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Laboratory handling of *Helicobacter pylori* critically influences the results of in-vitro metronidazole resistance determination

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ABSTRACT

In-vitro metronidazole resistance rates of *Helicobacter pylori* determined by Etest are high, and the predictive value of metronidazole resistance is low. It was hypothesised that altered laboratory methods could reduce the overestimation of resistance and improve the predictive value of the Etest. Pre-treatment isolates ($n = 150$) of *H. pylori* from 150 patients were investigated by Etest with incubation for 72 h. Treatment with metronidazole, tetracycline and bismuth for 10 days failed to eradicate *H. pylori* in 23 patients. After isolate storage for 3 years, resistance determination results by agar dilution and Etest, with incubation for 72 and 31 h, were compared. The rate of metronidazole resistance was reduced significantly during storage, and instability of resistance was associated significantly with treatment outcome. Isolates that retained in-vitro resistance had significantly (p 0.008) higher treatment failure rates ($n = 13$; 42%) than isolates that lost resistance ($n = 3$; 9%). The reproducibility achieved by dual testing with agar dilution and Etest was 41% and 70% for ± 1 and ± 2 $\log_2$ dilutions, respectively, after incubation for 72 h, and 85% and 92%, respectively, after incubation for 31 h. Thus, the predictive value was improved from 25% to 50% by the altered laboratory conditions (p 0.04). MIC values of 2–8 mg/L signified an intermediate risk of treatment failure.

Keywords Etest, *Helicobacter pylori*, metronidazole, MICs, resistance

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INTRODUCTION

Although the prevalence of in-vitro metronidazole resistance in *Helicobacter pylori* varies worldwide, it is generally found to be very high [1–10]. The association between in-vitro metronidazole resistance and failure to eradicate *H. pylori* following triple-drug therapy is weak [1–9,11]. Eradication therapy is therefore commonly prescribed, and large treatment studies are conducted without determination of metronidazole resistance [10–15].

The extent to which particular laboratory procedures might affect the outcome of resistance determination with the Etest has not been elucidated. Although reproducibility of in-vitro resistance tests is good when the same procedure is repeated with a single suspension [16–21], the influence of variations in laboratory handling, including the impact of storage on the test's predictive value, is not known. Therefore, in-vitro metronidazole resistance was examined by performing Etests on pre-treatment isolates pre- and post-storage, and relating the results to the outcome of triple-drug therapy. Furthermore, the impact of using different periods of incubation (72 h vs. 31 h) was assessed. The possible existence of a concentration zone of intermediate resistance was also investigated. The standard reference method, agar dilution, was used for comparison.

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MATERIALS AND METHODS

Between September 1995 and March 1996, 248 patients were included in a Norwegian multicentre study and treated for *H. pylori*-related disorders with a 10-day course of bismuth subnitrate 150 mg three-times-daily, oxytetracycline 500 mg three-times-daily, and metronidazole 400 mg three-times-daily. None of the patients had received anti-*H. pylori* treatment previously [22]. Before treatment, gastric specimens were harvested and sent to the Department of Microbiology, Østfold Central Hospital, Fredrikstad, Norway, where resistance tests were performed and the isolates stored. For comparison of test procedures, the resistance of the stored specimens was determined later, as described below, in three laboratories using different methods.

H. pylori eradication failed in 34 patients. Pre-treatment isolates from 23 of these patients were recoverable after storage. Metronidazole resistance tests were also performed with all three methods on 131 of 138 consecutive isolates from 214 patients treated successfully. Four of these were excluded because of lack of crucial data. Thus, test results from 150 (127 + 23) isolates were analysed. The strains originated from 72 males and 78 females, with a median age of 61.5 years (range 20–83 years).

Procedures for culture and resistance determination

Gastric specimens were ground and diluted in normal saline at Østfold Central Hospital. Suspensions were cultured on Columbia blood agar (Oxoid, Basingstoke, UK). *H. pylori* colonies, defined by appearance and by positive catalase, urease and oxidase reactions, were tested for in-vitro metronidazole resistance as described below, and stored at −70 °C [22]. If initial growth was insufficient, isolates were subcultured once on Columbia blood agar.

After storage for 3 years, isolates were cultured on three Columbia blood agar plates. After 3 days, two plates of the recovered isolates were sent, in micro-aerobic jars at 37 °C, to separate laboratories within a 5-h travelling radius.

The pre-storage Etest (AB Biodisk, Solna, Sweden) for in-vitro metronidazole resistance determination was performed at Østfold Central Hospital with chocolate agar plus Vitox (Oxoid). A heavy inoculum of *c.* 4 MacFarland standards was used, and CampyGen (Oxoid) was used to produce a micro-aerobic atmosphere. Plates were incubated for 72 h [22].

In-vitro metronidazole resistance was determined for post-storage isolates by three methods. First, a post-storage Etest was performed, as described above, by the same investigator who conducted the pre-storage test. Second, an Etest with a shorter incubation period was performed at the Department of Microbiology, Sentralsykehuset i Vestfold, Tønsberg, Norway, using Vestfold Charcoal Medium. This medium was based on charcoal agar (Oxoid) and contained serum 10% v/v and IsoVitaleX 1% v/v (Becton Dickinson, Franklin Lakes, NJ, USA) [16,23,24]. BR 38 (Oxoid), without catalyst, was used to produce a micro-aerobic atmosphere [25]. Plates were flooded with 2 mL of a 0.5 MacFarland standard. All extra fluid was removed immediately, and the plates were allowed to dry before Etest strips were applied. Plates were incubated for 31 h [17]. Third, an agar dilution test was performed on Wilkinson–Chalgreen agar (Oxoid) at Telelab, Skien, Norway [26]. After subcultivation on chocolate agar (with BR 38, but without catalyst), isolates were diluted in LB broth (Oxoid) to a density of 3 MacFarland standards. Inoculation was performed with the Scan 400 multipoint inoculator (Mast Laboratories, Bootle, UK), which transferred 0.3 μL/spot. Plates were incubated at 37 °C for 72 h in a micro-aerobic atmosphere and read manually.

When colonies were found within the inhibition zone by any of the methods, the isolate was recorded as being highly metronidazole-resistant (MIC > 32 mg/L).

Statistics

Median MIC values before and after storage were compared by use of the Mann–Whitney U-test. The occurrence of treatment failures among isolates that lost or retained their pre-treatment metronidazole resistance (MIC > 8 mg/L) during storage was compared using the chi-square test with Yates' correction. The statistical significance of the change in resistance identified with the Etest with 72 h of incubation was evaluated with McNemar's test for paired observations [27]. The rate of treatment failure among isolates with high MIC values (> 8 mg/L), i.e., the positive predictive value, was calculated for each of the procedures. The significance of the change in predictive value of the Etest was evaluated with the chi-square test for trends [27], using the following ordering: pre-storage Etest with 72 h of incubation, post-storage Etest with 72 h of incubation, and, finally, post-storage Etest with a shorter incubation time. Based on studies of recipient operating characteristic (ROC) curves, the existence of an MIC range of intermediate sensitivity was identified.

RESULTS

The median MIC value was reduced significantly during storage from 5.8 mg/L to 0.8 mg/L (p 0.001). Unstable resistance, i.e., a change in resistance status during storage, was found in 40 (27%) isolates. Thirty isolates lost in-vitro resistance (defined as an MIC > 8 mg/L) during storage, while ten isolates gained resistance (p 0.002). The ability of isolates to retain resistance throughout storage was associated significantly with treatment outcome. Treatment failure was thus significantly more prevalent among isolates with retained resistance than among isolates that lost their resistance ($\chi^2 = 7.2$; p 0.008) (Table 1).

There was a major difference between the two Etest methods with respect to their ability to reproduce results obtained with the agar dilution method (Table 2). The proportion of reproducible results, within both ±1 and ±2 double dilution gradients, was significantly higher with the shorter-incubation Etest method than with the standard Etest ($p < 0.0005$).

Treatment failure, i.e., the positive predictive value of the test, was numerically higher among patients with isolates that were resistant after

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Table 1. Impact of storage: the association between conserved vs. lost in-vitro metronidazole resistance (MIC > 8 mg/L) and the outcome of eradication therapy

	Resistance conserved	Resistance lost
Outcome of therapy		
Failure	13	3
Success	18	29

p 0.008; $n = 63$.

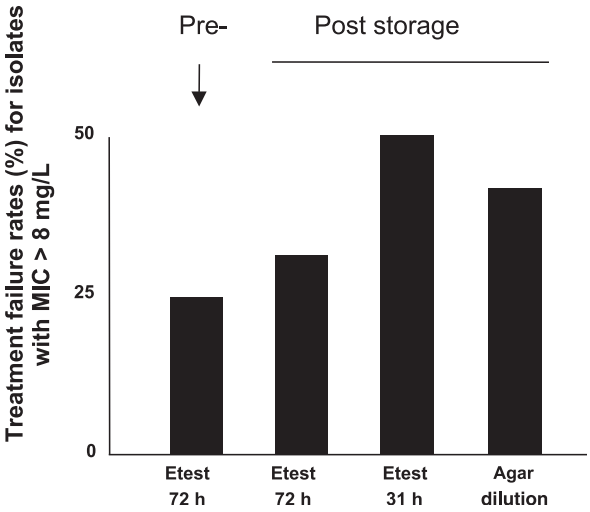

Fig. 1. Predictive value for eradication failure with four different test procedures.

storage compared to before, but this was not significant (35% and 25%, respectively; p 0.43). However, the predictive value was improved significantly with the shorter-incubation Etest (p 0.04) (Fig. 1). Hence, the best prediction of treatment failure (50%) was achieved with the shorter-incubation Etest on post-storage isolates.

In-vitro resistance was defined throughout the study as an MIC > 8 mg/L. According to the ROC curves, the post-storage Etest, the shorter-incubation Etest and the agar dilution method had additional breakpoints at 3, 2 and 2 mg/L, respectively (Fig. 2). MIC values in the interval between these breakpoints and 8 mg/L were associated with treatment failure rates that were intermediate between failure rates at high and low MIC values (Table 3). For the agar dilution method and the modified Etest, treatment failure rates were significantly lower for isolates with MICs ≤ 2 mg/L when compared to those with intermediate sensitivity (MIC>2–8 mg/L) (p 0.005 and 0.007, respectively).

DISCUSSION

In-vitro metronidazole resistance was found to be unstable, as the sensitivity status altered in 27% of isolates during storage at −70 °C. Furthermore, this loss of resistance was selective, as 29 (62%) isolates that were eradicated lost in-vitro resistance, compared to three (19%) treatment-resistant isolates (p 0.008). Stability of in-vitro resistance during storage was thus an important indicator of potential failure with triple-drug therapy.

It is known that the Etest may overestimate metronidazole resistance [16,28]. However, reduced overestimation following storage has not been reported previously, and could be associated with loss of resistance heterogeneity. In this context, resistance heterogeneity occurs when individual bacteria express MIC values that differ from those of the main proportion of the population [29,30]. Such resistance heterogeneity has been found in 33% of isolates [29], and individual bacteria with a four-fold increase in the metronidazole MIC were reported to occur at a rate of up to 5×10^{-5} in one study [31]. As the number of colonies that can be recovered from the frozen state and cultured on agar plates is normally $< 1 \times 10^{3}$ [25], the number of colonies obtained on agar plates may be too small to regularly transmit heterogeneous resistance. Hence, even at a high rate of occurrence, there is

Table 2. Comparison of Etest results after incubation for 31 and 72 h, respectively, with agar dilution results for metronidazole resistance determination with 150 *Helicobacter pylori* isolates

	No. of Etest determinations within indicated number of $\log_2$ dilutions											Agreement (%)	
Duration of incubation	> − 4	− 4	− 3	− 2	− 1	0	1	2	3	4	> 4	± 1 $\log_2$ dilution	± $\log_2$ dilution
31 h		2	6	8	34	71	23	2	1	1	2	85.3%	92%
72 h	1	7	19	31	25	30	13	6	3	5	10	41.3%	70%

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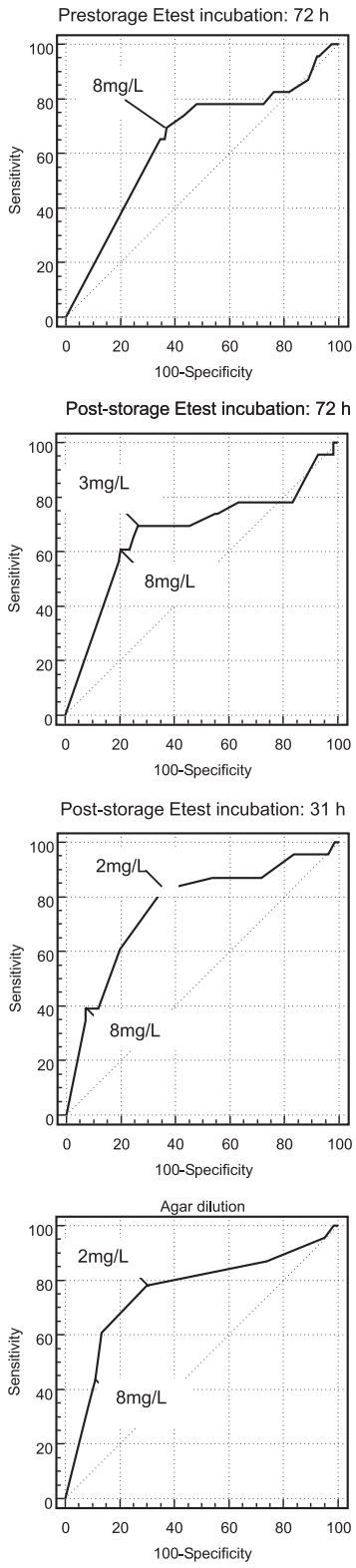

Fig. 2. ROC curves for the studied test procedures. Position of 8 mg/L and the alternative breakpoints are indicated.

a substantial potential for loss of such heterogeneity during storage.

Resistance heterogeneity predicts failure of monotherapy [19,31–33]. However, there is no evidence for an association between the occurrence of a few colonies within the metronidazole inhibition zone and failure of triple-drug therapy, although this has been assumed in a number of studies [4,18,22,34]. It is of note that, although a proper lawn of strong bacterial growth around a distinct inhibition zone is seen after incubation for 31 h under optimal conditions, single colonies within the inhibition zone are not observed at this point. Therefore, heterogeneity, expressed as single colonies growing within the inhibition zone, was not observed with the best-performing method in this study, i.e., the shorter-incubation Etest.

A diversity of agars has been used for resistance testing, in spite of the fear that different chemicals and nutrients may affect the test result [1,4,5,26]. Charcoal media are generally avoided for this reason, but determination of metronidazole resistance is exceptional, as the results of dual metronidazole resistance testing on charcoal and chocolate agars are in full agreement [16]. The only medium tested for resistance determination of *H. pylori* after incubation for 31 h contains charcoal [17]. Wilkinson–Chalgreen's agar has been recommended and is commonly used for resistance testing in the agar dilution method, as in the present study, although the potential of this medium to cause altered metronidazole MIC values has been raised [26].

Several factors may contribute to the problems involved in determination of resistance in *H. pylori*, including chemicals and nutrients found in the different culture atmospheres and media used [4,16,25], and their impact will depend entirely upon the duration of incubation. Interestingly, in this and other studies [4,16], improved reproducibility was associated with a short period of incubation. For most bacterial species, correct and short incubation periods are important for the standardisation of resistance testing procedures [32,33,35–37]. Little has been done to assess the negative effect of prolonged incubation (commonly 72 h) of *H. pylori*, as this is a fastidious species, and it is believed that a shorter incubation time is both impractical and not required [19–21,31–33]. However, the reproducibility of readings is not optimal, even containing major

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Table 3. Comparison of three methods for metronidazole resistance determination by pre-treatment MIC values and treatment outcomes

	Etest								
	Incubation for 72 h			Incubation for 31 h			Agar dilution		
MIC (mg/L)	ER	NE	Failure rate	ER	NE	Failure rate	ER	NE	Failure rate
< 2	86	7	7/100 (7%)	59	3	4/86 (5%)	33	3	5/94 (5%)
2	3	0		23	1		56	2	
3	4	0		20	5	10/46 (22%)	0	0	8/32 (25%)
4	2	1	2/9 (22%)	10	5		21	4	
6	2	1		5	0		0	0	
8	3	0		1	0		3	4	
12	1	0	14/41 (34%)	0	1	9/18 (50%)	0	0	10/24 (42%)
16	1	1		0	0		3	2	
≥ 32	25	13		9	8		11	8	

ER, eradicated; NE, not eradicated.
Failure rates are given for low, intermediate and high MIC values. The higher breakpoint value was defined as 8 mg/L according to common criteria; the lower breakpoint value was defined by ROC curves (Fig. 2).

errors, when incubation for 48–72 h is used with the disk diffusion method [18,21,28]. The reproducibility of results obtained with this method is improved significantly by limiting the incubation time to 31 h [17].

In the present study, the agar dilution method appeared to perform optimally with an incubation time of 72 h (Table 3), and this is in accordance with previous observations [18,28]. It has been more difficult to define the optimal time conditions for metronidazole resistance determination with the Etest [4,16,18,28]. In one study, it was found that incubation for 72 h gave optimal reproducibility of test results when the size and age of inocula were optimised and standardised [38]. It was also observed previously that test reproducibility was significantly improved when the incubation period was reduced from 72 to 48 h [16]. In the present study, there were a number of methodological differences between the two Etest procedures, so interpretations should be made with care, but one possible explanation for the improved reproducibility observed with a shorter incubation time is that 72 h is too long a period of incubation for the Etest (Table 2).

Redox potential variations are known to alter the outcome of in-vitro metronidazole resistance tests. Anaerobic preincubation of culture plates, and incubation at very low oxygen tension, are known to reduce MICs of metronidazole [34]. In the present study, the same method was used to produce the culture atmosphere before and after storage, and anaerobic preincubation was not applied. Therefore, there is no reason to assume that the observed shift in resistance during storage could be attributed to changes in atmospheric conditions.

For *H. pylori*, the gap between failure rates for high and low MIC values has been so small that intermediate resistance rates have not been an issue. In the present study, the difference between failure rates with high and low MIC values before storage was only 17%. With improved procedures for resistance determination, the difference increased, which supports the assumption that intermediate resistance also exists for *H. pylori*. This is in accordance with a previous error rate analysis on post-storage isolates, which found that the occurrence of major errors was reduced when an intermediate category was included in the susceptibility classification scheme [38]. For patients harbouring bacteria with MICs in this area of critical sensitivity, the type of treatment given in addition to metronidazole, as well as therapy length and compliance, may be of particular importance.

In conclusion, metronidazole resistance, as determined with the Etest, reduced significantly during storage. Dual testing by Etest and agar dilution yielded poor reproducibility, but this was improved significantly by modification of the Etest procedure. Alteration of the laboratory conditions led to an improvement in the predictive value of the Etest from 25% to 50%. In addition, rather than appearing at one specified breakpoint value, the risk of treatment failure was found to grow gradually, thus forming a level of intermediate resistance for MIC values within the range 2–8 mg/L.

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