Clinical Microbiology and Infection xxx (xxxx) xxx

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious *Haemophilus* spp.

Y. Sierra [1], F. Tubau [1, 2], A. González-Díaz [1, 2], A. Carrera-Salinas [1], J. Moleres [4], P. Bajanca-Lavado [3], J. Garmendia [2, 4], M. Ángeles Domínguez [1, 5, 6], C. Ardanuy [1, 2, 6, **], S. Martí [1, 2, *]

[1] *Microbiology Department, Hospital Universitari de Bellvitge, IDIBELL-UB, Barcelona, Spain*
[2] *Research Network for Respiratory Diseases (CIBERES), ISCIII, Madrid, Spain*
[3] *Haemophilus Influenzae Reference Laboratory, Department of Infectious Diseases, National Institute of Health, Lisbon, Portugal*
[4] *Instituto de Agrobiotecnología, CSIC-Gobierno, Navarra, Spain*
[5] *Spanish Network for Research in Infectious Diseases (REIPI), ISCIII, Madrid, Spain*
[6] *Department of Pathology and Experimental Therapeutics, Faculty of Medicine, University of Barcelona, Barcelona, Spain*

ARTICLE INFO

Article history:
Received 4 October 2019
Received in revised form
20 November 2019
Accepted 21 November 2019
Available online xxx

Editor: F. Allerberger

Keywords:
Antimicrobial susceptibility testing methods
clinical resistance breakpoint
EUCAST
Haemophilus influenzae
Haemophilus parainfluenzae
Resistance-related determinants
Trimethoprim-sulfamethoxazole

ABSTRACT

Objectives: To compare the determinants of trimethoprim-sulfamethoxazole resistance with established susceptibility values for fastidious *Haemophilus* spp., to provide recommendations for optimal trimethoprim-sulfamethoxazole measurement.

Methods: We collected 50 strains each of *Haemophilus influenzae* and *Haemophilus parainfluenzae* at Bellvitge University Hospital. Trimethoprim-sulfamethoxazole susceptibility was tested by microdilution, E-test and disc diffusion using both Mueller–Hinton fastidious (MH-F) medium and *Haemophilus* test medium (HTM) following EUCAST and CLSI criteria, respectively. Mutations in *folA*, *folP* and additional determinants of resistance were identified in whole-genome-sequenced isolates.

Results: Strains presented generally higher rates of trimethoprim-sulfamethoxazole resistance when grown on HTM than on MH-F, independent of the methodology used (average MIC 2.6-fold higher in *H. influenzae* and 1.2-fold higher in *H. parainfluenzae*). The main resistance-related determinants were as follows: I95L and F154S/V in *folA*; 3- and 15-bp insertions and substitutions in *folP*; acquisition of *sul* genes; and FolA overproduction potentially linked to mutations in -35 and -10 promoter motifs. Of note, 2 of 19 *H. influenzae* strains (10.5%) and 9 of 33 *H. parainfluenzae* strains (27.3%) with mutations and assigned as resistant by microdilution were inaccurately considered susceptible by disc diffusion. This misinterpretation was resolved by raising the clinical resistance breakpoint of the EUCAST guidelines to $\leq$30 mm.

Conclusions: Given the routine use of disc diffusion, a significant number of strains could potentially be miscategorized as susceptible to trimethoprim-sulfamethoxazole despite having resistance-related mutations. A simple modification to the current clinical resistance breakpoint given by the EUCAST guideline for MH-F ensures correct interpretation and correlation with the reference standard method of microdilution. **Y. Sierra, Clin Microbiol Infect 2019;▪:1**

Introduction

Antimicrobial susceptibility testing (AST) methods require standardized conditions to provide reproducible results between clinical centres. Their use and interpretation are standardized by organizations such as the American Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial

* Corresponding author. S. Martí, Microbiology Department, Hospital Universitari de Bellvitge, Feixa Llarga s/n, L'Hospitalet de Llobregat, Barcelona, 08907, Spain.
** Corresponding author. C. Ardanuy, Microbiology Department, Hospital Universitari de Bellvitge, IDIBELL-UB, Barcelona, Spain.
E-mail addresses: c.ardanuy@bellvitgehospital.cat (C. Ardanuy), smartinm@bellvitgehospital.cat (S. Martí).

https://doi.org/10.1016/j.cmi.2019.11.022
1198-743X/

Susceptibility Testing (EUCAST) [1,2], with the broth microdilution as reference standard [3,4] and the disc diffusion assay as a benchmark in routine clinical practice [5]. Major organizations recommend using Mueller–Hinton (MH) as reference medium because of satisfactory growth of most non-fastidious microorganisms and excellent antibiotic diffusion through its surface. When used with fastidious microorganisms, such as *Haemophilus influenzae*, this medium must be supplemented for bacterial growth, prompting the introduction of two MH-derived media, the MH Fastidious (MH-F) based on EUCAST criteria and the *Haemophilus* Test Medium (HTM) based on CLSI criteria [1,2]. These supplemented media are not expected to interfere with the tested antimicrobials and should not produce false-negative or false-positive results. Evaluating the activity of different antimicrobials on different media for fastidious and non-fastidious microorganisms is essential for methodological validation. Previous studies have already shown discrepancies in trimethoprim-sulfamethoxazole susceptibility results [6,7] due to differences in the composition of the substrate supplements. This was mainly associated with an excess of thymidine in the media, which subsequently affected bacterial metabolic activity [8,9].

Trimethoprim-sulfamethoxazole is a bacteriostatic agent composed of trimethoprim and sulfamethoxazole that is normally manufactured in a 1:5 or 1:19 ratio [10]. Its action is based on the inhibition of tetrahydrofolate formation as the limiting compound for pyrimidine synthesis. This process is carried out by selective inhibition of the dihydrofolate reductase (DHFR), which is encoded by the *folA* gene (also named *folH*, *dhfr* or *dhf*), that catalyses the reduction of dihydrofolate into tetrahydrofolate. Trimethoprim interferes in this step as a competitive analogue of dihydrofolate, binding to DHFR and inhibiting dihydrofolate recycling and *de novo* formation. In addition, the dihydropteroate synthase enzyme, encoded by *folP*, catalyses the formation of pteridine diphosphate and para-aminobenzoic acid. Sulfamethoxazole covalently attaches to pteridine diphosphate and competes with para-aminobenzoic acid for the active binding-site, thereby diverting its metabolic flux [11]. In *Haemophilus* spp., point mutations in *folA* [12–14], mutations or short-insertions in *folP* [15], plasmid-mediated acquisition of *sul* genes [16], and DHFR overproduction potentially linked to mutations in the promoter regions [17] are the most common resistance mechanisms. Existing evidence also relates *H. influenzae* resistance to thymidine auxotrophy caused by loss-of-function mutations in the thymidylate synthase encoding gene *thyA* [18,19].

Being a relatively inexpensive drug, trimethoprim-sulfamethoxazole is widely prescribed as the initial antibacterial treatment for acute otitis media, non-severe pneumonia and exacerbations of chronic obstructive pulmonary disease. This has led to selection pressures that have contributed to the emergence of resistant strains among common respiratory pathogens, such as *H. influenzae* [19]. Indeed, resistance levels exceeding 20% are common in *H. influenzae*, despite a lack of significant connection between trimethoprim-sulfamethoxazole use and the development of resistance. This is especially true of non-typable clinical isolates, contrasting with the low resistance rates observed in capsulated strains [20,21]. Although there has been little research into *Haemophilus parainfluenzae*, current data indicate a growth in the identification of multidrug-resistant clinical isolates with resistance mechanisms accumulating against several antimicrobial agents, including trimethoprim-sulfamethoxazole [22]. We have recently detected discrepancies in trimethoprim-sulfamethoxazole susceptibility when using different media (MH-F and HTM) and AST methods during routine hospital analysis, significantly affecting the validation of fastidious *H. influenzae* and *H. parainfluenzae*. Bacteria with MICs close to the resistance breakpoint were difficult to classify because of persistent contradictions in the susceptibility results. We therefore recognized the need for deeper study to identify the most accurate methodology. In this study, we identify and compare the determinants of resistance with the trimethoprim-sulfamethoxazole susceptibility values obtained by microdilution and disc diffusion and propose a more relevant breakpoint for trimethoprim-sulfamethoxazole resistance.

Methods

Study design and growth conditions

Fifty *H. influenzae* and 50 *H. parainfluenzae* strains were obtained from the routine microbiology stock collection at Bellvitge University Hospital. The selection was based on trimethoprim-sulfamethoxazole susceptibility rates for *H. influenzae* and *H. parainfluenzae* obtained in previous studies [23,24] as well as strains collected in our hospital. This built large and well-balanced groups for each pathogen, with similar numbers of susceptible and resistant strains based on disc diffusion following the EUCAST guideline. All information about the strains, susceptibility outcomes and whole-genome sequencing results can be consulted in the Supplementary material (Table S2). *Haemophilus influenzae* ATCC49247 (NZ_LR134171.1) was included as the standard control for susceptibility testing.

Antimicrobial susceptibility testing

Trimethoprim-sulfamethoxazole (1:19 ratio) susceptibility was tested simultaneously by microdilution, E-test (bioMérieux, Marcy-l'Étoile, France) and disc diffusion (Bio-Rad, Hercules, CA, USA). Commercial MH-F and HTM agar media were used for the disc diffusion and E-test methods based on EUCAST and CLSI guidelines, respectively [1,2]. Manufactured MH-F broth and commercial HTM broth were used to perform microdilution according to EUCAST and CLSI guidelines, respectively [1,3] (see Supplementary material, Table S1 for the final composition of the media used). Experiments were performed in duplicate on two different days and AST results were independently read by two experienced clinical analysts to reduce the bias induced by inaccurate reading.

Whole-genome sequencing

Genomic DNA from *H. parainfluenzae* strains was sequenced on an Illumina MiSeq Platform (Illumina Inc., San Diego, CA, USA) and assembled with the INNUca v4.2 pipeline (https://github.com/B-UMMI/INNUca) through ummidock/innuca:3.2-01, as previously described [22]. In addition, we included pre-sequenced isolates of *H. parainfluenzae* obtained from González-Díaz et al. [22], and of *H. influenzae* obtained from Moleres et al. [23] and Pinto et al. [24] (see Supplementary material, Table S2 and Data set S1). *In silico* screening of mutations targeting genes involved in antibiotic resistance was performed with Geneious R9 (Biomatters, Auckland, New Zealand), using the closed genomes of *H. parainfluenzae* T3T1 (NC_015964) and *H. influenzae* Rd KW20 (NC_002516.2) for reference. The acquired resistance mechanisms were screened using Abricate v0.8.0 (https://github.com/tseemann/abricate) through flowcraft/abricate:0.8.0-3 Docker image for ResFinder v3.2 [25]. Multiple sequence alignments of *folA* (including the upstream region) and *folP* were constructed in MEGA v6.0, highlighting all the changes at the nucleotide and amino acid levels (see Supplementary material, Data set S1). Susceptibility outcomes obtained through disc diffusion, E-test and microdilution methods, tested with both MH-F and HTM media, were compared with the mutations identified in the *folA* and *folP* genes to uncover discrepancies that may lead to clinical misinterpretation.

Please cite this article as: Sierra Y et al., Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious *Haemophilus* spp., Clinical Microbiology and Infection, https://doi.org/10.1016/j.cmi.2019.11.022

Results

Discrepancies among growth media and AST methods

Clinical isolates of *H. influenzae* and *H. parainfluenzae* were tested using two growth media (MH-F and HTM) and three AST methodologies (microdilution, E-test and disc diffusion). Significant differences were observed when evaluating both media, independent of the methodology used. Strains were generally more resistant to trimethoprim-sulfamethoxazole when grown on HTM instead of MH-F (see Supplementary material, Table S2). Although these differences were not clinically relevant (i.e. would not affect treatment selection) for the highly susceptible or highly resistant strains, they could produce discordant readings among strains with MICs close to the breakpoint limits.

The disc diffusion and E-test methods were compared against the reference standard microdilution method using a four-quadrant chart to define major errors. For *H. influenzae* (Fig. 1), only two and nine strains were found within the very major error quadrants and identified as false-susceptible in the MH-F media (Fig. 1c) and HTM media (Fig. 1a), respectively. MICs obtained by the E-test and microdilution methods were hardly comparable, with substantially lower values obtained by the E-test, independent of the growth medium used (see Supplementary material, Table S2). This finding was corroborated by the presence of values clearly above the estimated correlation line between the MICs (Figs. 1b,d). For *H. parainfluenzae*, although the correlation between the disc diffusion and microdilution methods was better for the HTM medium (Fig. 2), the E-test method presented a non-suitable correlation with the microdilution (Figs. 2b,d). In this case, nine strains were false-susceptible in the MH-F medium (Fig. 2c), compared with only two strains in the HTM medium (Fig. 2a).

Resistance-related determinants

The discrepancies found among the growth media and AST methods prompted a detailed genetic analysis of the genes involved in trimethoprim-sulfamethoxazole resistance. Table S2 (see Supplementary material) summarizes the susceptibility outcomes and all mutations identified in the *folA* and *folP* genes by comparison with the RdKW20 reference strain. These data also show the correlations of trimethoprim-sulfamethoxazole resistance with mutations for analysis of the accuracy of both growth media. There was an overestimation of trimethoprim-sulfamethoxazole resistance in the HTM medium, especially for *H. influenzae*, with seven resistant strains identified that had no known mutations. The strains were then classified by groups according to the main amino acid substitutions associated with resistance, as shown in Table 1: Group I included susceptible strains with no mutations; Group II was assigned to strains that only presented mutations in *folA* and/or the promoter site, despite having a wide range of MICs; Group III comprised resistant strains with mutations in *folA* (including the promoter region) and *folP* genes; and Group IV was linked to the presence of the mobile sulphonamide-resistance (*sul2*) gene, but was only identified in *H. parainfluenzae* strains.

Based on the microdilution results obtained on MH-F media (Table 1), resistance among *H. influenzae* strains was more likely with mutations in the *folA* gene that caused amino acid modifications in the central (I95L) or C-terminal (F154S) domains. Additionally, mutations and a 15-bp nucleotide insertion in the *folP* gene were responsible for the high resistance to trimethoprim-sulfamethoxazole, with MIC values rising above 4 mg/L. This applied to all but one susceptible *H. influenzae* strain that had an MIC of 0.25 mg/L and that lacked the initial mutations in *folA*.

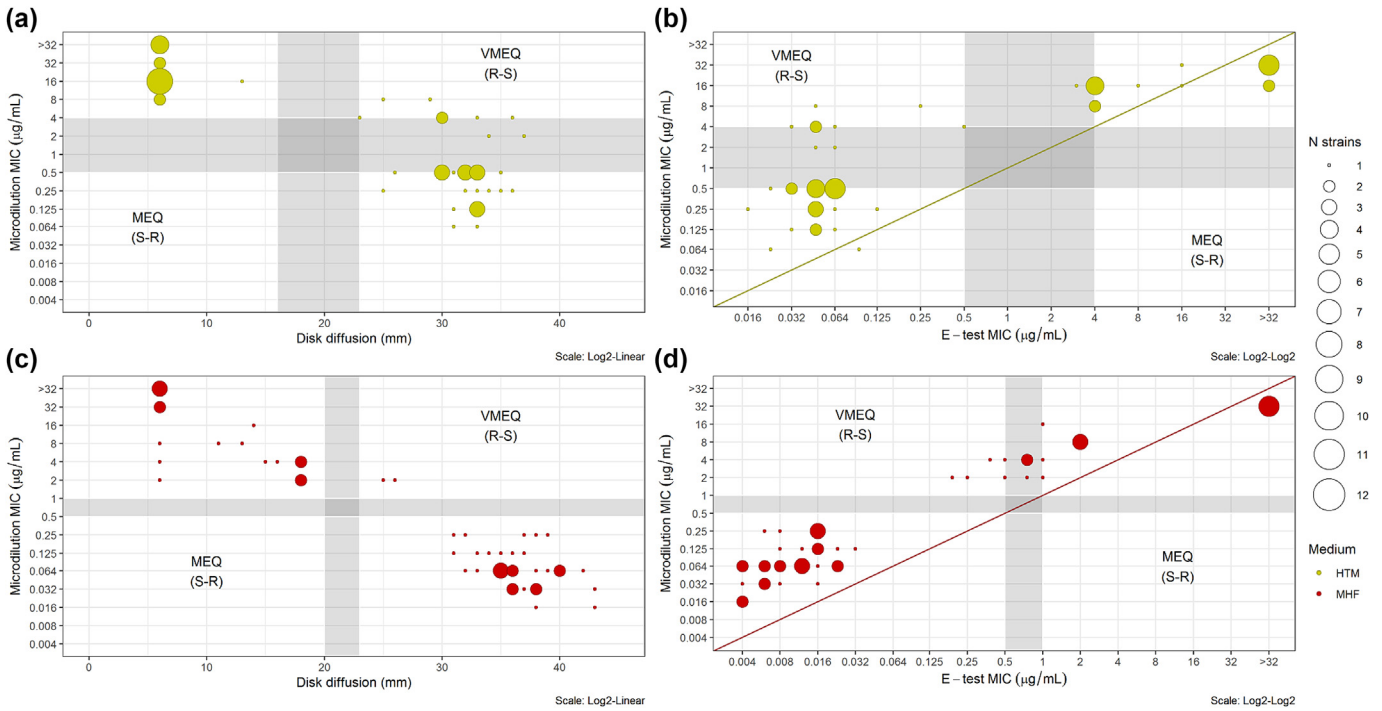

Fig. 1. Trimethoprim-sulfamethoxazole susceptibility in *Haemophilus influenzae* by testing method and media. Microdilution (log2) was correlated with the disc diffusion method in both HTM and MH-F media (a and c, respectively). Microdilution (log2) was correlated with the E-test method (log2) in both HTM and MH-F media (b and d, respectively). The coloured lines represent ideal correlation. Grey areas represent clinical breakpoints for trimethoprim-sulfamethoxazole, expressed as the trimethoprim concentration (a and b following the CLSI guideline, and c and d following the EUCAST guideline). Plot areas are represented as follows: VMEQ, Very major error quadrant (R–S, false-susceptible); MEQ, major error quadrant (S-R, false-resistant); R-R, non-error quadrant (resistant); S–S, non-error quadrant (susceptible). Abbreviations: CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; HTM, *Haemophilus* Test Medium; MH-F, Mueller–Hinton Fastidious.

Please cite this article as: Sierra Y et al., Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious *Haemophilus* spp., Clinical Microbiology and Infection, https://doi.org/10.1016/j.cmi.2019.11.022

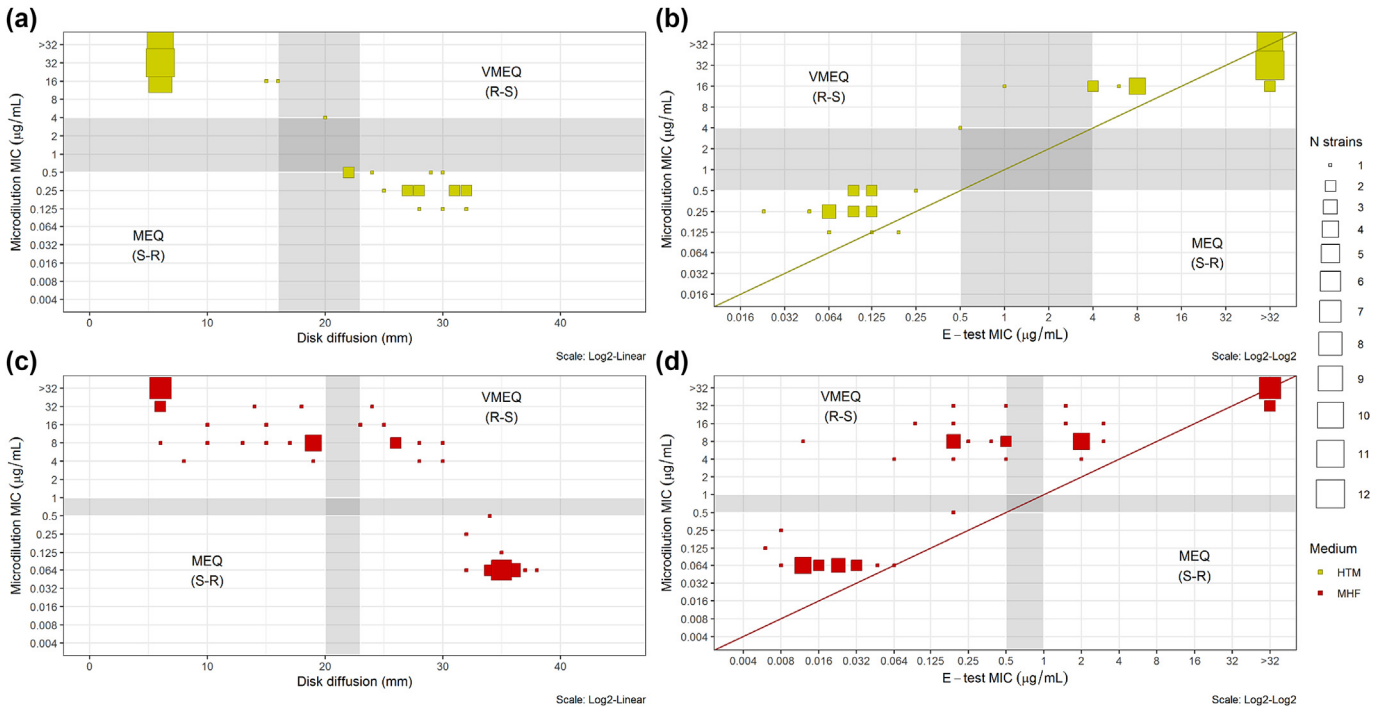

Fig. 2. Trimethoprim-sulfamethoxazole susceptibility in *Haemophilus parainfluenzae* by testing method and media. Microdilution (log2) was correlated with the disc diffusion method in both HTM and MH-F media (a and c, respectively). Microdilution (log2) was correlated with the E-test method (log2) in both HTM and MH-F media (b and d, respectively). The coloured lines represent ideal correlation. Grey areas represent clinical breakpoints for trimethoprim-sulfamethoxazole, expressed as the trimethoprim concentration (a and b following the CLSI guideline, and c and d following the EUCAST guideline). Plot areas are represented as follows: VMEQ, Very major error quadrant (R–S, false-susceptible); MEQ, major error quadrant (S-R, false-resistant); R-R, non-error quadrant (resistant); S–S, non-error quadrant (susceptible). Abbreviations: CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; HTM, *Haemophilus* Test Medium; MH-F, Mueller–Hinton Fastidious.

Although trimethoprim-sulfamethoxazole-associated mutations have not been described for *H. parainfluenzae* strains, analogous mutations to those identified for *H. influenzae* were also observed in *folA* (I95L and F154S/V), together with the acquisition of *sul2* genes linked to high-level resistance. Indeed, we identified only one susceptible strain without mutations in either *folA* or *folP*.

Resistant *H. influenzae* strains presented mutations in the promoter region upstream of the *folA* gene (Fig. 3a), with a mutation within the -35 motif (ATGAAA→ATGACA) and in the nucleotide position -24 (T→C) compared with the reference *H. influenzae* KW20 strain. By contrast, resistance-associated mutations in the promoter region of *H. parainfluenzae folA* (Fig. 3b) were mainly identified within the -10 motif (TATAGT→TATAAT).

Discrepancies in trimethoprim-sulfamethoxazole resistance reports in routine clinical testing

The previously observed discrepancies among AST methods were compared with the determinants of resistance, and the results supported the accuracy of the MIC values against the disc diffusion methodology. Among *H. influenzae* (Table 1a), two Group II strains identified as resistant by microdilution (2 mg/L) were classified as susceptible by disc diffusion despite presenting mutations in the *folA* promoter region and/or the I95L substitution in FolA. Among the *H. parainfluenzae* strains (Table 1b), differences were even greater. Seven Group II and two Group III strains were identified as susceptible by disc diffusion, with MIC values ranging from 4 to 32 mg/L. They all had a mutation in the -10 motif of the promoter region and additional mutations in the *folA* gene. Group III strains also presented modifications in FolP.

According to these data, 11 resistant strains (two for *H. influenzae*, 10.5%; and nine for *H. parainfluenzae*, 27.3%) would be inaccurately considered susceptible based on the disc diffusion results. Consequently, the breakpoint for disc diffusion may benefit from slight modification to accommodate the results of genomic analysis for *folA* and *folP*. Simply raising the clinical resistance breakpoint of the EUCAST guideline to ≤30 mm for both *H. influenzae* and *H. parainfluenzae* may be sufficient to rectify this issue (Fig. 4).

Discussion

Our study provides an updated evaluation about trimethoprim-sulfamethoxazole susceptibility of *H. influenzae* and *H. parainfluenzae* strains assessed using different AST methods on HTM (CLSI) and MH-F (EUCAST) media. Previous studies have already reported the potential for false interpretation with disc diffusion methods depending on the medium used [7,26]. Over time, the stability of growth media has increased, with a consequent improvement in the reproducibility of results. Nevertheless, Jacobs et al. [6] described that differences in the tested media resulted in low reproducibility of susceptible *H. influenzae* strains. According to our results, MH-F proved to be the most reliable medium for determining trimethoprim-sulfamethoxazole susceptibility, showing better correlation among the different methodologies and with the genetic modifications associated with trimethoprim-sulfamethoxazole resistance. In addition, the MH-F medium was associated with optimal growth of both *Haemophilus* species, overcoming the frequent complaints about clinical isolates that fail to grow on HTM [27], and produced results that were easier to read because of better definition of the inhibition zone, corroborating previous observations [6].

Regarding the mutation analyses, De Groot et al. [17] suggested that trimethoprim resistance in *H. influenzae* was due to alterations

Table 1
Trimethoprim-sulfamethoxazole susceptibility results tested by microdilution, E-test, and disc diffusion in *Haemophilus influenzae* (a) and *Haemophilus parainfluenzae* (b) strains grown over Mueller–Hinton-Fastidious medium (EUCAST criteria)

(a)	***H. influenzae* (*n* = 50)**											
		Mueller–Hinton fastidious medium (MH-F)		**FolA amino-acid changes**		**FolP amino-acid changes**			**Additional resistance-related determinants**			
Group[a]	***n***	**Microdilution (mg/L)**	**Disc (mm)**	**I95**	**F154**	**P64_N65ins**	**N65**	**G189**	***sul***	***folA* promoter changes**		
										-35 motif	**-24 bp**	**-4 bp**	**-3 bp**
I	30	0.016–0.25	31–43										
II	1	2	25							A→C			
	6	2–4	6–26	Leu						A→C			
	1	8	13		Ser					A→C	T→C		
	1	16	14		Ser						T→C		G→T
	1	32	6	Leu	Val					A→C			
III	2	0.25–4	6–31			Asn	Asp	Cys					
	1	4	15			Asn	Asp	Cys			T→C	G→A	
	3	4–8	6–16	Leu		Asn	Asp	Cys		A→C			
	2	≥32	6	Leu		Ser-Phe-Leu-Tyr-Asn	Asp	Cys		A→C			
	1	>32	6	Leu		Ser-Phe-Leu-Tyr-Asn	Asp	Cys		A→C	T→C	G→A	
	1	>32	6	Leu		Asn	Asp	Cys					
(b)	***H. parainfluenzae* (*n* = 50)**											
		Mueller–Hinton fastidious medium (MH-F)		**FolA amino-acid changes**		**FolP amino-acid changes**			**Additional resistance-related determinants**			
Group[a]	***n***	**Microdilution (mg/ml)**	**Disc (mm)**	**I95**	**F154**	**P64_M65ins**	**A66_E67ins**	**G189**	***sul***	***folA* promoter changes**		
										-10 motif		
II	1	4	28	Leu						G→A		
	8	4-8	6–30		Ser					G→A		
	2	8	26–28	Leu	Ser					G→A		
	8	8–32	6–26	Leu	Val					G→A		
III	2	8	10–13	Leu	Val		Ala			G→A		
	2	8	15–19		Ser	Ile		Cys		G→A		
	2	16	23–25	Leu		Ile		Cys		G→A		
	1	>32	6	Leu	Val	Ile		Cys		G→A		
IV	1	0.5	34						*sul2*			
	1	32	6		Ser				*sul2*			
	1	>32	6	Leu	Val				*sul2*	G→A		
	1	>32	6	Leu		Ile		Cys	*sul2*	G→A		
	3	>32	6	Leu	Val		Ala		*sul2*	G→A		
	1	>32	6		Ser	Ile		Cys	*sul2*	G→A		

Data are shown as a range of values for microdilution (mg/L) and disc diffusion (mm).
EUCAST clinical breakpoints (year 2019); MIC: ≤0.5 mg/L (susceptible), >1 mg/L (resistant); Disc diffusion: ≥23 mm (susceptible), <20 mm (resistant).
[a] Strains were classified by groups according to resistance determinants pattern. Group I, No mutations; Group II, Mutations in *folA*; Group III, Mutations in *folA* and *folP*; Group IV, Strains carrying *sul2* gene.

in the species-specific *folA* genes rather than the horizontal transfer of resistance genes from other bacterial species. They reported that the greatest effects on susceptibility were from point mutations in *folA* and a possible overproduction of DHFR due to mutations in the *folA* promoter region [17]. Among our tested strains, the I95L substitution was identified as the most relevant modification accounting for the rise of MIC values. This was followed by changes at position 154, mainly among *H. parainfluenzae* strains, suggesting a role of secondary structure alterations as leading resistance mechanisms against trimethoprim-sulfamethoxazole. Conversely, the previously described C-terminal change at position 135 seemed to have no relevance, being identified among susceptible *H. influenzae* strains (E135K) and all *H. parainfluenzae* strains (S135N). Regarding FolP modifications, asparagine insertion (P64_N65ins) [16] seemed to have no impact on the MIC by itself, rather acting in an interconnected manner initiated with *folA* mutations, the main mechanism of trimethoprim-sulfamethoxazole resistance. Conversely, the 15-bp insertion found in *H. influenzae*, as well as the acquisition of *sul2* [15,16] among *H. parainfluenzae*, may have resulted in the higher MIC values.

We consider analysis of the genetic determinants of resistance essential to identifying the optimal methodological conditions. As is already known [26,28], we showed that MICs obtained by the E-test method were hardly comparable with those obtained by microdilution, giving substantially lower values that underestimate the level of resistance. Although this devalued the suitability of the E-test method, the inaccuracies observed with the disc diffusion method were even more alarming. Given the widespread adoption of disc diffusion instead of microdilution in routine clinical practice, our results indicate that a significant number of strains could be potentially miscategorized as susceptible despite presenting resistance-related mutations. A slight modification to the current resistance breakpoint could accommodate these strains that are phenotypically resistant by microdilution but susceptible by disc diffusion. Although no discordances between microdilution and disc diffusion were found with the HTM medium for *H. parainfluenzae* strains, we still advocate using the MH-F medium for both species because it showed lower risk of growth failure, easier susceptibility result assessment and better outcomes.

Our study had some statistical limitations, mainly due to the small population size, which could be corrected by including a larger collection of strains in the future. Additional mutagenesis studies may be required to clarify the involvement of minor changes among *H. parainfluenzae* strains as well as mutations in the *folA* upstream promoter region that were identified among resistant strains. The involvement of this region with resistance was unclear because many susceptible strains also presented these mutations.

Please cite this article as: Sierra Y et al., Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious *Haemophilus* spp., Clinical Microbiology and Infection, https://doi.org/10.1016/j.cmi.2019.11.022

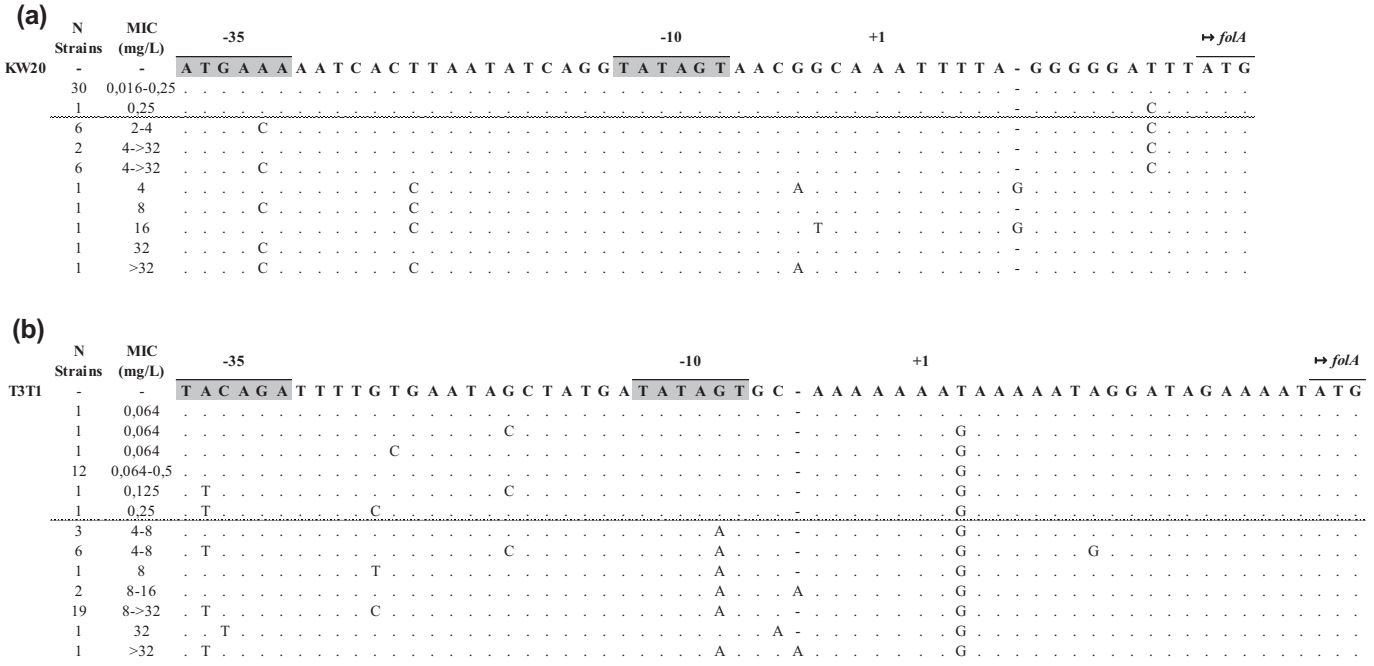

Fig. 3. Upstream nucleotide sequence of the promoter region of *folA* gene in *Haemophilus influenzae* (a) and *Haemophilus parainfluenzae* (b). Strains were grouped by sequence pattern and MIC result by microdilution on MH-F (EUCAST criteria). The -35 and -10 promoter regions are shown in grey, with the arrow representing the *folA* start codon. The dotted line separates the strains by susceptibility validation as resistant or susceptible (EUCAST criteria). Abbreviations: EUCAST, European Committee on Antimicrobial Susceptibility Testing; HTM, *Haemophilus* Test Medium; MH-F, Mueller–Hinton Fastidious.

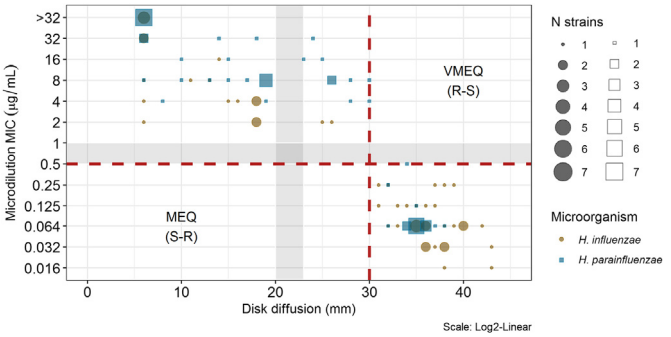

Fig. 4. *Haemophilus influenzae* and *Haemophilus parainfluenzae* trimethoprim-sulfamethoxazole susceptibility tested on Mueller-Hinton Fastidious medium. Microdilution (log2) correlated with disc diffusion in MH-F. Grey areas represent clinical breakpoints (EUCAST) for trimethoprim-sulfamethoxazole expressed as the trimethoprim concentration. Red dashed line represents clinical breakpoints proposed based on the current study outcomes. Error areas are represented as follows: VMEQ, Very major error quadrant (R–S, false-susceptible); MEQ, major error quadrant (S-R, false-resistant).

In conclusion, we performed a challenging evaluation of the current clinical methodologies for trimethoprim-sulfamethoxazole susceptibility testing in combination with a genomic analysis. We identified various resistance-associated mutations and found that accuracy cannot be ensured with a disc diffusion breakpoint <30 mm. Consequently, our results suggest the need to modify the current clinical resistance breakpoint given by the EUCAST guideline to ensure the correct interpretation of the disc diffusion test. Moreover, where facilities allow, we recommend that this should be complemented by microdilution analysis for those strains that are difficult to evaluate.

Acknowledgements

We wish to thank the staff of the Microbiology Laboratory of Bellvitge University Hospital who contributed to this project on a daily basis, and the CERCA Programme/Generalitat de Catalunya for institutional support.

Transparency Declaration

The authors have no conflicts of interest to disclose.

Funding

This study has been funded by the Fundación Española del Pulmón SEPAR 418/2017 and the Instituto de Salud Carlos III through the Projects from the Fondo de Investigaciones Sanitarias 'PI16/00977' to SM, and CIBER de Enfermedades Respiratorias (CIBERES—CB06/06/0037), co-funded by the European Regional Development Fund/European Social Fund (ERDF/ESF, 'Investing in your future'), and the Ministerio de Ciencia, Innovación y Universidades through the Projects SAF2015-66520-R and RTI2018-096369-B-I00 to JG.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2019.11.022.

References

[1] CLSI. In: Performance standards for antimicrobial susceptibility testing: 29 th ed. CLSI supplement document M100. Wayne, PA: CLSI; 2019.
[2] The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. 2019. Available at:, Version 9.0. http://www.eucast.org.
[3] European Committee on Antimicrobial Susceptibility. EUCAST reading guide for broth microdilution. EUCAST Read Guid Broth Microdilution 2019;1.0.
[4] Van Belkum A, Dunne WM. Next-generation antimicrobial susceptibility testing. J Clin Microbiol 2013;51:2018–24.
[5] Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 2014;20:255–66.

[6] Jacobs MR, Bajaksouzian S, Windau A, Appelbaum PC, Lin G, Felmingham D, et al. Effects of various test media on the activities of 21 antimicrobial agents against *Haemophilus influenzae*. J Clin Microbiol 2002;40:3269–76.
[7] Traub WH, Leonhard B. Agar disk diffusion (Bauer–Kirby) tests with various fastidious and nonfastidious reference (ATCC) strains: comparison of several agar media. Chemotherapy 1994;40:374–83.
[8] Koch AE, Burchall JJ. Reversal of the antimicrobial activity of trimethoprim by thymidine in commercially prepared media. Appl Microbiol 1971;22:812–7.
[9] Ferone R, Bushby SRM, Burchall JJ, Moore WD, Smith D. Identification of Harper Cawston factor as thymidine phosphorylase and removal from media of substances interfering with susceptibility testing to sulfonamides and diaminopyrimidines. Antimicrob Agents Chemother 1975;7:91–8.
[10] Cockerill FR, Edson RS. Trimethoprim-sulfamethoxazole. Mayo Clin Proc 1991;66:1260–9.
[11] Palmer AC, Kishony R. Opposing effects of target overexpression reveal drug mechanisms. Nat Commun 2014;1:4296.
[12] Groot RDE, Donald T, Kuehn M, Smith AL. Trimethoprim resistance in *Haemophilus influenzae* is due to altered dihydrofolate reductase(s). Biochem J 1991;274:657–62.
[13] Cornere B, Menzies R. In vitro resistance of *Haemophilus influenzae* to co-trimoxazole and trimethoprim. N Z Med J 1975;26:292–4.
[14] May JR, Davies J. Resistance of *Haemophilus influenzae* to trimethoprim. Br Med J 1972;3:376–7.
[15] Mohd-Zain Z, Kamsani NH, Ahmad N. Molecular insights of co-trimoxazole resistance genes in *Haemophilus influenzae* isolated in Malaysia. Trop Biomed 2013;30:584–90.
[16] Enne VI, King A, Livermore DM, Hall LMC. Sulfonamide resistance in *Haemophilus influenzae* mediated by acquisition of *sul2* or a short insertion in chromosomal *folP*. Antimicrob Agents Chemother 2002;46:1934–9.
[17] De Groot R, Sluijter M, De Bruyn AD, Campos J, Goessens WHF, Smith AL, et al. Genetic characterization of trimethoprim resistance in *Haemophilus influenzae*. Antimicrob Agents Chemother 1996;40:2131–6.
[18] Platt DJ, Guthrie AJ, Langan CF. The isolation of thymidine-requiring *Haemophilus influenzae* from the sputum of chronic bronchitic patients receiving trimethoprim. J Antimicrob Chemother 1983;11:281–6.
[19] Rodríguez-Arce I, Martí S, Euba B, Fernández-Calvet A, Moleres J, López-López N, et al. Inactivation of the thymidylate synthase *thyA* in non-typeable *Haemophilus influenzae* modulates antibiotic resistance and has a strong impact on its interplay with the host airways. Front Cell Infect Microbiol 2017;20:266.
[20] Kärpänoja P, Nyberg ST, Bergman M, Voipio T, Paakkari P, Huovinen P, et al. Connection between trimethoprim-sulfamethoxazole use and resistance in *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis*. Antimicrob Agents Chemother 2008;52:2480–5.
[21] Tsang RSW, Shuel M, Whyte K, Hoang L, Tyrrell G, Horsman G, et al. Antibiotic susceptibility and molecular analysis of invasive *Haemophilus influenzae* in Canada, 2007 to 2014. J Antimicrob Chemother 2017;72:1314–9.
[22] González-Díaz A, Tubau F, Pinto M, Sierra Y, Cubero M, Càmara J, et al. Identification of polysaccharide capsules among extensively drug-resistant genitourinary *Haemophilus parainfluenzae* isolates. Sci Rep 2019;9:1–8.
[23] Moleres J, Fernández-Calvet A, Ehrlich RL, Martí S, Pérez-Regidor L, Euba B, et al. Antagonistic pleiotropy in the bifunctional surface protein *fadI* (OmpP1) during adaptation of *Haemophilus influenzae* to chronic lung infection associated with chronic obstructive pulmonary disease. MBio 2018;9:1–23.
[24] Pinto M, González-Díaz A, Machado MP, Duarte S, Vieira L, Carriço JA, et al. Insights into the population structure and pan-genome of *Haemophilus influenzae*. Infect Genet Evol 2019;67:126–35.
[25] Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012;67:2640–4.
[26] Griffith R, Creely D, Revell P, Dunne WM, Shortridge D. Comparison of three reference methods for testing susceptibility of staphylococci to trimethoprim-sulfamethoxazole. J Clin Microbiol 2009;47:3726–8.
[27] Barry AL, Fuchs PC, Brown SD. Identification of β-lactamase-negative, ampicillin-resistant strains of *Haemophilus influenzae* with four methods and eight media. Antimicrob Agents Chemother 2001;45:1585–8.
[28] Luber P, Bartelt E, Genschow E, Wagner J, Hahn H. Comparison of broth microdilution, E-test, and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. J Clin Microbiol 2003;41:1062–8.

Please cite this article as: Sierra Y et al., Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious *Haemophilus* spp., Clinical Microbiology and Infection, https://doi.org/10.1016/j.cmi.2019.11.022