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Comparison of Two Standardizations Used in Antibiotic Susceptibility Testing in *Helicobacter pylori*

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Authors' contributions

This work was carried out in collaboration among all authors. Author SUP collected data and wrote the article. Author BRB wrote the article. Author LEM critically reviewed the article. Author EN assisted in data collection. Author JCP guided work and critically reviewed the article. All authors read and approved the final manuscript.

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

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Place and Duration of Study: Department of Gastroeterology, Hospital de Clínicas de Porto Alegre (Brazil), between January 2014 and July 2014.
Methodology: 50 isolates of *Helicobacter pylori* stored at -80℃ 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 standardizations were equivalents (κ=1.0000; p<0.001). However, the sensitivity of the British methodology was lowest (Sensitivity=85%). 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.

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

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

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

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

CDS Method breakpoints establish that *H. pylori* samples are susceptible to amoxicillin when MIC ≤ 1 µg/mL and susceptible to clarithromycin when MIC ≤ 0.5 µg/mL [5].

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

3. RESULTS

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

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

4. DISCUSSION

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

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

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

Another favorable aspect of this quantitative methodology described in the CDS Method is the incubation time: 3 days in microaerophilic atmosphere are needed, while an incubation of 5 days is suggested in the BSAC. This longer period is recommended for the visualization of bacteria subpopulations that may alter the interpretation of the susceptibility result. In our study, an importance of this late reading was evidenced: a *H. pylori* subpopulation resistant to

clarithromycin was not observed in 3 days of incubation in BSAC, being only evidenced after 4 days of incubation. On the other hand, it was possible to determine the MIC of this same sample in 3 days of incubation through the other technique. Hence, the CDS Method quantitative standardization is apparently more advantageous by also enabling a trustable reading in only 3 days of incubation.

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

Contrary to the criteria used in this study, there are other studies in which no standardization was 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℃ 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].

5. 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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