

Original Research Article

Comparison of two standardizations used in antibiotic susceptibility testing in *Helicobacter pylori*

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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.

25 **Aims:** The aim of this study was to compare the *Helicobacter pylori* susceptibility
 26 results obtained by two international standardization indicating the more reliable
 27 methodology to be used by laboratories.

28 **Study design:** Transversal study.

29 **Place and Duration of Study:** Department of Gastroenterology, Hospital de Clínicas de
 30 Porto Alegre (Brazil), between January 2014 and July 2014.

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

34 **Results:** The minimum inhibitory concentration (MIC) values for amoxicillin and
 35 clarithromycin in both standardizations were equivalent ($\kappa=1.0000$; $p<0.001$).
 36 However, the sensitivity of the British methodology was lowest (Sensitivity=85%). The
 37 Australian technique promoted more intense growth of *H. pylori* on the agar surface,
 38 allowing a more accurate reading of the inhibition zones of antibiotics (MIC).

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

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

43

44 INTRODUCTION

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

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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 (Calibrated Dichotomous Sensitivity Method), seeking to establish the most easily performed and interpretable test.

MATERIAL AND METHODS

In this study, 50 isolates of *H. pylori* previously identified by molecular methods were analyzed, which were stored in the Hospital de Clínicas de Porto Alegre (Brazil), 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 Columbia Chocolate (Oxoid, United Kingdom) and incubated at 37°C in a microaerophilic atmosphere (Microaerobac®, Probac, Brazil) for 72 hours. Afterwards, a 100 µL of BHI broth was added to the bacterial growth on Agar Columbia Chocolate, spreading it on the surface with a sterile Drigalski spatula. Then, plates were again incubated under the same conditions for 72 hours. A great number of colony forming units (CFU) was possible to obtain from each *H. pylori* isolate, which were used thereafter in the execution of the susceptibility tests preconized by the British Society for Antimicrobial Chemotherapy (BSAC), British origin, and CDS Method of Australian origin.

In accordance with the British Society for Antimicrobial Chemotherapy (BSAC), *H. pylori* colonies were suspended in distilled water until the growth reached a turbidity equal to 3 McFarland standard (9×10^8 CFU/mL) (adjusted in Densimat®, Biomeriéux). The suspension was then plated on plates (90 mm of diameter) containing Mueller-Hinton agar with 10% of

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77 horse blood. After the surface of the agar was dry, one E-test[®] amoxicillin and one
78 clarithromycin strip was applied on each plate and incubated at 35°C in a microaerophilic
79 atmosphere for up to 5 days. The minimum inhibitory concentration (MIC) was defined as the
80 zone of complete inhibition of bacterial growth (4).

81 Susceptibility or resistance definition obeyed the breakpoints determined by BSAC: *H. pylori*
82 isolate was susceptible when MIC $\leq$ 1 µg/mL for amoxicillin and/or clarithromycin and resistant
83 when MIC $>$ 1µg/mL for amoxicillin and/or clarithromycin (4).

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

95 CDS Method breakpoints establish that *H. pylori* samples are susceptible to amoxicillin when
96 MIC $\leq$ 1 µg/mL and susceptible to clarithromycin when MIC $\leq$ 0.5 µg/mL (5).

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

101 RESULTS

102 Minimum values of MIC for amoxicillin in both methods were $<$ 0.16 µg/mL, and the maximal
103 MIC was equal to 1.5 µg/mL in the BSAC and 2 µg/mL in the CDS Method. There was no
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104 alteration in the category “resistant” to amoxicillin for the *H. pylori* isolate, in which these
105 disagreeing MIC were observed, and the statistical analysis revealed the maximal degree of
106 agreement (Kappa) between the CDS Method and BSAC methods ($\kappa=1.0000$; $p<0.001$).

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

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115 **DISCUSSION**

116 Simultaneously execution of tests by both standardizations allowed perceiving that CDS
117 Methods permitted a clearer and evident reading of the E-test (MIC) ellipse. A fact attributed to
118 the intense growth of *H. pylori* on the agar surface. This finding is possibly related with the use
119 of BHI broth for the *H. pylori* inoculum preparation in the CDS Method, while in the BSAC the
120 inoculum was prepared in sterile distilled water. Nutritional support offered by the BHI broth, a
121 buffered medium containing calf brain infusion, beef heart infusion, proteose peptone, glucose,
122 sodium chloride and disodium phosphate (6) might have cooperated to a more confluent
123 growth, allowing an adequate MIC interpretation when compared to the growth of the inoculum
124 suspended only in distilled water.

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

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

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

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

150 Contrary to the criteria used in this study, there are other studies in which no standardization
151 was employed. For instance, in a research developed in Colombia, *H. pylori* sensitivity was
152 tested in Mueller-Hinton horse blood agar supplemented with 2% Isovitalex (8). After proper
153 incubation (microaerophilic atmosphere, 37°C for 2 to 3 days), reading was performed with the
154 use of breakpoints described in another susceptibility profile study (9), which denotes the lack of
155 standardization for test preparation and its interpretation (8).

156 Similarly, other incomplete or incongruent information were observed. In a Brazilian study,
157 performed in 2011, the adopted McFarland standard for the evaluation of *H. pylori* susceptibility

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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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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

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182 Simone Ulrich Picoli collected data and wrote the article. Bruna Rafaella Becker wrote the
 183 article. Luiz Edmundo Mazzoleni critically reviewed the article. Erli Neuhauss assisted in data
 184 collection. João Carlos Prolla guided work and critically reviewed the article.

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