accessed 18 September 2012). On disc diffusion testing for \textit{S. pyogenes} using SXT discs in a ratio of 1:19, a zone size of \( \geq 18 \text{ mm} \) is
susceptible and < 15 mm is resistant. Using these breakpoints, a total of 358 tests were performed, with 5 confirmed resistant strains (1.4%) (http://mic.eucast.org, last accessed 18 September 2012). This can be contrasted with US based literature using the CLSI methods, where AST for *S. pyogenes* is performed on agar supplemented with defibrinated sheep blood and SXT is not routinely tested as *S. pyogenes* are considered universally resistant (51). Defibrinated sheep blood is utilised as the haemolytic reactions of beta-haemolytic streptococci on blood agar containing sheep blood are deemed “true” (1).

The *in vitro* results reported in the current study confirming the susceptibility of *S. pyogenes* to SXT suggest treatment of SSTI with SXT is worth considering. Our current RCT to assess non inferiority of SXT to the standard treatment with benzathine penicillin G (BPG) for impetigo will provide the necessary clinical evidence to inform guidelines. It is based on a pilot study of 13 participants which indicated that both BPG and SXT were efficacious in healing impetigo (48). There is one study published comparing these agents for *S. pyogenes* infection in tonsillitis, which reported a 70% treatment efficacy for SXT compared to 88% for penicillin, a non-statistically significant difference (52).

The infrequent reports of susceptibility of *S. pyogenes* to SXT demonstrate resistance rates ranging from 0% to 100% depending on which medium and testing conditions are used (Table 6). Although the variation in results may relate to the particular strains included, or the local prescribing patterns of SXT, it is most likely related to the methodology of testing. All of the studies reporting high resistance rates either used media known to have high concentrations of thymidine, or did not provide details of...
the medium used. As standardisation to ensure low thymidine concentration of Mueller-Hinton medium was only introduced in 2006, it is likely that, unless low thymidine-media were specified (2, 14, 18, 22, 26, 44, 54), publications prior to this may not have controlled for thymidine content.

Alongside this, *S. pyogenes* has remained 100% susceptible *in vitro* to penicillin. Hence there has been no pressing need to understand SXT susceptibility as an alternative antibacterial in the public health approach to treatment of *S. pyogenes* infections. However, this is changing in the context of rising MRSA rates for SSTI.

Our results show that testing of *S. pyogenes* for susceptibility to SXT on MHS gives a higher MIC than all of the other agars, although the organism remains in the susceptible range. This could possibly be due to the availability of thymidine or other inhibitory substances in this medium. However, thymidine concentrations of the various media utilised were not assessed. Alternatively, the absence of an enzyme to reduce the inhibition in sheep blood as compared to horse blood may be the explanation.

The original paper describing the identification of the Harper Cawston Factor (25) as thymidine (21) reports an interesting observation that this study has partially demonstrated. Only lysed horse blood contains thymidine phosphorylase to convert thymidine to thymine, and hence overcome the inhibition of folate metabolism that occurs in the presence of thymidine. No other mammalian blood contains this enzyme, which is a possible reason for the higher MICs reported on MHS than those agars containing horse blood. In the original paper, the presence of thymidine at
concentrations of 1.6µg/ml was sufficient to completely prevent inhibition by the drugs. The inclusion of lysed horse blood restored the inhibition. This has also been shown by Coll et al (11). However, the MIC of S. pyogenes isolates tested on MHLHBA was higher than those tested on MHF (Study 2), MHA (Studies 1 and 2) or MHBA (Study 1) which suggests other factors at play.

A limitation of this study is the reliance upon a single method for susceptibility testing, the Etest®, which is a commercially derived method. Further work using broth or agar dilution methods would add to our understanding of the susceptibility of S. pyogenes to SXT. From Table 6, when these additional methods have been assessed, the susceptibility of S. pyogenes ranges from 0% (54) to 3.3% (18) resistant, similarly low resistances to those reported in this study.

Reading MICs for SXT can be challenging due to haze. In particular, only faint growth of S. pyogenes was achieved on MHA (due to the absence of blood) and this made reading endpoints more difficult. MHLHBA and MHF had similar problems to MHA with respect to haze. Despite this in Study 2, the MICs were reproducible between readers with a high level of inter-rater reliability within 1 MIC gradation.

**Conclusions**

The widespread belief that SXT is ineffective for S. pyogenes infections because of inherent antimicrobial resistance is a fallacy due to technical limitations in laboratory methodology: namely, the use of media containing high concentrations of thymidine which inhibits the action of sulphur antibacterials. When media containing low concentrations of thymidine and/or high concentrations of the enzyme thymidine...
phosphorylase are used, resistance rates are low in most cases, although this must be monitored over time and may vary with local epidemiology and antibacterial prescribing patterns. This study provides justification to proceed to clinical trials of SXT for *S. pyogenes* infections. Corroboration with clinical trial data may convince clinicians that SXT can safely and appropriately be used for infections involving *S. pyogenes*. The Skin Sore Trial will answer the clinical applicability of this current *in vitro* study. In the era of rising MRSA prevalence, more clinical trials of SXT for treatment of SSTI where *S. pyogenes* and *S. aureus* are frequently co-pathogens, are needed.

**Acknowledgements:**

The work of the research assistants in Study 1 and the Skin Sore Trial team (Irene O’Meara, Jane Nelson, Tammy Fernandes, Melita McKinnon, Dianne Halliday, Colleen Mitchell, Valerie Coomber, and Christine Francais) in recruiting participants for Study 2 has made this research possible. Study scientists who carried out this work include RL, PW and Vanya Hampton. Mark Chatfield provided assistance with the statistical analysis. The authors also acknowledge all of the participants and their families who have participated in the Skin Sore Trial and Rheumatic Heart Disease studies.

This work was supported through a National Health and Medical Research Council (NHMRC) Project Grant (545234) on which AB, ST, MM, BC and JC are all investigators. AB is the recipient of a NHMRC scholarship for PhD research (605845) as well as an Australian Academy of Sciences Douglas and Lola Douglas scholarship. ST is the recipient of a NHMRC Early Career Fellowship (605829).
Conflicts of Interest:
None to declare

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antimicrobials against bacterial species associated with upper respiratory tract 

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Table 1: Antibiotic Susceptibility Testing Methods and agar used.

<table>
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<th>Method</th>
<th>Study</th>
<th>Agar used</th>
<th>Abbreviation</th>
</tr>
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<tbody>
<tr>
<td>CLSI (8)</td>
<td>2</td>
<td>Mueller Hinton agar supplemented with 5% sheep blood</td>
<td>MHS</td>
</tr>
<tr>
<td>EUCAST (20)</td>
<td>2</td>
<td>Mueller Hinton agar supplemented with 5% defibrinated horse blood + β-NAD</td>
<td>MHF</td>
</tr>
<tr>
<td>Experimental</td>
<td>1 &amp; 2</td>
<td>Mueller Hinton Agar</td>
<td>MHA</td>
</tr>
<tr>
<td>Experimental</td>
<td>1 &amp; 2</td>
<td>Mueller Hinton Agar containing 5% lysed horse blood</td>
<td>MHLHBA</td>
</tr>
<tr>
<td>Experimental</td>
<td>1</td>
<td>Mueller Hinton Agar containing horse blood</td>
<td>MHBA</td>
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Table 2: SXT Etest® susceptibility results for Study 1

<table>
<thead>
<tr>
<th>Results</th>
<th>MHBA</th>
<th>MHA</th>
<th>MHLHBA</th>
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</thead>
<tbody>
<tr>
<td>Susceptible (MIC $\leq$1mg/L) (%)</td>
<td>100</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Resistant (MIC $&gt;$2mg/L) (%)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Geometric Mean MIC (mg/L)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>SD (log)</td>
<td>1.52</td>
<td>2.20</td>
<td>1.78</td>
</tr>
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</table>


Table 3: Difference in MIC in Study 1. Pair wise comparisons of geometric mean MIC of various media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>MHA</th>
<th>MHLHBA</th>
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</thead>
<tbody>
<tr>
<td>MHBA</td>
<td>MHBA lower by 7%</td>
<td>MHBA lower by 25%*</td>
</tr>
<tr>
<td></td>
<td>(-9% to 20%)</td>
<td>(13% to 35%)</td>
</tr>
<tr>
<td>MHA</td>
<td>MHA lower by 20%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3% to 33%)</td>
<td></td>
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</tbody>
</table>

95% CI in parentheses. * indicates significance with P value <0.05.
Table 4: SXT Etest® susceptibility results for Study 2

<table>
<thead>
<tr>
<th>Results</th>
<th>MHF</th>
<th>MHS</th>
<th>MHA</th>
<th>MHLHBA</th>
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</thead>
<tbody>
<tr>
<td>Susceptible (MIC ≤1mg/L) (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Geometric Mean</td>
<td>0.07</td>
<td>0.16</td>
<td>0.07</td>
<td>0.09</td>
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<tr>
<td>MIC (mg/L)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SD (log)</td>
<td>2.05</td>
<td>1.79</td>
<td>2.03</td>
<td>2.30</td>
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Table 5: Difference in MIC in Study 2. Pair wise comparisons of geometric mean MIC of various media.

<table>
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<th>Medium</th>
<th>MHF</th>
<th>MHA</th>
<th>MHLHBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHS</td>
<td>MHS higher by 128%*&lt;br&gt;(107% to 150%)</td>
<td>MHS higher by 132%*&lt;br&gt;(113% to 153%)</td>
<td>MHS higher by 92%*&lt;br&gt;(70% to 116%)</td>
</tr>
<tr>
<td>MHF</td>
<td>MHF higher by 2%&lt;br&gt;(-12% to 7%)</td>
<td>MHF lower by 16%*&lt;br&gt;(3% to 27%)</td>
<td></td>
</tr>
<tr>
<td>MHA</td>
<td></td>
<td>MHA lower by 17%*&lt;br&gt;(6% to 27%)</td>
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</table>

95% CI in parentheses. * indicates significance with P value <0.05.
Table 6: Summary of published results of *S. pyogenes* in vitro susceptibility to SXT.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Year</th>
<th>Country</th>
<th>Method &amp; Medium used</th>
<th>Findings</th>
<th>Resistance</th>
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<td>Yourassowsky et al</td>
<td>1974</td>
<td>Belgium</td>
<td>AST determined by the agar dilution method on Wellcotest agar supplemented with 5% lyzed horse blood</td>
<td>59/59 strains of <em>S. pyogenes</em> susceptible to TMP, SMZ and SXT</td>
<td>0%</td>
</tr>
<tr>
<td>Finland et al</td>
<td>1976</td>
<td>USA</td>
<td>Plate dilution method on a modified thymidine-deficient Mueller-Hinton medium containing 5% laked blood</td>
<td>35/35 strains of <em>S. pyogenes</em></td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32/35 strains of <em>S. pyogenes</em> susceptible to SXT</td>
<td></td>
</tr>
<tr>
<td>Darrell et al</td>
<td>1968</td>
<td>UK</td>
<td>MIC determined by the plate dilution method on diagnostic sensitivity agar containing 5% lyzed horse blood</td>
<td>14/14 strains of <em>S. pyogenes</em> susceptible to TMP with MIC ≤ 1µg/ml</td>
<td>0%</td>
</tr>
<tr>
<td>Liebowitz et al</td>
<td>2003</td>
<td>South Africa</td>
<td>MICs determined by the broth microdilution method according NCCLS guidelines</td>
<td>66/66 <em>S. pyogenes</em> strains susceptible to SXT</td>
<td>0%</td>
</tr>
<tr>
<td>Hartman et al</td>
<td>1949</td>
<td>USA</td>
<td>Wilson’s method (56, 57) a semi-solid medium with low sulphonamide antagonist</td>
<td>94/96 strains of <em>S. pyogenes</em> susceptible to</td>
<td>1.9%</td>
</tr>
<tr>
<td>Study</td>
<td>Year</td>
<td>Location</td>
<td>Methodology</td>
<td>S. pyogenes Strains</td>
<td>Susceptibility</td>
</tr>
<tr>
<td>---------------</td>
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<td>-----------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Schultz et al</td>
<td>1958</td>
<td>USA</td>
<td>Wilson’s method (56, 57) a semi-solid medium with low sulphonamide antagonist content.</td>
<td>84/86 strains of S. pyogenes</td>
<td>2.3%</td>
</tr>
<tr>
<td>Eliopoulos et al</td>
<td>1997</td>
<td>USA</td>
<td>MICs determined by agar dilution methods on Mueller Hinton II agar + 5% lysed horse blood or thymidine phosphorylase at 0.2IU/ml added</td>
<td>58/60 S. pyogenes</td>
<td>3.3%</td>
</tr>
<tr>
<td>Berger-Rabinowitz et al</td>
<td>1970</td>
<td>Israel</td>
<td>Wilson’s method (56, 57) a semi-solid medium with low sulphonamide antagonist content.</td>
<td>849/890 S. pyogenes strains</td>
<td>4.6%</td>
</tr>
<tr>
<td>Dhanda et al</td>
<td>2011</td>
<td>India</td>
<td>Kirby Bauer disc diffusion test as per CLSI guidelines. Agar not specified.</td>
<td>24/26 S. pyogenes</td>
<td>6.7%</td>
</tr>
<tr>
<td>Bushby</td>
<td>1973</td>
<td>USA</td>
<td>Disc diffusion using TMP/SMZ discs 1.25/23.75µg on various media</td>
<td>699/757 S. pyogenes strains</td>
<td>7.7%</td>
</tr>
<tr>
<td>Lakshmy et al</td>
<td>2011</td>
<td>India</td>
<td>Kirby Bauer disc diffusion test as per CLSI guidelines. Agar not specified.</td>
<td>95/119 S. pyogenes strains</td>
<td>21.8%</td>
</tr>
<tr>
<td>Dumre et al</td>
<td>2009</td>
<td>Nepal</td>
<td>Kirby Bauer disc diffusion test</td>
<td>11/38 S. pyogenes</td>
<td>71%</td>
</tr>
<tr>
<td>Reference</td>
<td>Year</td>
<td>Location</td>
<td>Methodology</td>
<td>Results</td>
<td></td>
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<tr>
<td>Traub and Leonard (54)</td>
<td>1997</td>
<td>Germany</td>
<td>Agar disc diffusion using NCCLS criteria on sheep blood MHA.</td>
<td>0/63 strains of <em>S. pyogenes</em> susceptible to SXT 100%</td>
<td></td>
</tr>
<tr>
<td>Kaplan <em>et al</em> (34)</td>
<td>1999</td>
<td>USA</td>
<td>Etest performed on MHA containing 5% sheep blood</td>
<td>0/169 strains of <em>S. pyogenes</em> susceptible to SXT with MIC $\geq$ 32 µg/ml 100%</td>
<td></td>
</tr>
</tbody>
</table>