

Role of screening agar plates for in vitro susceptibility testing of *Helicobacter pylori* in a routine laboratory setting

V J Warburton-Timms, C A M McNulty

Abstract

Background—Resistance of *Helicobacter pylori* to the more frequently used antibiotics (metronidazole and clarithromycin) reduces eradication rates even with triple treatment. Determining the antibiogram profile of *H pylori* can take up to 14 days and delays appropriate treatment.

Aims—To determine the role of screening agar plates for more rapid in vitro susceptibility of *H pylori* to metronidazole, amoxicillin, and clarithromycin.

Methods—Routine gastric biopsy specimens from 507 dyspeptic patients were inoculated on to 10% lysed blood agar plates containing metronidazole (8 µg/ml), clarithromycin (2 µg/ml), or amoxicillin (0.5 µg/ml). The minimum inhibitory concentration (MIC) of the 90 isolates was determined using the E test.

Results—Metronidazole resistance was detected in 28 of 90 isolates by E test and nine of 98 by screening agar. The screening agar detected none of the four clarithromycin resistant isolates detected by the E test.

Conclusions—The screening agar method is not sufficiently sensitive to be used alone.

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Keywords: *Helicobacter pylori*; metronidazole; clarithromycin; susceptibility testing

The bacterium *Helicobacter pylori* infects about 40% of the population and is responsible for the development of duodenal ulcer disease.¹ The infection also has a causative role in gastric ulcer disease, non-ulcer dyspepsia, and gastric adenocarcinoma, and is classed as a type 1 carcinogen by the World Health Organisation. The treatment of *H pylori* involves the use of a proton pump inhibitor and two or three antibiotics, usually metronidazole, amoxicillin, or clarithromycin. Treatment efficacy can drop from 86% to 58% if the infecting strain is resistant to metronidazole or clarithromycin.² Metronidazole and clarithromycin resistant *H pylori* strains are increasing. The prevalence of primary metronidazole resistance varies from 7% to 90%,³ with the developing world having the highest prevalence. Primary resistance to clarithromycin is between 4% and 7% and rising,⁴ and recently amoxicillin resistance has been reported.⁵ Previous antibiotic use of these agents increases the likelihood of a resistant *H pylori* infection.⁶

Detecting antibiotic resistant isolates by traditional methods (disc, epsilometer (E) test, or agar dilution) takes between 10 and 14 days; therefore, empirical treatment is often started before sensitivity results are known. Primary sensitivity testing on antibiotic containing medium could reduce reporting time to three to five days. Preliminary data on this screening agar method reported by Glupczynski *et al* suggest that 84% of metronidazole resistant isolates can be detected.⁷ The aim of our study was to confirm the usefulness of this screening agar method in detecting resistance to metronidazole, clarithromycin, and amoxicillin. Our study was run in parallel with a multicentre European study determining the prevalence of *H pylori* resistance across Europe by the E test.⁸ The E test was used as the gold standard because it correlates well with agar dilution, allowing Chaves *et al* to use it as a reference method.⁹

Methods

PATIENTS

Five hundred and seven consecutive routine biopsy samples from patients (> 18 years) attending for upper gastrointestinal endoscopy at Gloucestershire Royal NHS Trust endoscopy clinic over a six month period were used in our study. Patients who had been treated previously with nitroimidazoles and/or macrolides were excluded.

ISOLATION OF *H PYLORI* AND INOCULATION OF SCREENING AGAR

Biopsy specimens for culture were sent in normal saline to the Gloucester Public Health Laboratory. Biopsy specimens were inoculated on to a chocolate agar plate (Columbia agar base CM331; Oxoid, Basingstoke, UK), three screening agar plates, and an *H pylori* selective agar (Dent; Oxoid). The biopsy specimens were not crushed before inoculation because a large series has shown that this is not warranted.¹⁰ The three screening agar plates contained metronidazole (8 µg/ml), amoxicillin (0.5 µg/ml), and clarithromycin (2 µg/ml) with 10% lysed horse blood and Columbia agar base (CM331; Oxoid).⁷ To control for growth, the biopsy specimen was smeared on the screening agar plates in between the two culture plates. There were only four occasions when there was no growth on the *H pylori* selective agar when *H pylori* had grown on chocolate agar; in three of these cases growth on chocolate agar was scanty. *Helicobacter pylori* type strain NCTC11637 and a recently isolated strain

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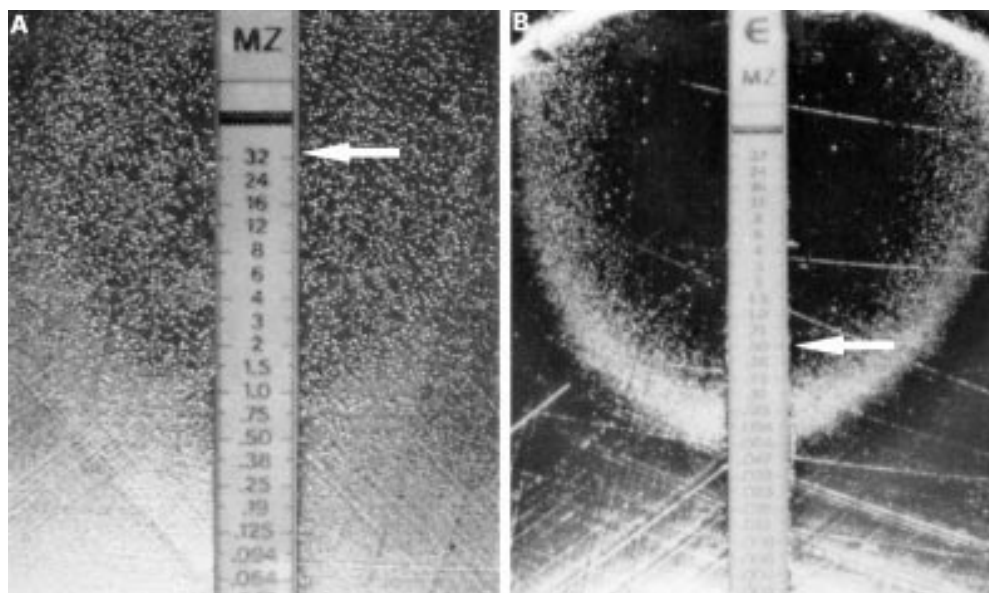


Figure 1 Reading guide for E tests. (A) Colonies of a metronidazole resistant subpopulation in the ellipse minimum inhibitory concentration (MIC) > 32; (B) trailing of microcolonies at the end point MIC 0.5 µg/ml.

were incubated with the isolates to control for changes in atmospheric conditions. Plates were incubated in a microaerobic atmosphere (6% O₂, 10% CO₂), obtained by evacuation of air to 500 mm Hg and replacement with the appropriate gas mixture (10% H₂, 10% CO₂, 80% N₂), at 37°C for five to seven days. After five days the plates were checked for growth. If there was no growth the plates were incubated for a further two days. All agar plates were scored as scanty, moderate, or profuse growth with *H pylori* (confirmed by urease, catalase, and Gram film), or no growth.

E TEST

The method agreed by the European *H pylori* study group was used.⁸ Primary isolates of *H pylori* were subcultured from the non-selective or Dents primary culture plates for the E test. A 48 hour fresh *H pylori* culture was harvested into 5 ml peptone water tubes. The inoculum density was adjusted to four on the MacFarland scale (approximately 10⁸ colony forming units/ml). Mueller Hinton agar (CM337; Oxoid) with 10% horse blood was used and the pH of the medium was adjusted to between 7.2 and 7.4. All plates were warmed at room temperature before use. Three plates were inoculated for each strain, one plate for each E test strip. A lawn of inoculum was produced with the suspension using a sterile swab. Plates were allowed to dry for 10 minutes before applying the E test strips. The plates were incubated at 37°C in a microaerobic atmosphere (6% O₂, 10% CO₂) for three days. The minimum inhibitory concentration (MIC)

from the E test was taken as the point at the intersection between the edge of the inhibition zone and the graduations on the E test strip. Isolates were considered resistant if their MIC was > 8 µg/ml for metronidazole, > 2 µg/ml for clarithromycin, and > 0.5 µg/ml for amoxicillin, as suggested by the European *H pylori* study group.⁸ In line with E test reading guides (AB Biodisk; Solna, Sweden), isolated colonies of *H pylori* growing inside the inhibition zone were noted as resistant mutants (fig 1A and B). MIC values of three reference strains from the culture collection of the University of Gotenberg (CCUG), sent by the European *H pylori* study group, were determined by E test, using the same method described above, for amoxicillin, clarithromycin, and metronidazole on three separate occasions (at the start, halfway, and at the end of the study). The control strains were kept viable by freezing at -70°C in glycerol broth, rather than repeated subculture, to avoid the emergence of possible strain modifications.

Results

One hundred and twenty eight of the 507 gastric biopsy specimens were positive for *H pylori* by the biopsy urease test (BUT).¹⁰ Of these, 123 were culture positive. E-tests were only available for 90 isolates because there was a supply problem with the E test strips during our study. Screening agar results are available for 98 isolates, because the remaining biopsy specimens arrived in the laboratory when the research scientist was absent, so that plates could not be inoculated. Resistance to metronidazole was detected in nine of the 98 isolates by the screening agar (table 1). Using a cut off point of 8 µg/ml, 28 of 90 of isolates were metronidazole resistant by the E test. Seventy isolates had sensitivity test results by both methods. The screening agar failed to detect 16 of the 21 isolates found to be metronidazole resistant by E test. Two plates were overgrown

Table 1 Resistant isolates detected by screening agar and E test

	Total tested	Metronidazole resistant >8 µg/ml	Clarithromycin resistant >2 µg/ml	Amoxicillin resistant >0.5 µg/ml
E test	90	28	4	0
Screening agar	98	9	0	0
Screening agar + E test	70	21*	4**	0

*Detected in five isolates by screening agar.

**None detected by screening agar.

Table 2 E test MICs for CCUG reference strains performed at the beginning, midway, and at the end (runs 1–3) of our study

QC strain	Clarithromycin MIC µg/ml	Metronidazole MIC µg/ml	Amoxicillin MIC µg/ml
Resistance breakpoint	>2	>8	>0.5
CCUG 38770			
Expected result	0.012–0.25	>256	0.015
Run 1	0.38	>256	0.016
Run 2	0.38	>256	0.016
Run 3	0.25	>256	0.016
CCUG 38771			
Expected result	0.03	0.5	<0.015
Run 1	0.023	1	<0.016
Run 2	0.023	0.19	<0.016
Run 3	0.023	0.19*	<0.016
CCUG 38772			
Expected result	2	32–64	<0.015
Run 1	6	>256	<0.016
Run 2	0.75	128	<0.016
Run 3	6	64	<0.016

*Mutant colonies inside the zone.
CCUG, culture collection of the University of Gotenberg; MIC, minimum inhibitory concentration.

with other gastric flora and four isolates only yielded scanty growth on the non-selective agar. Four isolates were clarithromycin resistant (MIC > 2 µg/ml by E test); three of these were resistant subcolonies (fig 1B). The screening agar method failed to detect any clarithromycin resistant isolates. No amoxicillin resistant isolates were detected by E test or screening agar.

Table 2 shows the reference strain E test results. Twenty one of the 27 tests were within one dilution of the expected result. Two E test clarithromycin results would have been reported incorrectly as resistant; the expected MIC of CCUG 38772 lay on the breakpoint at 2 µg/ml and our test results were reported at 6 µg/ml.

Discussion

The prevalence of primary metronidazole and clarithromycin resistance in west Gloucestershire is 31% and 5%, respectively. Amoxicillin resistance was not detected. The prime aim of our project was to determine the reliability of using a screening agar method at primary isolation to detect resistant *H pylori*. However, we only detected five of the 21 metronidazole resistant and none of the four clarithromycin resistant strains. In their preliminary report Glupczynski *et al* correctly detected 84% of metronidazole resistant isolates.⁷ We cannot explain this discrepancy and we have checked that we were using the same antibiotic concentrations and agar. They found that isolates reported falsely as sensitive yielded a very low density culture of *H pylori*, or that plates were contaminated by non-*H pylori* flora. In our study, contamination or low density culture was only recorded in six of the 16 resistant isolates not detected. The use of only one biopsy specimen for culture in our study might have yielded a lighter growth. However, only eight of the 98 cultures on the *H pylori* selective agar inoculated after the screening plates yielded a lighter growth than the chocolate agar. No growth was recorded in four cases; resistance was recorded in two of these isolates by the E test. This suggests that using only one biopsy

may miss 7–10% of resistant strains. Glupczynski's group⁷ grind biopsy specimens with an electric grinder; we do not grind our specimens because large studies in our laboratory have not found an improvement in primary isolation rates after grinding. However, it could be that this makes a difference with this screening agar.

The screening agar would be more cost effective if primary screening agar plates were only set up when a BUT inoculated in endoscopy was positive at arrival in the laboratory. This method is now used successfully by Glupczynski *et al* and may explain why this group's detection rate was much higher. If only specimens with a positive BUT are inoculated, this would reduce the number of plates inoculated by 70%. However, this does rely on staff in the endoscopy department informing staff in the microbiology department of their results.

Oderda *et al* have presented preliminary results on 45 strains using a multisector screening agar plate containing Dent's selective agar and metronidazole, clarithromycin, or amoxicillin in each sector. Concordance between E tests and the multisector plates was seen in all 10 metronidazole and 15 of the 16 clarithromycin resistant isolates.¹¹ This method looks promising; however, the multisector plates would be costly to produce in terms of staff time and the method needs further evaluation.

The prevalence of metronidazole resistance in west Gloucestershire has risen in the past 10 years from 10% in 1989, 22% in 1992, to 31% in 1999. This is of concern given the widespread use of metronidazole in the treatment of *H pylori* infection and the lower eradication rate in the presence of metronidazole resistant strains.² Because studies have shown that metronidazole resistance is not reversible, culture for sensitivity testing should now be encouraged and treatment regimens adjusted according to local prevalence data.

Clarithromycin resistance was seen predominantly as subcolonies within the E test ellipse (fig 1A). These might be difficult to detect on the screening agar plates and may explain why we failed to identify these resistant strains by the screening agar method. Clarithromycin resistance has a greater impact on the outcome of infection than metronidazole resistance, and prevalence is now high in some European countries, especially in children.¹² The quality control strain CCUG 38772 would have been reported incorrectly as resistant to clarithromycin (reported MIC, 6 µg/ml; expected MIC, 2 µg/ml). When the expected MIC lies on the breakpoint, the results can be unreliable. When the result lies close to the breakpoint it is probably better to report the MIC value. Although amoxicillin resistance was not detected, continuing surveillance for amoxicillin is still necessary because it can cause a small increase in the numbers of resistant subpopulations.

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