Efficacy of Disc Diffusion and Agar Dilution Methods in Evaluating *Helicobacter pylori* Susceptibility to Antibiotics

Fariborz Mansour-Ghanaei[1], Gelareh Poostizadeh[2], Farahnaz Joukar[1], Farideh Siavoshi[2,*]

[1] Gastrointestinal and Liver Diseases Research Center (GLDRC), Guilan University of Medical Sciences, Rasht, Iran
[2] Department of Microbiology, School of Biology, University College of Sciences, Tehran University, Tehran, Iran

[*] **Corresponding Author:**
Farideh Siavoshi, PhD
Department of Microbiology, School of Biology, University College of Sciences, University of Tehran, Enghelab Avenue, Tehran 14176-14411, Iran.
Tel :+ 9821 61112460
Fax :+ 98 21 66492992
Email: farideh.siavoshi@yahoo.com

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ABSTRACT

BACKGROUND:
In this study, efficacy and consistency of disc diffusion (DD) and agar dilution (AD) methods in determining *Helicobacter pylori* susceptibility to antibiotics were evaluated using Brucella blood agar (BBA) in both methods and tetrazolium egg yolk agar (TEYA) in AD.

METHODS:
Twenty *H. pylori* isolates were tested for susceptibility to nine antibiotics; metronidazole (MTZ), clarithromycin (CLR), amoxicillin (AMX), tetracycline (TET), ofloxacin (OFX), levofloxacin (LVX), ciprofloxacin (CIP), furazolidone (FRZ), and rifampin (RIF). Antibiotics solutions were impregnated into blank paper disks on BBA in the DD method or added to BBA (ADB) or TEYA (ADT) media in the AD method. Suspensions of *H. pylori* isolates were surface or spot inoculated on solid media. Plates were incubated in CO_2 incubator at 37°C for 5-7 days.

RESULTS:
The highest rate of susceptibility to MTZ (65%) was determined by DD method compared with AD method (ADB: 40%, ADT: 30%). Both methods showed similar CLR (85%) and AMX (100%) susceptibility rates. Susceptibility to remaining antibiotics, determined by DD and ADB/ADT media were in respective order as 95%, 75% / 75% for TET, 100%, 95% / 85% for FRZ, 85%, 85% / 75% for OFX, 90%, 95% / 85% for LVX, 90%, 85% / 85% for CIP, and 100%, 85% / 75% for RIF.

CONCLUSION:
DD and AD methods showed consistency in determining 161 (89.4%) susceptibility and resistance and inconsistency in determining 19 (10.6%) susceptibility and resistance ($P < 0.05$). DD is recommended as a cheap and easy method with the efficacy and precision comparable to the AD method in determining *H. pylori* susceptibility to antibiotics.

KEYWORDS:
Helicobacter pylori, Antibiotic susceptibility, Disc diffusion, Agar dilution

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INTRODUCTION

Reports from different parts of the world indicate different rates of *Helicobacter pylori* resistance to currently used antimicrobials. These differences could result from differences in local patterns of antibiotic prescription and consumption[1] or variations in the methods used for

performing susceptibility tests.[2] It has been suggested that *H. pylori* requirement for supplements added to the medium and long incubation under microaerobic conditions might be the reasons for difficulties in designing a reliable method for evaluating bacterial susceptibility to antibiotics.[3]

Bacterial susceptibility test by agar dilution (AD) method has been approved as the gold standard method by the National Committee for Clinical Laboratory Standards (NCCLS).[4] The advantage of the AD method is that a large number of strains can be tested at the same time. However, this method is time-consuming and laborious, and solid media with a given antibiotic should be used fresh. When testing *H. pylori* for susceptibility to antibiotics by the AD method, the bacterium produces colorless colonies on Brucella blood agar (BBA), which are difficult to observe. In several studies, the addition of triphenyltetrazolium chloride (TTC) to a solid medium containing egg yolk as a color indicator facilitated visualization of small *H. pylori* colonies as red spots on the surface of bright-yellow tetrazolium egg yolk agar (TEYA).[5] Growing bacteria produce red colonies due to the reduction of the soluble colorless TTC into a red insoluble formazan complex.[6] TEYA has been used for testing susceptibility of *H. pylori* isolates to metronidazole (MTZ) and clarithromycin (CLR) by the AD method.[7]

Epsilometer test (E-test) method has been approved for susceptibility testing of many bacteria but not *H. pylori*. However, this method has been used in several studies on the antibiotic susceptibility of *H. pylori*. E-test is rather expensive but with less labor, compared with AD method. Furthermore, by E-test method, bacterial susceptibility, as well as minimum inhibitory concentration (MIC) of antibiotics, can be simultaneously estimated. The accuracy of E-test for examining of *H. pylori* susceptibility to MTZ has not been confirmed by NCCLS. On the one hand, E-test and AD methods have been found reliable for testing susceptibility of *H. pylori* to amoxicillin (AMX) and CLR but not for MTZ.[8] On the other hand, E-test has been used as a suitable alternative to the AD method, and both methods showed satisfactory results of *H. pylori* susceptibility or resistance to CLR and MTZ in most (93%) of the studied cases.[9] It has been demonstrated that disc diffusion (DD) can be used as an alternative method to E-test for *in vitro* antibiotic susceptibility tests because the DD method is simple, easy to perform, and economical.[10, 11] However, while E-test has been suggested for *H. pylori* susceptibility tests due to a stable pattern of antibiotic release and tolerating prolonged incubation,[12] the DD method has not been recommended for testing slow-growing bacteria because long incubation could affect the pattern of antibiotic release from the disc.[13,14]

In this study, susceptibility of 20 *H. pylori* isolates to nine currently prescribed antibiotics was examined by AD and DD methods, using two solid media; BBA and TEYA. Results were compared to evaluate the efficacy and consistency of the two methods in determining the antibiotic susceptibility of *H. pylori* isolates.

MATERIALS AND METHODS

Helicobacter pylori Isolates

Helicobacter pylori isolates used in this study were cultured from the gastric biopsy of 20 patients with dyspepsia (13 men and 7 women aged 25-86 years) who were referred to the endoscopy unit of Shariati Hospital, Tehran, Iran. All patients signed informed consent, and the study was approved by the research ethics committee of Tehran University of Medical Sciences. Bacterial isolates were cultivated on BBA (Pronadisa, Spain) under a microaerobic atmosphere at 37°C for 3-5 days and identified as *H. pylori* according to their microscopic and biochemical characteristics. Bacterial isolates produced glistening pin-pointed colonies on BBA, and gram-stained smears showed gram-negative spirals when examined by the light microscope. They also exhibited catalase, oxidase, and urease activities.

Testing Susceptibility of *Helicobacter pylori* Isolates to Antibiotics by AD and DD Methods

Concentrated solutions of antibiotics were prepared in dimethyl sulfoxide and added to BBA or TEYA medium in AD method or impregnated into the blank paper disks deposited on BBA in DD method. The appropriate concentration of each antibiotic was used to reach its MIC (µg/mL): MTZ (8), CLR (2), AMX (1), tetracycline (TET 0.5), ofloxacin (OFX 1), levofloxacin (LVX 1), ciprofloxacin (CIP 1), furazolidone (FRZ 0.5),[15] and rifampin (RIF 4)

(unpublished data). Fresh cultures of *H. pylori* isolates were used for the preparation of bacterial suspensions with the turbidity of McFarland unit No.2.

AD Method with BBA (ADB) and TEYA (ADT) Culture Media

For preparing ADB medium, antibiotics were added to cool 45°C BBA to reach their MIC. For ADT, filter-sterilized TTC was added (40 mg/L) to cool sterile Brucella agar; then antibiotics were added to reach their final MIC. Homogenized egg yolk was added in the last step to reach the final concentration of 10%. Eggs were washed with soap and soaked in ethanol for 2 hours before use. Finally, a 10-µL volume of each bacterial suspension was spot-inoculated onto the surface of BBA or TEYA containing antibiotics.

DD Method

A 100-µL of each bacterial suspension was spread on the surface of BBA, using a sterile glass rod. Sterile blank paper discs were superimposed on the surface of plates and impregnated with 10 µL of each antibiotic solution.

Control Plates

BBA/TEYA plates without antibiotics were used as controls for *H. pylori* growth. All the plates were incubated under a microaerobic atmosphere at 37°C and examined for bacterial growth after 5-7 days. In the AD method, bacterial growth was considered positive when wet or red spots appeared on BBA or TEYA, respectively. In the DD method, the diameter of the inhibition zone (DIZ) on BBA was measured for each antibiotic in millimeters. DIZ ≥ 20 mm was considered susceptible.

Statistical Analysis

The difference between the results was determined by Fisher exact and chi-square tests using SPSS software, and $P < 0.05$ was considered significant.

RESULTS

***Helicobacter pylori* Susceptibility to Antibiotics Determined by DD and AD (ADB/ADT) Methods**

MTZ susceptibility rates of 20 *H. pylori* isolates determined by DD, ADB/ADT were 65%, 40% /30% and resistance rates were 35%, 60%/70%, respectively ($P < 0.0001$). The highest rate of susceptibility to MTZ (65%) was determined by the DD method and the highest resistance rates by the AD method (ADB: 60% and ADT: 70%). Both methods showed similar susceptibility rates to CLR (85%) and AMX (100%). TET susceptibility was determined as 95% by DD and 75% by AD (ADB/ADT). The highest susceptibility to FRZ (100%) was determined by the DD method, compared with AD (ADB: 95% and ADT: 85%). Almost similar rates of susceptibility to quinolones, OFX, LVX, and CIP, were determined in respective order by DD (85%, 90%, and 90%) and AD (ADB: 85%, 95%, and 85%, ADT: 75%, 85%, and 85%). The highest susceptibility rate to RIF (100%) was determined by the DD method, compared with those determined by AD (ADB: 85% and ADT 75%) (Figure1). Statistical analyses showed no significant differences between the results ($P > 0.05$).

Efficacy of DD and AD Methods in Determining Antibiotic Susceptibility/Resistance of *Helicobacter pylori* Isolates

Results of susceptibility testing of 20 *H. pylori* isolates to nine antibiotics showed that out of total 180 susceptibility/resistance counts by each method, the highest count of susceptibility (162/180, 90%) and the lowest count of resistance (18/180, 10%) were determined by the DD method. Furthermore, lower susceptibility counts were determined by ADB (149/180, 82.8%) and ADT (139/180, 77.2%) with higher resistance counts of 31/180 (17.2%) and 41/180 (22.8%), respectively. However, no significant difference was found between the results ($P = 0.239$, Figure 1).

Consistency between DD and AD Methods in Determining Antibiotic Susceptibility/Resistance of *Helicobacter pylori* Isolates

When considering 180 antibiotic susceptibility or resistance counts of 20 *H. pylori* isolates determined by DD and AD, the two methods showed consistency in determining 134/180 (74%) susceptibility and 27/180 (15%) resistance. Furthermore, both methods showed inconsistency in determining 19/180 (10.6%) susceptibility or resistance ($P = 0.0001$, Figure 2). The most inconsistency within the results of the two methods was related to MTZ (10/180, 5.5%) and OFX

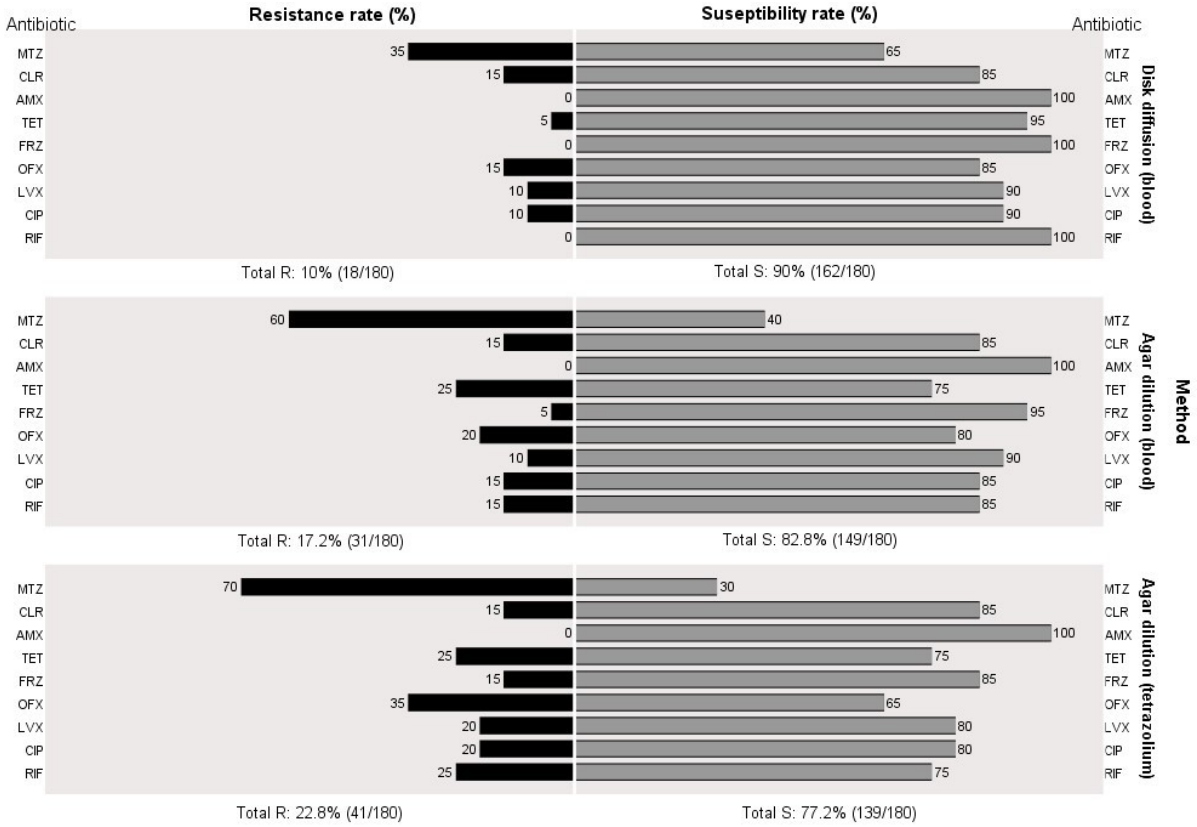

Fig. 1: Efficacy of disc diffusion and agar dilution (blood/tetrazolium) methods in determining susceptibility or resistance of 20 *H. pylori* isolates to nine currently-used antibiotics. R: resistant, S: susceptible.

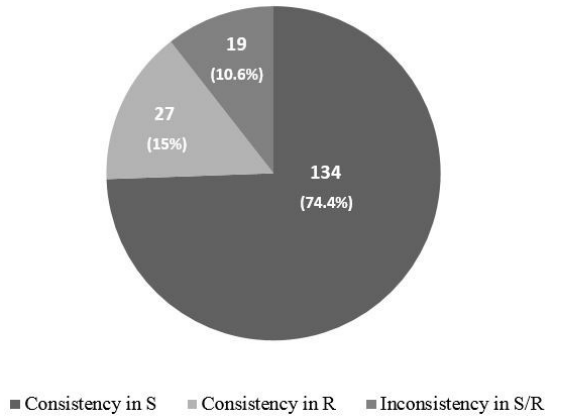

Fig. 2: Consistency of disc diffusion and agar dilution methods in determining susceptibility or resistance of 20 *H. pylori* isolates to nine currently-used antibiotics. The two methods showed consistency in determining 134/180 (74%) susceptibility and 27/180 (15%) resistance to antibiotics. The two methods also showed inconsistency in determining 19/180 (10.6%) susceptibility or resistance to antibiotics. S: susceptibility, R: resistance.

(5/180, 2.25%) groups. In other words, the two methods showed a considerable consistency in determining *H. pylori* susceptibility (65%-100%) or resistance (0%-35%) to the remaining seven antibiotics.

DISCUSSION

First reports on inhibition of bacterial growth due to diffusion of fungal products in 1874[16] and 1876[17] inspired investigators to use diffusion-based methods for testing the effectiveness of antibiotics in the inhibition of bacterial growth. After the discovery of penicillin in 1924, Fleming and Wright introduced well diffusion method for facilitating the diffusion of antimicrobials in the medium[18] that was followed by the production of the first paper disc impregnated with penicillin.[19] Later in 1947, the DD method with filter paper discs impregnated with different antibiotics was used for testing bacterial susceptibility in different laboratories. Around 1940s, the direct addition of antibiotics to the growth medium, called the AD method, was introduced.[20] Despite some limitations in DD and AD methods, since similar results were obtained with both techniques, they were recognized as appropriate methods for testing the susceptibility of bacterial isolates to antibiotics.[21] Improvements in the DD method by Ericsson et al in 1954[22] and later by Bauer et al in 1966[23] led to the introduction of the DD method as a standard method for

susceptibility testing in 1975.[24] Eventually, a modified DD method was introduced as E-test in 1977.[25] Despite being cost-effective and easy to perform, the DD method has been considered unreliable for susceptibility testing of slow-growing *H. pylori* by some investigators because they believe results should be recorded within 18-24 hours.[26-28]

In this study susceptibility/resistance rate of 20 *H. pylori* isolates to currently used antibiotics was determined by DD and AD (ADB/ADT) methods. The susceptibility (S) or resistance (R) rates of *H. pylori* isolates to MTZ determined by DD (S: 65%, R: 35%) and AD (S: 30%/40%, R: 70%/60%) methods showed a considerable difference between the results of two methods ($P < 0.05$). Reports have suggested long incubation in microaerobic conditions as the possible reason for the discrepancy in the results of MTZ susceptibility tests by DD and AD methods.[29] However, in the present study, differences between the results of the two methods that used similar culture media and incubation conditions indicated the impact of the type of method used. Our results also showed that the presence of egg yolk in TEYA, as the source of cholesterol, did not significantly enhance *H. pylori* resistance to antibiotics compared with blood in BBA. Accumulation of cholesterol in *H. pylori* cell membrane has been implicated in the maintenance of bacterial spiral shape, normal membrane permeability, bacterial virulence, and resistance to antibiotics.[30]

While the highest rate of susceptibility (65%) was obtained for MET by the DD method, similar susceptibility rates were determined by both methods for the rest of antibiotics ($P < 0.05$). CLR (85%) and AMX (100%) by both methods. Susceptibility to TET was 95% by DD and 75% by AD method. The highest (100%) susceptibility to FRZ was found by DD, followed by 85%-90% by AD. Similar susceptibility rates were obtained for quinolones by DD (85%-90%) and AD (75-95%) methods. The highest susceptibility to RIF was obtained by DD (100%), followed by AD (75%-85%). In a study from Brazil, susceptibility of 77 *H. pylori* isolates to AMX, CLR, MET, TET, and FRZ was performed, comparing DD and E-test methods with the gold standard AD method. E-test showed a better agreement with AD; however, DD showed major errors and high disagreement with AD.[31] In a study from China using 301 *H. pylori* isolates, a high susceptibility agreement (>95%) was found between E-test and DD for MTZ, CLR, and LVX. However, such an agreement was not found for AMX, TET, and FRZ. Accordingly, the performance of the DD method was recommended for routine testing of *H. pylori* susceptibility to MTZ, CLR, and LVX in clinical laboratories.[32]

Evaluation of the efficacy and consistency of DD and AD methods in determining susceptibility/resistance of *H. pylori* to nine antibiotics showed that although the highest susceptibility rates were determined by the DD method, the results showed no significant difference when compared with those determined by the AD method. Moreover, the two methods showed consistency in estimating 74% of susceptibility and 15% of resistance rates to antibiotics. The most inconsistency within the results of the two methods was related to MTZ (5.5%) and OFX (2.25%) groups. In other words, the two methods showed a considerable consistency in determining *H. pylori* susceptibility (65%-100%) or resistance (0-35%) to the remaining seven antibiotics.

Results of this study showed the efficacy of the DD method as an appropriate method for susceptibility testing of *H. pylori,* with the results comparable to AD for all antibiotics except MTZ for which it works better. Using a small number of bacterial isolates here might show the limitation of our study, suggesting susceptibility testing for a larger number of isolates. Some investigators have regarded the DD method as an unreliable method for testing *H. pylori* susceptibility to antibiotics,[13,14] while others found the efficacy of the DD method equivalent to E-test method.[10,11] Furthermore, neither AD nor E-test has been regarded as sufficiently accurate methods in determining *H. pylori* susceptibility to antibiotics due to inter- and intra-test variability of these methods.[33] Accordingly, the DD method may be recommended as a reliable, cheap, and easy alternative method to AD or E-test for performing *H. pylori* susceptibility tests in clinical laboratories.

ETHICAL APPROVAL

There is nothing to be declared.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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