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

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Background: Several discrepancies were found in clinical routine regarding trimethoprim-sulfamethoxazole (SXT) susceptibility determination depending on antimicrobial susceptibility (AST) method used and growth media. We aimed to compare the determinants of SXT resistance with established susceptibility values for fastidious *Haemophilus* spp., in order to provide recommendations for optimal SXT measurement.

Materials/methods: We collected 50 strains each of *Haemophilus influenzae* and *Haemophilus parainfluenzae* at Bellvitge University Hospital. SXT susceptibility was tested by microdilution, E-test, and disc diffusion using both Mueller-Hinton Fastidious (MH-F) 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 SXT 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 mechanisms were as follows: I95L and F154S/V in FolA; 3 and 15 base pair 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 ≤30 mm.

Conclusions: Given the routine use of disc diffusion, a significant number of strains could potentially be miscategorised as susceptible to SXT despite having resistance-related mechanisms. A simple modification to the current clinical resistance breakpoint given by the EUCAST guideline for MH-F ensures correct interpretation and correlation with the gold-standard method of microdilution.