

Original Research Article**Comparison of two standardizations used in antibiotic susceptibility testing in *Helicobacter pylori*****ABSTRACT**

Helicobacter pylori is a bacterium that is widespread in the world's population and constitutes a risk factor for the development of gastric cancer. One possible cause of treatment failure is antimicrobial resistance, indicating the importance of susceptibility testing.

Aims: This study compared the *Helicobacter pylori* susceptibility results obtained by two international standardization: British Society for Antimicrobial Chemotherapy (BSAC) and CDS Method. Both aiming to indicate a more reliable methodology to be used by laboratories in order to minimize possible errors when mapping the susceptibility profiles of *Helicobacter pylori*.

Study design: Transversal study.

Place and Duration of Study: Department of Gastroenterology, Hospital de Clínicas de Porto Alegre, between January 2014 and July 2014.

Methodology: 50 isolates of *Helicobacter pylori* stored at -80 °C were used in the execution of the susceptibility tests preconized by the British Society for Antimicrobial Chemotherapy (BSAC), British origin, and CDS Method of Australian origin.

Results: The minimum inhibitory concentration (MIC) values for amoxicillin and clarithromycin in both methods were equivalents ($\kappa=1.0000$; $p<0.001$). However, there was a discrepancy in the results for clarithromycin in a bacterial sample, which was categorized as

sensible by the BSAC and by CDS Method as resistant, revealing the lowest sensitivity of British methodology on the test (Sensitivity=85%). Moreover, the simultaneous execution of the tests using the two standardization allowed to establish that the Australian technique promoted more intense growth of *H. pylori* on the agar surface, allowing a more accurate reading of the inhibition zones of antibiotics (MIC).

Conclusion: Thus, CDS Method offered greater sensitivity and clarity in the interpretation of MIC in only three days of incubation.

Keywords: Helicobacter pylori; Microbial Sensitivity Tests; Reference Standards, Minimum Inhibitory Concentration, Amoxicillin, Clarithromycin

INTRODUCTION

Helicobacter pylori is a gram-negative bacterium capable of colonizing the human stomach, causing inflammation which may latter originate a superficial or chronic gastritis, gastric atrophy, peptic, duodenal ulcer and gastric cancer. This pathogen is of great global importance, since it affects about 50% of the world population (1, 2).

There are numeral causes that prevent the bacterium eradication, even if the patient properly follows the treatment protocol. One of the most likely causes is the resistance to antimicrobials. Therefore, in order to minimize treatment failures associated with this event, it is necessary to determine *H. pylori* susceptibility profile to antibiotics (3).

Different techniques for these susceptibility tests are described in the literature, however some may not offer accurate results due to non-use of a validated standardization. Thus, this study aimed to compare the results generated by the performance of susceptibility tests according to the international standardizations British Society for Antimicrobial Chemotherapy (BSAC) and CDS Method, seeking to establish the most easily performed and interpretable test.

MATERIAL AND METHODS

In this study, 50 isolates of *H. pylori* were analyzed, which were stored at -80°C Brain Heart Infusion (BHI) (Himedia, India) broth supplemented with 10% of fetal bovine serum and 5% of dimetilsulfoxide (DMSO) (Sigma Aldrich, Germany). For thawing, an aliquot of each sample was plated onto Agar

51 Columbia Chocolate (Oxoid, United Kingdom) and incubated at 37°C in a microaerophilic atmosphere
52 (Microaerobac[®], Probac, Brazil) for 72 hours. Afterwards, a 100 µL of BHI broth was added to the
53 bacterial growth on Agar Columbia Chocolate, spreading it on the surface with a sterile Drigalski
54 spatula. Then, plates were again incubated under the same conditions. A great number of colony
55 forming units (CFU) was possible to obtain from each *H. pylori* isolate, which were used thereafter in
56 the execution of the susceptibility tests preconized by the British Society for Antimicrobial
57 Chemotherapy (BSAC), British origin, and CDS Method of Australian origin.

58 In accordance with the British Society for Antimicrobial Chemotherapy (BSAC), *H. pylori* colonies
59 were suspended in distilled water until the growth reached a turbidity equal to 3 McFarland standard
60 (9×10^8 CFU/mL) (adjusted in Densimat[®], Biomeriéux). The suspension was then plated on plates (90
61 mm of diameter) containing Mueller-Hinton agar with 10% of horse blood. After the surface of the agar
62 was dry, one E-test[®] amoxicillin and one clarithromycin strip was applied on each plate and incubated
63 at 35°C in a microaerophilic atmosphere for up to 5 days. The minimum inhibitory concentration (MIC)
64 was defined as the zone of complete inhibition of bacterial growth (4).

65 Susceptibility or resistance definition obeyed the breakpoints determined by BSAC: *H. pylori* isolate
66 was susceptible when MIC ≤ 1 µg/mL for amoxicillin and/or clarithromycin and resistant when MIC >
67 1µg/mL for amoxicillin and/or clarithromycin (4).

68 In agreement with the CDS Method (2013), a bacterial suspension equivalent to 2 McFarland
69 standard (adjusted in Densimat[®], Biomeriéux) was prepared in BHI broth from colonies with 72 hours
70 of growth. An abundant inoculum was plated on the surface of plates (90 mm of diameter) containing
71 Agar Columbia Chocolate and, immediately after complete drying of the plate surface, an
72 antimicrobial strip was applied on each plate. The technique advocates the use of amoxicillin and
73 clarithromycin antibiotic disks, however, these were substituted by E-Test[®] (bioMérieux, France) strips
74 for MIC determination. This alteration was due to the unavailability in the Brazilian market of 2 µg
75 amoxicillin and 5 µg erythromycin disks recommended by the CDS Method. Incubation of plates was
76 performed in a microaerophilic atmosphere (Microaerobac[®], Probac of Brazil), at 35°C for 3 days. The
77 complete growth inhibition zone defined MIC reading criteria (5).

78 CDS Method breakpoints establish that *H. pylori* samples are susceptible to amoxicillin when MIC $\leq$ 1
79 $\mu\text{g/mL}$ and susceptible to clarithromycin when MIC $\leq$ 0.5 $\mu\text{g/mL}$ (5).

80 The results were organized on Excel spreadsheets (2014) and analyzed by SPSS program 18.0
81 (SPSS Inc., Chicago, IL). Susceptibility to antibiotics was obtained by calculating the percentage. The
82 degree of agreement between methods (standardization) was evaluated by the kappa (κ) index and
83 the significance level was established at $\alpha=0.05$.

84 **RESULTS**

85 Minimum values of MIC for amoxicillin in both methods were $< 0.16 \mu\text{g/mL}$, and the maximal MIC was
86 equal to 1.5 $\mu\text{g/mL}$ in the BSAC and 2 $\mu\text{g/mL}$ in the CDS Method. There was no alteration in the
87 category "resistant" to amoxicillin for the *H. pylori* isolate, in which these disagreeing MIC were
88 observed, and the statistical analysis revealed the maximal degree of agreement (Kappa) between
89 the CDS Method and BSAC methods ($\kappa=1.0000$; $p<0.001$).

90 For clarithromycin, MIC values oscillated from $< 0.016 \mu\text{g/mL}$ to $> 256 \mu\text{g/mL}$ in both standardizations,
91 BSAC and CDS Method respectively. The resistance to this antibiotic was visibly detected in 7/50
92 (14%) *H. pylori* isolates by the CDS Method (Sensitivity=100%, Specificity=100%), while BSAC
93 detected it in 6/50 (12%) isolates (Sensitivity=85%, Specificity=100%), evidencing the highest
94 sensitivity observed by the CDS Method. However, statistical analysis demonstrated a very
95 satisfactory degree of agreement (Kappa) between the results found by the CDS Method and BSAC
96 ($\kappa=0.913$; $p<0.001$).

97 **DISCUSSION**

98 Simultaneously execution of tests by both standardizations allowed perceiving that CDS Methods
99 permitted a clearer and evident reading of the E-test (MIC) ellipse. A fact attributed to the intense
100 growth of *H. pylori* on the agar surface. This finding is possibly related with the use of BHI broth for
101 the *H. pylori* inoculum preparation in the CDS Method, while in the BSAC the inoculum was prepared
102 in sterile distilled water. Nutritional support offered by the BHI broth, a buffered medium containing
103 calf brain infusion, beef heart infusion, proteose peptone, glucose, sodium chloride and disodium

104 phosphate (6) might have cooperated to a more confluent growth, allowing an adequate MIC
105 interpretation when compared to the growth of the inoculum suspended only in distilled water.

106 Another aspect that may have favored a more abundant growth of *H. pylori* through the CDS Method
107 was the use of Agar Columbia Chocolate, which is a more nutritional medium than Mueller-Hinton
108 Blood agar used in BSAC. The base of Agar Columbia Chocolate contains peptone, corn starch,
109 sodium chloride and agar (6), in addition to hemin and hematin, which are essential compounds for
110 the growth of fastidious microorganisms, released from horse blood cells during their addition to
111 warmed Columbia Agar Base (80°C) (7).

112 Therefore, the combination of two nutritional aspects (BHI broth and Agar Columbia Chocolate)
113 provided a better development of *H. pylori* in the susceptibility test following the CDS Method when
114 compared to the other quantitative standardization (BSAC).

115 Another favorable aspect of this quantitative methodology described in the CDS Method is the
116 incubation time: 3 days in microaerophilic atmosphere are needed, while an incubation of 5 days is
117 suggested in the BSAC. This longer period is recommended for the visualization of bacteria
118 subpopulations that may alter the interpretation of the susceptibility result. In our study, an importance
119 of this late reading was evidenced: a *H. pylori* subpopulation resistant to clarithromycin was not
120 observed in 3 days of incubation in BSAC, being only evidenced after 4 days of incubation. On the
121 other hand, it was possible to determine the MIC of this same sample in 3 days of incubation through
122 the other technique. Hence, the CDS Method quantitative standardization is apparently more
123 advantageous by also enabling a trustable reading in only 3 days of incubation.

124 When evaluating the susceptibility profile of a particular microorganism to antibiotics it is essential to
125 choose a standardized methodology, which requires a set of procedures that must be followed to yield
126 the real result. This standardization should explain all the execution steps, since the preparation of the
127 bacterial inoculum, culture medium used, specific incubation conditions as time, temperature, in
128 addition to interpretation breakpoints for each of the antimicrobials.

129 Contrary to the criteria used in this study, there are other studies in which no standardization was
130 employed. For instance, in a research developed in Colombia, *H. pylori* sensitivity was tested in

Mueller-Hinton horse blood agar supplemented with 2% Isovitalex (8). After proper incubation (microaerophilic atmosphere, 37°C for 2 to 3 days), reading was performed with the use of breakpoints described in another susceptibility profile study (9), which denotes the lack of standardization for test preparation and its interpretation (8).

Similarly, other incomplete or incongruent information were observed. In a Brazilian study, performed in 2011, the adopted McFarland standard for the evaluation of *H. pylori* susceptibility on plates was not mentioned (10). Furthermore, another research conducted in Brazil (Recife) makes no reference to any standardization for susceptibility to clarithromycin, using randomly a very dense inoculum (4 McFarland standard in BHI broth), Mueller-Hinton agar with another type of blood (calf blood) and antibiotic breakpoint without a reference (11).

At last, other four different studies only report the use of E-test strips in their methodologies, without any reference regarding the inoculum concentration used, nor (not even) the reading standard of the tests (12, 13, 14, 15). In the same context, some authors only describe the resistance index found in their research, not mentioning the technique and further relevant methodology information (16).

CONCLUSION

Although the results of *H. pylori* antibiotics susceptibility were quite similar between both standardizations, a better performance in the CDS method was evidenced. This methodology offered maximum sensitivity in the assays, combined with other advantages, for instance, clearness in the MIC reading at shorter incubation time, which collaborates for an earlier start of treatment. Thus, the use of this standardization in studies determining *H. pylori* susceptibility profile to antibiotics is recommended.

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