Research Paper

Antimicrobial susceptibility testing for *Helicobacter pylori* isolates from Brazilian children and adolescents: Comparing agar dilution, E-test, and disk diffusion

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Abstract

Antimicrobial susceptibility testing for *Helicobacter pylori* is increasingly important due to resistance to the most used antimicrobials agents. Only agar dilution method is approved by CLSI, but it is difficult to perform routinely. We evaluated the reliability of E-test and disk diffusion comparing to agar dilution method on *Helicobacter pylori* antimicrobial susceptibility testing. Susceptibility testing was performed for amoxicillin, clarithromycin, furazolidone, metronidazole and tetracycline using E-test, disk-diffusion and agar dilution method in 77 consecutive *Helicobacter pylori* strains from dyspeptic children and adolescents. Resistance rates were: amoxicillin - 10.4%, 9% and 68.8%; clarithromycin - 19.5%, 20.8%, 36.3%; metronidazole - 40.2%33.7%, 38.9%, respectively by agar dilution, E-test and disk diffusion method. Furazolidone and tetracycline showed no resistance rates. Metronidazole presented strong correlation to E-test (r = 0.7992, p < 0.0001) and disk diffusion method (r=-0.6962, p < 0.0001). Clarithromycin presented moderate correlation to E-test (r = 0.6369, p < 0.0001) and disk diffusion method (r=-0.5656, p < 0.0001). Amoxicillin presented weak correlation to E-test (r = 0.3565, p = 0.0015) and disk diffusion (r=-0.3565, p = 0.0015). Tetracycline presented weak correlation with E-test (r = 0.2346, p = 0.04) and furazolidone to disk diffusion (r=-0.0288, p = 0.8038). E-test presented better agreement with gold standard. It is an easy and reliable method for *Helicobacter pylori* susceptibility testing. Disk diffusion method presented high disagreement and high rates of major errors.

Key words: *Helicobacter pylori*, E-test, disk diffusion, susceptibility test.

Introduction

Consensus recommends triple therapy to eradicate *Helicobacter pylori* in infected children (Chey & Wong, 2007; Fock *et al.*, 2009; Jones *et al.*, 2005; Malfertheiner *et al.*, 2012). However in developing countries increasingly antimicrobial resistance, mainly to metronidazole and clarithromycin, is observed (Alvarez *et al.*, 2009; Mendonça *et al.*, 2000; Ogata *et al.*, 2013; Sherif *et al.*, 2004; Wong *et al.*, 2003) and empirical treatment presents the risk of eradication failure and/or development of secondary resistance (Kalach *et al.*, 2002; Molina-Infante & Gisbert, 2013; Nguyen *et al.*, 2012; Wong *et al.*, 2003). Thus, antimicrobial susceptibility testing for *Helicobacter pylori* is growing in importance (Chey & Wong, 2007; Graham & Fischbach, 2010; Kalach *et al.*, 2002; Malfertheiner *et al.*, 2012; Mégraud, 2004; Sykora & Rowland, 2011).

Therefore, it is necessary to validate a reproducible *in vitro* susceptibility test to support the choice of antimicrobials drugs to eradicate *H. pylori*. The Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, Approved standard M7-A5, Informational supplement M100-S10, 2000) has approved only agar dilution method, but it is time-consuming, labor-intensive, and useful to simultaneously test a large number of strains. It is not adaptable to small numbers of strains,

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becoming impractical to perform routinely (Mégraud & Lehours, 2007). Two alternative methods have been reported, the Epsilometer test (E-test) and the disk diffusion method. The E-test is a plastic strip containing increasing gradients of antimicrobial concentration (AB Biodisk, Solna, Sweden), and the MIC is read directly from the intersection of the elliptical zone of growth inhibition. This is the current recommended phenotypic method, due to a stable pattern of antibiotic release and a tolerance to prolonged incubation, being an accurate method to test a fastidious organism like *H. pylori*. This is the main reason for why E-test, and not disk diffusion method, has been recommended for *Helicobacter pylori* susceptibilty testing. But it is more expensive (US$ 2.60 *vs.* US$ 0.026) (Mishra *et al.*, 2006).

The disk diffusion uses a disk with a known concentration of antimicrobial, it is simple and easy to perform, economical, and adaptable to clinical practice. But its a qualitative method, results can be only susceptible or resistant. And it is not standardized.

The difficulty to recommend one of these tests are the discrepancies between the methods and a lack of standardization to laboratory tests (Alarcon *et al.*, 1998; Best *et al.*, 2003; Glupczynski *et al.*, 2002; Grignon *et al.*, 2002; Hachem *et al.*, 1996; Lang & Garcia, 2004; Midolo *et al.*, 1997; Mishra *et al.*, 2006). The purpose of this study was to assess the reliability of E-test and disk diffusion comparing to the agar dilution method to evaluate *Helicobacter pylori* antimicrobial susceptibility.

Patients and Methods

From February 2008 to August 2009, 77 consecutive *Helicobacter pylori* isolates were obtained from children and adolescents (range: 3 - 20 years, mean age = 11.1 ± 3.9 y, Median = 10.8 years; M/F: 1:1.08) who underwent endoscopic examination to evaluate dyspeptic symptoms at Hospital São Paulo - Universidade Federal de São Paulo and Cândido Fontoura Children´s Hospital, located respectively at the south and east side of São Paulo City, Brazil.

The study was approved by the Institutional Review Board of Universidade Federal de São Paulo and the legal responsible signed an informed consent form.

Biopsy sampling and bacterial strains: Two antral biopsy specimens were collected and transported in Brain-Heart Infusion (BHI) broth + glycerol (10%) at 4 °C. Gastric biopsy specimens were homogenized and 5 μL of the solution was inoculated into selective BHI agar base (DIFCO - Lawrence, Kansas - USA) containing 7-10% defibrinated sheep blood, vancomycin (10 mg/L), trimethoprim (5 mg/L), cefsulodin (5 mg/L) and amphotericin B (5 mg/L) (*Helicobacter pylori* selective Medium - Dent Supplement Oxoid - Basingstoke, Hampshire - UK). All plates were incubated for 10 days at 37 °C in a microaerobic atmosphere (10% CO_2, 85% N_2, 5% O_2) at 95% humidity - Microaerobac - Probac do Brasil - São Paulo, Brazil). *Helicobacter pylori*

INCQS 00380 - ATCC 43504 strain was used as control. *H. pylori* isolates were confirmed based on colony morphology, positive urease, oxidase, and catalase reaction, and Gram staining. Isolates were stored at -70 °C in BHI broth containing glycerol 30% until performing susceptibility test.

Susceptibility tests were performed using five mL of frozen isolates that were subcultured on to BHI agar containing 10% defibrinated sheep blood and incubated for 3 days at 37 °C under microaerophilic conditions. The colonies were suspended in BHI broth, adjusted to McFarland 4 turbidity standard (approximately 1 x 10^8 cfu/mL), and then inoculated into Mueller-Hinton agar, according each method. The plates were incubated at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2 at 95% humidity) for 72 h.

Agar dilution method was performed according to the Clinical Laboratory Standards Institute (CLSI) protocols (CLSI (2006) M7-A5). A multipoint replicating device was used to deliver 2 μL of inoculums in each plate. Breakpoint to antimicrobials drugs recommendation was selected based on CLSI or literature citations. The final concentrations of clarithromycin (Abbott Laboratories - Chicago, Illinois - USA), tetracycline, furazolidone and amoxicillin (Sigma Aldritch Chemie - Steinheim, Germany) ranged from 0.015 to 64 μg/mL, while metronidazole (Sigma Aldritch Chemie - Steinheim, Germany) concentration ranged from 0.015 to 256 μg/mL. The MICs values were the lowest antibiotic concentration that completely inhibited visible growth of the bacteria.

E-test was performed to clarithromycin, tetracycline, metronidazole and amoxicillin. Antimicrobial concentration ranges from 0.016 to 256 μg/mL. After inoculation the plates were dried at 37 °C, then plastic strips (E-test) containing increasing gradient of antimicrobial concentration (AB Biodisk, Solna, Sweden) were applied onto surface of agar plate. The MICs values were read as the interception of the elliptical zone of inhibition with the graded strip for the E test after 72 h.

MIC interpretative criteria: There is no standardized MIC breakpoint for *H. pylori*, except to clarithromycin (MIC ≥ 1 μg/mL, and intermediary if MIC = 0.5 μg/mL). The MIC breakpoint for other antimicrobials was based on the literature (MIC ≥ 2 μg/mL to amoxicillin and furazolidone; ≥ 4 μg/mL to tetracycline, and, ≥ 8 μg/mL to metronidazole).

Disk diffusion uses disks containing 2 μg of clarithromycin, furazolidone, metronidazole and amoxicillin that were placed on the dried and inoculated Mueller-Hinton agar plates, results were read based on the diameters of the zone growth inhibition. Metronidazole standard by CLSI to disk diffusion: susceptible ≥ 21 mm, intermediary 16-21 mm, and resistant < 16 mm (Chaves *et al.*, 1999). The breakpoint to other antimicrobials was based on previous studies with similar methodology: amoxicillin susceptible

≥ 25 mm and amoxicillin resistant < 25 mm, clarithromycin susceptible ≥ 21 mm and clarithromycin resistant < 21 mm, and furazolidone susceptible ≥ 13 mm and furazolidone resistant < 21 mm.

Statistical analysis

The disagreement among the 3 tests was analysed by McNemars test. MIC values analysis between E-test and agar dilution, and MIC values analysis of agar dilution and the disk diffusion inhibition zone diameter was performed by Spearmans correlation test. Susceptibility tests were examined by error categorization.

Results

Figures 1-4 show the scattergrams comparing agar dilution and E-test MICs to metronidazole, amoxicillin, tetracycline and clarithromycin, scattergram comparing the diameter of inhibition zone of disk diffusion and agar dilution MIC to metronidazole, clarithromycin, amoxicillin and furazolidone is showed in figure 5-8. The overall rate of clarithromycin, furazolidone, metronidazole, tetracycline, and amoxicillin resistance is shown in Table 1. Metronidazol showed the highest resistance level: 40.2%, 33.7% and 39.9% by agar dilution method, E-test and disk diffusion, respectively. The MIC$_{90}$ value for agar dilution and E-test was similar (64 μg/mL, 3 log$_2$ over recommended breakpoint). There was a strong correlation (r = 0.7992, p < 0.0001) with agar dilution and E-test, 5 (6.5%) isolates were discordant. When compared to disk diffusion, disagreement occurred in

10%. The intermediary susceptibility to metronidazole by disk difusion was observed in 27/77 (35%); these results were exluded because difficult in comparison to agar dilution method. Nevertheless, strong correlation (r = -0.6962, p < 0.0001) was observed with disk diffusion and agar dilution (Table 2).

Clarithromycin presented resistance rate of 19.5% by agar dilution method and by E-test (20.8%); MIC$_{90}$ was observed near the breakpoint (≤ 1 μg/mL). Disk diffusion showed resistance in 38.9% (Table 1). Evaluating disagreement to E-test, clarithromycin presented one intermediary result by agar dilution that was resistant by E-test (1.3%) but correlation was moderate (r = 0.6369, p < 0.0001) and disagreement with disk diffusion occurred in 11.7%, 9 strains were susceptible by agar dilution and resistant by disk diffusion, but correlation was moderate (r= -0.5656, p < 0.0001) (Table 2).

Amoxicillin showed 10.4% of resistance rate by agar dilution and 9% by E-test, but presented the highest resistance rate by disk diffusion (68.8%) (Table 1). Disagreement between E-test and agar dilution occurred in one strain, that was susceptible by agar dilution and resistant by E-test (1.3%), but E-test showed a weak correlation (r = 0.3565, p = 0.0015). Agar dilution presented the highest disagreement (58.4%) and a weak correlation too (r = -0.3565, p = 0.0015) (Table 2).

Tetracycline presented no resistant strains (0%) by agar dilution method and by E-test, but correlation was weak (r = 0.2346, p = 0.04). Furazolidone showed no resistant strains (0%) by agar dilution and by disk diffusion,
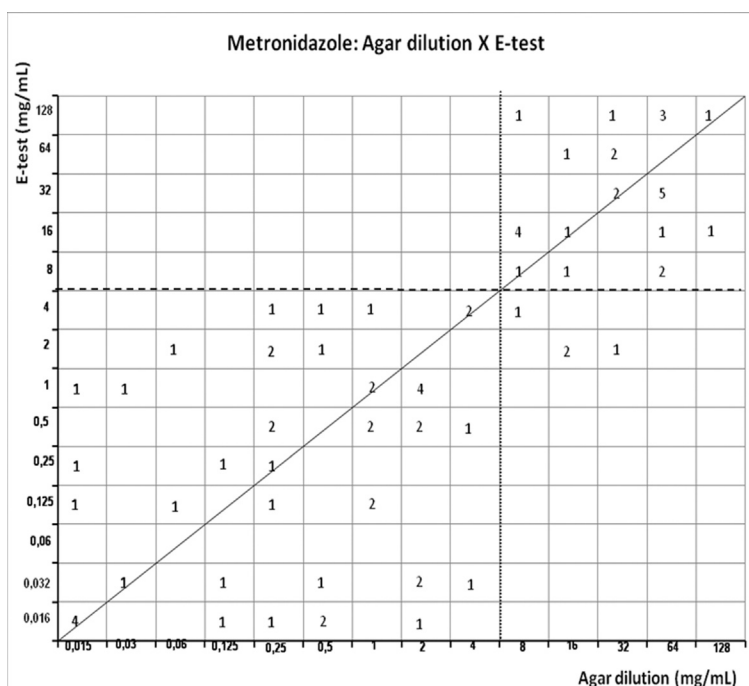
Figure 1 - Scattergram demonstrating the correlation between agar dilution reference metronidazole MIC (resistant ≥ 8 μg/mL - dotted line) and E-test metronidazole MIC (resistant ≥ 8 μg/mL - dashed line) determined on Muller-Hinton agar.
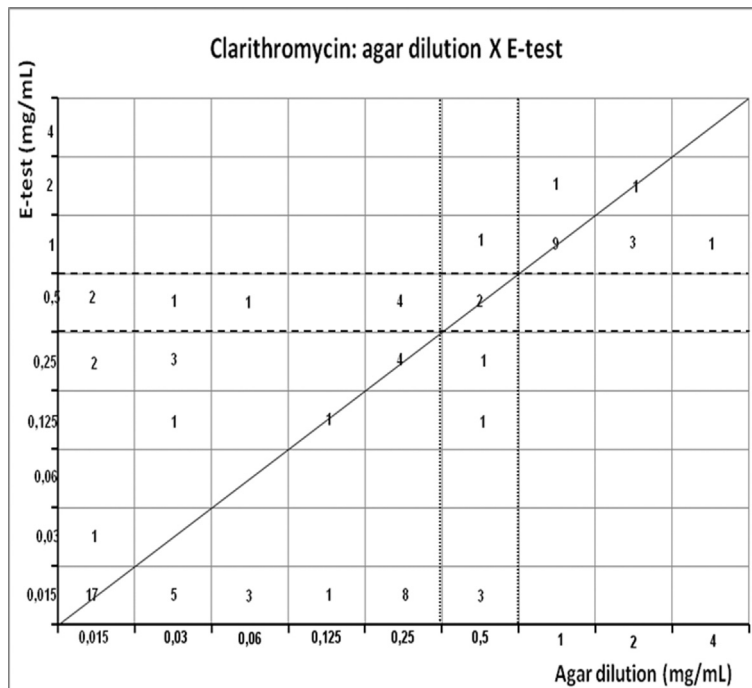
Figure 2 - Scattergram demostrating the correlation between agar dilution reference clarithromycin MIC (resistant ≥ 1 μg/mL, and intermediary = 0.5 μg/mL - dotted line) and E-test clarithromycin MIC (resistant ≥ 1 μg/mL, and intermediary = 0.5 μg/mL - dashed line) determined on Muller-Hinton agar.
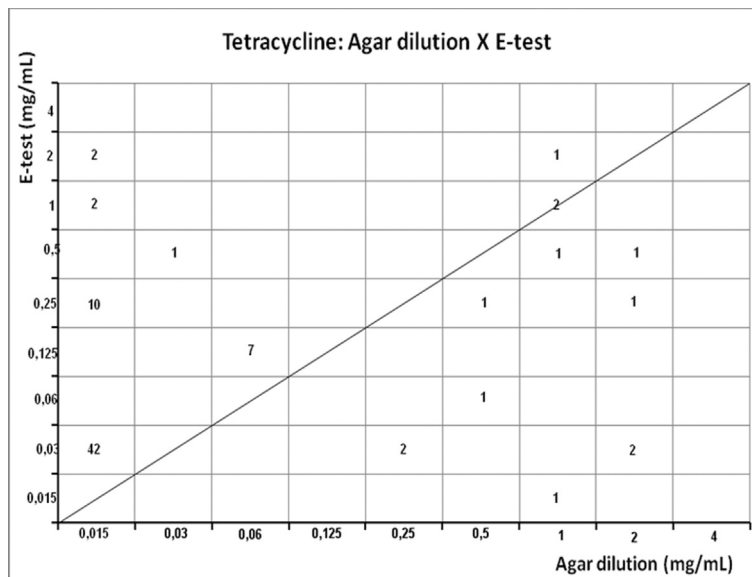
Figure 3 - Scattergram demostrating the correlation between agar dilution reference tetracycline MIC (resistant ≥ 4 μg/mL) and E-test tetracycline MIC (resistant ≥ 4 μg/mL) determined on Muller-Hinton agar.

and presented weak correlation too (r = -0.0288, p = 0.8038).

Interpretative error rate analysis (amoxicillin, clarithromycin and metronidazole)

Very major error (VME)

That occurred when the bacteria was resistant (agar dilution) and disk-diffusion method or E-test results were susceptible. In this situation the patients are treated with the antimicrobial, but eradication failure can occur;

### *Major error (ME)*

When the bacteria was susceptible (agar dilution) and diffusion test disk or E-test showed resistance. In this situation the patient doesn't use the antimicrobial, but the treatment could work;
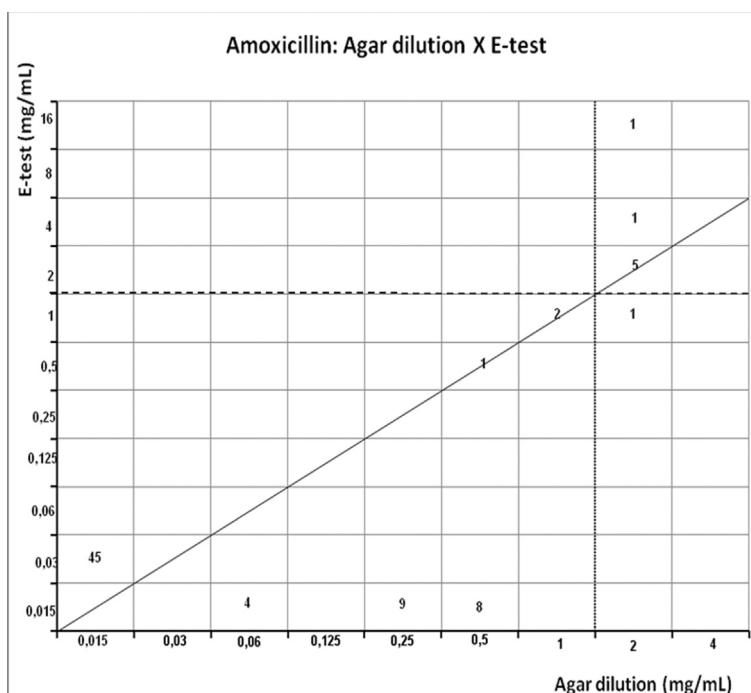
Figure 4 - Scattergram demonstrating the correlation between agar dilution reference amoxicillin MIC (resistant ≥ 2 µg/mL - dotted line) and E-test amoxicillin MIC (resistant ≥ 2 µg/mL - dashed line) determined on Muller-Hinton agar.
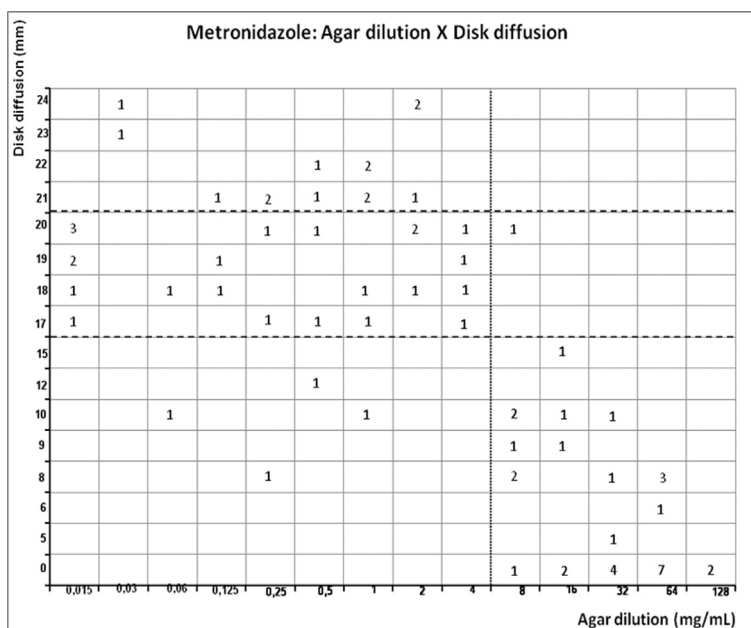
Figure 5 - Scattergram demonstrating the correlation between agar dilution reference metronidazole MIC (resistant ≥ 8 µg/mL - dotted line) and disk diffusion metronidazole inhibition zone (susceptible ≥ 21 mm, intermediary 16-21 mm, and resistant < 16 mm - dashed line) determined on Muller-Hinton agar.

Minor error (E)

The results where in the intermediary range of resistance.

Disk diffusion method presented 94/231 (40.7%) errors (59 ME and 35 E). E-test presented 13/107 (5.6%) errors. Amoxicillin shows 1 VME with E-test (1.3%) and 45 ME by disk diffusion method (58.4%). Clarithromycin showed 7 (9.1%) errors by E-test, 1 ME (1.3%) and 6 E (7.8%), by disk diffusion 17 (22.1%) errors occurred, 9 ME (11.7%) and 8 E (10.4%). Metronidazole presented the highest rate of VME by E-test - 5 (6.5%), and by disk diffusion - 5 ME (6.5%) and 27 E (41.5%).

Clarithromicyn: Agar dilution X Disk difusion

Disk difusion (mm) vs Agar dilution (mg/mL)

Disk difusion (mm)	0,015	0,03	0,06	0,125	0,25	0,5	1	2	4
37	1								
32	1				1				
31	1				1				
30	4	1			2				
29			1		1				
26		1			3				
25	2	3	1	1	1				
24	3				1				
23	1					1			
22	2	1	1		2				
21	5	1		1	3	1			
20	2	3	1		3	4	1		
19						3	1		
18						1			
17									1
16						3			
15						1	2		
14						1			
13							1		
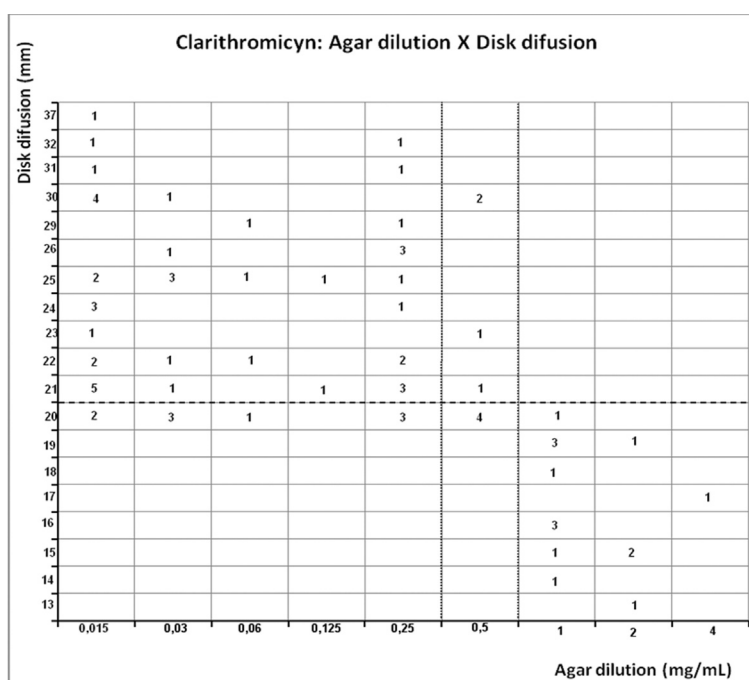
Figure 6 - Scattergram demonstrating the correlation between agar dilution reference clarithromycin MIC (resistant ≥ 1 μg/mL, and intermediary = 0.5 μg/mL - dotted line) and disk diffusion clarithromycin diameter of inhibition zone (resistant < 21 mm - dashed line) determined on Muller-Hinton agar.

Furazolidone: Agar dilution X Disk diffusion

Disk diffusion (mm) vs Agar dilution (mg/mL)

Disk diffusion (mm)	0,015	0,03	0,06	0,125	0,25	0,5	1	2	4
35	1	3			1				
31	1				1				
30	5								
29	1	1			1				
28	1		1						
26	9	1							
25	6	1			1				
24	3	1	3		1				
23	3	1			6				
22	7	3	1						
21	13	2			6				
20	17	2	1	25	1				
19	7	1	1				2	3	
18	1	1			9			5	
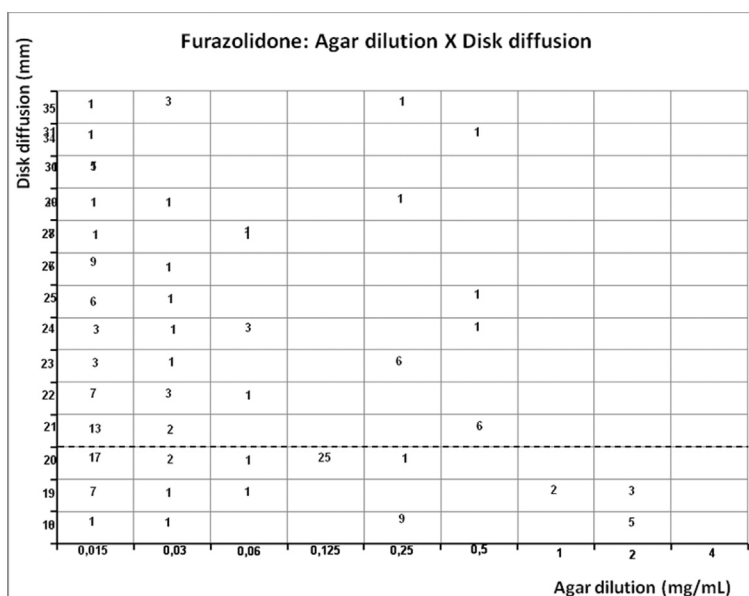
Figure 7 - Scattergram demonstrating the correlation between agar dilution reference furazolidone MIC (resistant ≥ 4 mg/mL) and disk diffusion furazolidone diameter of inhibition zone (susceptible ≥ 13 mm and resistant < 21 mm - dashed line) determined on Muller-Hinton agar.

Technical and economical aspects

The main differences were the price of each test (U$ / sample) and the time consumed to prepare each one.

Agar dilution needs a sequencial dilution of antimicrobials and uses, in our study, 11 plates to be performed. It cost U$ 37.26 and was the most laborious and time-consuming test to prepare. However, we can evaluate 20 samples in each plate then the values can decrease to U$ 1.86 / sample, but we need to collect all samples to perform the test.

E-test costs about U$ 45.60, but was the easiest and simplest test to prepare. And each sample costs U$ 23.49 to be evaluated by disk diffusion method. It is as simple to prepare as E-test.
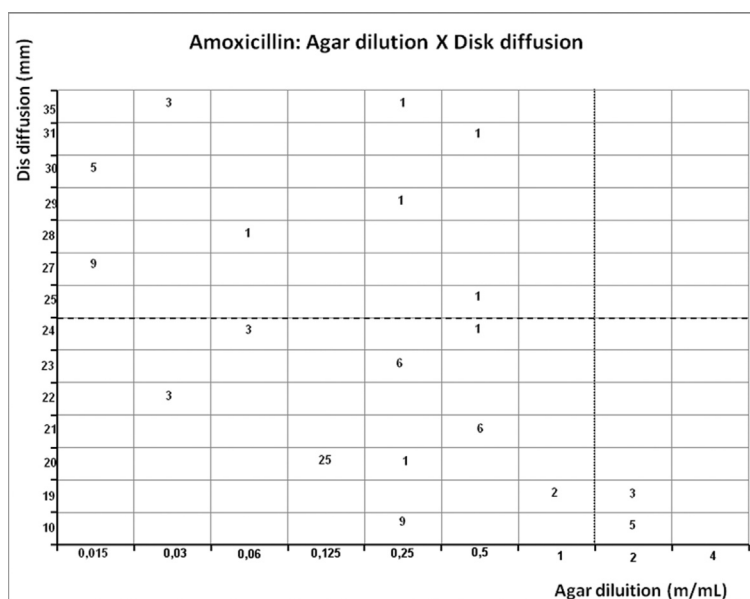
Figure 8 - Scattergram demostrating the correlation between agar dilution reference amoxicillin MIC (resistant ≥ 2 µg/mL - dotted line) and disk diffusion amoxicillin inhibition zone (resistant < 25 mm - dashed line) determined on Muller-Hinton agar.

Table 1 - Comparison of antimicrobials susceptibility test results by Agar dilution, E-test and Disk-diffusion method.

Method			Antimicrobial Agent				
			Amoxicillin	Clarithromycin	Furazolidone	Metronidazole	Tetracycline
Agar Dilution	MIC (µg/mL)	Range	0.015 - 2	0.015 - 4	0.015 - 0.06	0.015 - 256	0.015 - 2
		MIC_{50}	0.03	0.25	0.015	2.00	0.015
		MIC_{90}	1.00	1.00	0.03	64	1.00
	N (%) of isolates	Sensitive	69 (89.6)	62 (80.5)	77 (100)	46 (59.8)	77 (100)
		Resistant	8 (10.4)	15 (19.5)	0 (0)	31 (40.2)	0 (0)
E-test	MIC (µg/mL)	Range	0.016 - 16	0.016 - 2		0.016 - 256	0.016 - 2
		MIC_{50}	0.032	0.125		2	0.032
		MIC_{90}	1.00	1.00		64	0.5
	N (%) of isolates	Sensitive	70 (91)	61 (79.2)		51 (66.2)	77 (100)
		Resistant	7 (9)	16 (20.8)		26 (33.8)	0 (0)
Disk-Diffusion	mm	Range	10 - 35	15 - 37	18 - 35	0 - 24	
	N (%) of isolates	Sensitive	24 (31.2)	45[a] (63.7)	77 (100)	15[b] (18.2)	
		Resistant	53 (68.8)	24[a] (36.3)	0 (0)	35[b] (38.9)	

[a] Eight strains excluded because of intermediate results by agar dilution.
[b] Twenty-seven strains excluded because of intermediate results by disk-difusion.

Discussion

Our results suggest that E-test is the best option to routinely performing susceptibility test for *Helicobacter pylori*. The results are similar to other studies that consider E-test a good method to clarithromycin and amoxicillin (Best *et al.*, 2003; Glupczynski *et al.*, 1991; Hachem *et al.*, 1996), with good intra and interlaboratorial correlation (Glupczynski *et al.*, 2002). Despite the price.

Agreement and disagreement analysis of E-test and agar dilution method showed the best agreement to tetracycline (100%), followed by amoxicillin, clarithromycin and, metronidazole, all of them presented agreement rate over than 90%. Evaluation of MIC values presented low correlation to amoxicillin, moderate to clarithromycin, and high to metronidazole. These results show that despite good agreement to discriminate susceptible and resistant isolates, MIC can present a large range between both methods. Even a variation of 2 $\log_2$ in the MIC was not significant, not interfering in the interpretation of susceptible and resistant strains.

Table 2 - Analysis of disagreement and correlation of agar diffusion methods (E-test and Disk-diffusion) compared to Agar dilution method.

Antimicrobial agent	Method			
	E-test		Disk-diffusion	
	Disagreement - % (p)	Correlation (p)	Disagreement - % (p)	Correlation (p)
Amoxicillin	1.3 (1)	0.3565 (0.0015)	58.4 (0.0001)	-0.3565 (0.0015)
Clarithromycin	1.3 (1)	0.6369 (< 0.0001)	11.7 (0.0077)	-0.5656 (< 0.0001)
Furazolidone				-0.0288 (0.8038)
Metronidazole	6.5 (0.07)	0.7992 (< 0.0001)	10 (0.07)	-0.6962 (< 0.0001)
Tetracycline		0.2346 (0.04)		

Disagreement analysis - McNemar's test
Correlation analysis - Spearmans correlation test

Table 3 - Interpretative error rate analysis to E-test and disk-diffusion.

Antimicrobial (N)	Error	E-test N (%)	Disk-Diffusion N (%)	Total N (%)
Amoxicillin (77)	Very major	1 (1.3)	0	1 (2.2)
	Major	0	45 (58.4)	45 (97.8)
	Minor	0	0	46 (100)
		1 (1.3)	45 (58.4)	
Clarithromycin (77)	Very major	0	0	0
	Major	1 (1.3)	9 (11.7)	10 (41.7)
	Minor	6 (7.8)	8 (10.4)	14 (58.3)
		7 (9.1)	17(22.1)	24 (100)
Metronidazole (77)	Very major	5 (5.6)	0	5 (13.5)
	Major	0	5 (5.6)	5 (13.5)
	Minor	0	27 (35.9)	27 (73)
		5 (6.5)	32 (41.5)	37 (100)
Total	Very major	6 (2.6)	0	6 (5.6)
	Major	1 (0.4)	59 (25.5)	60 (56)
	Minor	6 (2.6)	35 (15.2)	41 (38.3)
231		13 (5.6)	94 (40.7)	107 (100)

Metronidazole presented good correlation between agar dilution and E-test method. However, literature shows conflicting results. As our study, some authors observed high correlation with the agar dilution method (Best *et al.*, 2003; Chaves *et al.*, 1999; Osato, 2000; Tankovic *et al.*, 2001). Chaves *et al.* (Chaves *et al.*, 1999) did not observed significant differences between MIC of metronidazole by agar dilution and E-test. But there was discrepancy in other studies (Alarcon *et al.*, 1998; Mégraud & Lehours, 2007). The results of one study (Glupczynski *et al.*, 2002) showed a good correlation to the antimicrobials, except to metronidazole that presented large range of inter and intralaboratorial values of MIC. This difference was large (> 2log$_2$) and authors recommend to test the isolates in parallel to improve the accuracy of E-test. The results need to be in acceptable level of MIC.

In opposition, disk diffusion method showed conflicting and inconsistent results to amoxicillin (breakpoint = 25 mm - 41.6%). The lack of standardization of the breakpoint is a difficulty, in addition to variations in the antimicrobial concentration in each disk. This study used disks containing 2 µg and breakpoint based on previous studies with similar methodology. However, agreement with gold-standard was lower than other studies (Lang & Garcia, 2004; Midolo *et al.*, 1997; Sung *et al.*, 2009).

Clarithromycin (breakpoint = 21 mm - 83.1%), is the unique antimicrobial standardized to disk diffusion (Grignon *et al.*, 2002; Hachem *et al.*, 1996; Midolo *et al.*, 1997; Warburton-Timms & McNulty, 2001). Comparing MIC by agar dilution and E-test we observed mild correlation, results that were comparable to other studies. When disk diffusion method used disks with 2 µg of clarithromycin concentration (MIC > 2 mg/L), discrepancies rarely occurred. The breakpoint = 22 mm to clarithromycin has the same discriminatory power than the MIC > 0.5 µg/L (Mégraud & Lehours , 2007).

Metronidazole showed agreement in 90% and mild correlation. Disk diffusion method to metronidazole was evaluated by Chaves *et al.* (Chaves *et al.*, 1999), significant difference to MIC by agar dilution or disk diffusion was not observed using criteria of susceptible, intermediate and resistant. E-test and disk diffusion presented good correlation (r = 0.94). Interpretative error rate analysis shows major rate of errors using disk diffusion method. There is not a good agreement with agar dilution method. E-test, by the other hand, showed a good correlation and agreement.

Conclusions

E-test method presented better agreement with gold standard. It is an easy and reliable method to test *Helicobacter pylori* susceptibility. While, disk-diffusion method presented high disagreement with gold-standard and high rates of major errors. Probably a lack of break-point standardization, and different concentration of antimicrobial in diffusion disks are confouding factors. However, studies with a larger number of isolates are necessary to evaluate disk diffusion method that is simpler and cheaper.

Financial Support Source

Conflict of Interest

No

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