E-Test or Agar Dilution for Metronidazole Susceptibility Testing of *Helicobacter Pylori:* Importance of the Prevalence of Metronidazole Resistance

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Research Article

Keywords: E-test, dilution, metronidazole susceptibility testing, Helicobacter pylori, prevalence of metronidazole resistance, infectious disease

Posted Date: July 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-677597/v1

Version of Record: A version of this preprint was published at Frontiers in Microbiology on March 14th, 2022. See the published version at https://doi.org/10.3389/fmicb.2022.801537.

Abstract

Background: A number of studies have shown that E-test overestimated the presence of Helicobacter pylori (*H. pylori*) resistance compared to agar dilution.

Objective: The purpose of this study was to explore whether E-test could be an alternative for agar dilution to detect the metronidazole susceptibility of *H. pylori*.

Method: E-test and agar dilution were used to assess susceptibility of *H. pylori* to metronidazole, clarithromycin and levofloxacin in 281 clinical isolates obtained from China where resistance was high. Cohen kappa analysis, McNemar test, essential and categorical agreement analysis were performed for these two methods.

Results: Overall, the result of E-test showed similar prevalence of resistance rate to all antibiotics compared with agar dilution. The essential agreement (EA) of E-test method and agar dilution in the evaluation susceptibility of *H. pylori* to clarithromycin and levofloxacin were moderate, with 89.0% and 79.7% respectively, but only 45.9% for metronidazole. Results showed categorical agreement (CA) between E-test and agar dilution were 100% for both clarithromycin and levofloxacin. As for metronidazole, the CA was 98.7%, no major error was identified, and rate of very major error was 1.8%.

Conclusion: E-test can be an alternative method to detect the metronidazole susceptibility of *H. pylori* in regions where high-level resistance is common.

Introduction

All successful infectious disease therapy is directly or indirectly susceptibility based. For regions where resistance is common, susceptibility-guided tailored treatment is typically required to achieve high cure rates. This is especially true with *Helicobacter pylori* (*H. pylori*) infections (1, 2). The worldwide increase in antibiotic resistant *H. pylori* has resulted in poor cure rates has resulted in relative poor cure rates with empiric therapy (3) However, even in regions with only modest levels of resistance antibiotic susceptibility testing prior to *H. pylori* treatment has been shown to increase the eradication rate compared to empirical treatment (4, 5). Metronidazole, clarithromycin and levofloxacin are among the most commonly used antibiotics in clinical treatment of *H. pylori* and the need to assess bacterial antibiotic susceptibility patterns before treatment has received increasing attention (6).

At present, there are three methods widely used for traditional microbial susceptibility testing, including agar dilution, E-test and disk diffusion. Agar dilution is believed to be the gold standard for *H. pylori* susceptibility testing, although this method is time-consuming and laborious (7).

E-test as an alternative method combines the principle of dilution and diffusion methods by placing a single strip containing an increased antibiotic concentration on the surface of agar medium and reading the intersection of bacterial growth zone and inhibition zone to determine the minimum inhibitory

concentration (MIC). At present, due to the convenience of the implementation of E-test, it is widely used in the clinical microbiological laboratory. However, some studies reported that it cannot be used for evaluating metronidazole susceptibility of *H. pylori* because of its inconsistent with agar dilution (8–10).

The purpose of this study was to perform the susceptibility tests by agar dilution method and E-test method to verify whether E-test could be an alternative way for detecting antibiotic susceptibility especially metronidazole.

Method

Study population

From July 2019 to December 2020, a total of 281 *H. pylori* isolates were obtained from patients who underwent endoscopy at Renji Hospital, School of Medicine, Shanghai Jiao Tong University, China.

H. pylori strains

During endoscopy, two biopsies were collected from antrum of stomach and cultured on BHI agar medium (Oxoid, Stoke, Basin, UK) containing 5% defibrinated sheep blood, 5mg/L trimethoprim, 10mg/L vancomycin, 20U/L polymyxin B and 10mg/L nalidixic acid under microaerophilic conditions (85% N2, 10% CO2, and 5% O2) at 37°C. Strains were confirmed according to Gram-negative, positive urease, oxidase, and catalase reaction, and its morphology was spiral or curved. The strains were collected in BHI broth with glycerol at 4°C and stored at -80°C. Before susceptibility test, bacteria were resuscitated and subcultured on BHI agar medium (Oxoid, Stoke, Basin, UK). ATCC43504 was used as quality control with MIC from 64 to 256 mg/L for metronidazole, from 0.016 to 0.125 mg/L for clarithromycin and from 0.032 to 0.125 mg/L for levofloxacin.

Agar dilution method

Agar dilution was performed based on the protocol represented by Clinical Laboratory Standards Institute. In brief, metronidazole, clarithromycin and levofloxacin were dissolved in dimethyl sulfoxide. The drug was added to agar medium to produce continuous twofold dilutions with concentration ranging from 0.032 to 256 mg/L for metronidazole, from 0.032 to 256 mg/L for clarithromycin and 0.032 to 32 mg/L for levofloxacin. Bacteria suspensions (0.5 McFarland) were prepared with sterile saline. Adjusted inoculum (2-5 ul) was then delivered to each plate by an inoculator (Sakuma Seisaku, Tokyo, Japan). After 3 days of incubating the plates in a microaerobic environment, the lowest concentration of a drug that prevented the visible growth of a bacterium (excluding single colony or multiple tiny colonies) was defined as minimum inhibitory concentration.

E-test method

One hundred microliter of *H. pylori* suspension (3 McFarland) was inoculated onto the agar plate without antibiotics, after standing for a few minutes, E-test strip was then placed on the center of the agar plate. The plates were then incubating under microaerobic for 72 hours. The endpoint of E-test was read as the interception of graded strips with the elliptical zone of inhibition. If the end point were not within the two-fold dilution range, it would be rounded up to the next highest two-fold dilution for MIC assessment.

Discrepancy analysis

isolates with inconsistent interpretation of susceptibility after initial testing by agar dilution and E-test were further tested four additional times. The most frequent results of agar dilution were considered as the MIC reference value of isolates and the most frequent results of E-test were used as the final MIC value which isolates tested by it.

Statistical analysis

Isolates were classified as resistance based on the breakpoint for each drug established by EUCAST (MIC ≥8mg/L for metronidazole, MIC ≥0.5mg/L for clarithromycin, MIC ≥1mg/L for levofloxacin). The disagreement of two tests was performed by Mc-Nemars test and Cohen's kappa analysis. Essential agreement (EA) was determined by calculating the percentage of isolates whose MIC produced by E-test were within ±1 doubling dilution that produced by agar dilution. Categorical agreement (CA) was determined by calculating the percentage of isolates occupied same susceptibility category tested by agar dilution and E-test. very major error (VME) was defined as isolates being resistance e by agar dilution and susceptible by E-test. Major error (ME) was defined as isolate being susceptible by agar dilution and resistance by E-test.

Results

Clinical characteristics

Table 1 showed the clinical information of strains. The median age of the hosts was 46 years (16-73), with 101 (35.9%) males and 180 (64.1%) females.

Agreement of susceptibility results

Table 2 showed resistance rates of the isolated H. pylori to metronidazole, clarithromycin and levofloxacin when assessed in terms a binary outcome (susceptible/resistant) were 71.5%, 88.6% and 80.4% respectively tested by agar dilution method. As for clarithromycin and levofloxacin, E-test showed same susceptibility pattern (McNemar test, P=1.00). For metronidazole, E-test showed slight difference (McNemar test, P=0.062) when compared with agar dilution method. Cohen's kappa analysis was further performed to determine the consistency and accuracy of E-test (Table 2) as agar dilution method was used as reference in this study. The kappa values indicated a substantial agreement for metronidazole (0.96; 95%CI: 0.92-1.00), clarithromycin (1.00; 95%CI: 1.00-1.00) and levofloxacin (1.00; 95%CI: 1.00-1.00) between E-test and agar dilution method.

Discrepancies analysis was shown in supplementary table 1.

Essential and categorical agreement

Figure 1 to 3 showed the distribution of MIC for agar dilution and E-test. EA for these two methods (Table 3) indicated a moderate correlation for testing susceptibility of clarithromycin (84%) and Levofloxacin (79.7%). However, for metronidazole, EA between these two methods was low (45.9%). On the contrary,

CA was high (>98%) for all three antibiotics susceptibility test comparison without VME, only 1.8% of the strains tested for metronidazole were observed ME between E-test and agar dilution.

Discussion

Accurate knowledge of the resistance pattern can effectively improve the success rate of treatment, avoid use of unnecessary antibiotics and improve the compliance (11, 12). Agar dilution is regarded as a gold standard for bacterial susceptibility test, although it is cumbersome and time consuming. E-test is often used as a substitution in clinical practice because of its convenience in detecting single or few isolates. However, the results with E-test are more difficult to interpret (13).

Previous studies have compared the efficacy of above two methods and found that the EA and CA of E-test and agar dilution were both high agreement for amoxicillin, clarithromycin and levofloxacin (14-16). However, for metronidazole, a class of nitroimidazole compounds, the EA of E-test and agar dilutions is generally considered to be low which often less than 60% in previous studies, and the CA remains controversial as some laboratories have reported that about 5% to 32% of bacteria existed a change in the pattern of metronidazole resistance between these two methods (9, 10, 13, 14, 16-19). Understanding the drug susceptibility pattern of metronidazole is of great significance for the treatment of *H. pylori*. Although it has been reported that metronidazole resistance in vitro does not necessarily indicate the failure of treatment. In practice, high dose of metronidazole can overcome drug resistance, but its side effects are large and patient compliance is poor. Besides, in our past study, we found that the metronidazole-containing therapy had the highest eradication rate when administered under the guidance of the drug susceptibility (20, 21).

Our results showed that the EA between E-test and agar dilution method for metronidazole susceptibility test was poor (45.9%), but the categorical agreement was high (98.2%). For 5 strains with changes in susceptibility pattern, all were metronidazole-susceptible strains identified by agar dilution, which were regarded as resistant by E-test. However, different from some previous studies (9, 19, 22), VME were not found in our work for testing metronidazole. At the same time, unlike clarithromycin and levofloxacin, the two methods showed obvious discrepancies in the MICs distribution for metronidazole. The values obtained by E-test were often 2-5 times higher than those by agar dilution, especially when MIC is higher than the clinical break point. This is consistent with some previous reports (13, 23, 24). Thus, the percentage of cases were E-test will overestimate resistance depends on the relative proportion with infections deemed susceptible by agar dilution.

Four replicates were performed on these five strains with MEs, and the results can be divided into two categories for discussion. For the first category, after the MIC of bacteria in this category was identified by agar dilution method, its value was around the break point of metronidazole. As mentioned earlier, due to the value of E-test were usually higher than that of agar dilution method, it may be difficult to determine accurately for such bacteria with a MIC near the break point. For the second category, these bacteria might have mixed infection. Due to the small amounts of bacteria inoculated by agar dilution method,

only monoclonal or tiny clones existed on the culture plate when determining the MIC, which was still considered as sensitive strain according to the protocol. However, in the E-test, the inoculation amounts of bacteria was larger than agar dilution, and there were still many scattered clones in the inhibition zone on the culture plate, thus we determined that it was drug-resistant bacteria interpretated by E-test, which leading to the discordance with agar dilution.

The MIC discrepancy between agar dilution and E-test is related in part to the details of placing the strips on the plates and the experience in interpretation of the results (24, 25). In the procedure of preparing agar dilution medium, variables including drug degradation, drug weighing error, heterogeneous mixing of drug and medium may influence the antibiotic activities. As for E-test, ambient temperature, humidity, and depth of medium which may affect the diffusion efficiency of drug on medium, may have effect on the results. In addition, cryopreservation, continuous subculture of bacteria before experiment and the motility of bacteria may also interfere the experimental results. Meanwhile, metronidazole itself is also affected by oxygen concentration. Takemoto *et al* reported that some bacteria whose MICs is overestimated by E-test had a MICs concordance to the agar dilution after 24 hours of pre-incubation in an anaerobic environment, however, the explanation for this phenomenon remains unexplained (25, 26). Considering that E-test has only a few discordant with agar dilution in determining the susceptibility pattern of the metronidazole, it is suggested that when E-test is used for metronidazole susceptibility testing, the prevalence of resistance strains may be overestimated, leading to a decrease in the effective use of metronidazole as well as an overestimation of the ability of a regimen to overcome metronidazole resistance. However, this is less of an issue in areas where metronidazole resistance is widespread and the drug would be commonly used for susceptible strains.

Conclusion

In general, although E-test may overestimate the susceptibility of *H. pylori* to metronidazole, this is only clinically important in regions where metronidazole resistance remains low and the error might eliminate a potentially useful antibiotic or suggest that the therapy was more useful with resistant strains that was the case(27, 28).

Abbreviations

H. pylori Helicobacter pylori

EA Essential agreement

CA Categorical agreement

VME Very major error

ME Major error

MIC Minimum inhibitory concentration

Declarations

Ethics approval and consent to participate

This study conformed to the Ethical Guidelines of the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects. The study was approved by The Ethics Committee of The Renji Hospital; Shanghai Jiaotong University School of Medicine.

competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the grant from the Shanghai Science and Technology Committee (grant number 19411970300).

Author contributions

Hong Lu: study concept and design. Jinnan Chen, Yu Huang and Zhaohui Ding: acquisition of data. Jinnan Chen and Yu Huang: analysis and interpretation of data. Jinnan Chen and Zhaohui Ding: drafting of the manuscript. Hong Lu: critical revision of the manuscript for important and obtained funding. Jinnan Chen: statistical analysis.

Availability of data and materials

The dataset for this study is available from the corresponding author, if reasonably requested.

Consent for publication

Not applicable.

Acknowledgements

Not applicable.

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Tables

Due to technical limitations, table 1-3 is only available as a download in the Supplemental Files section.

Figures

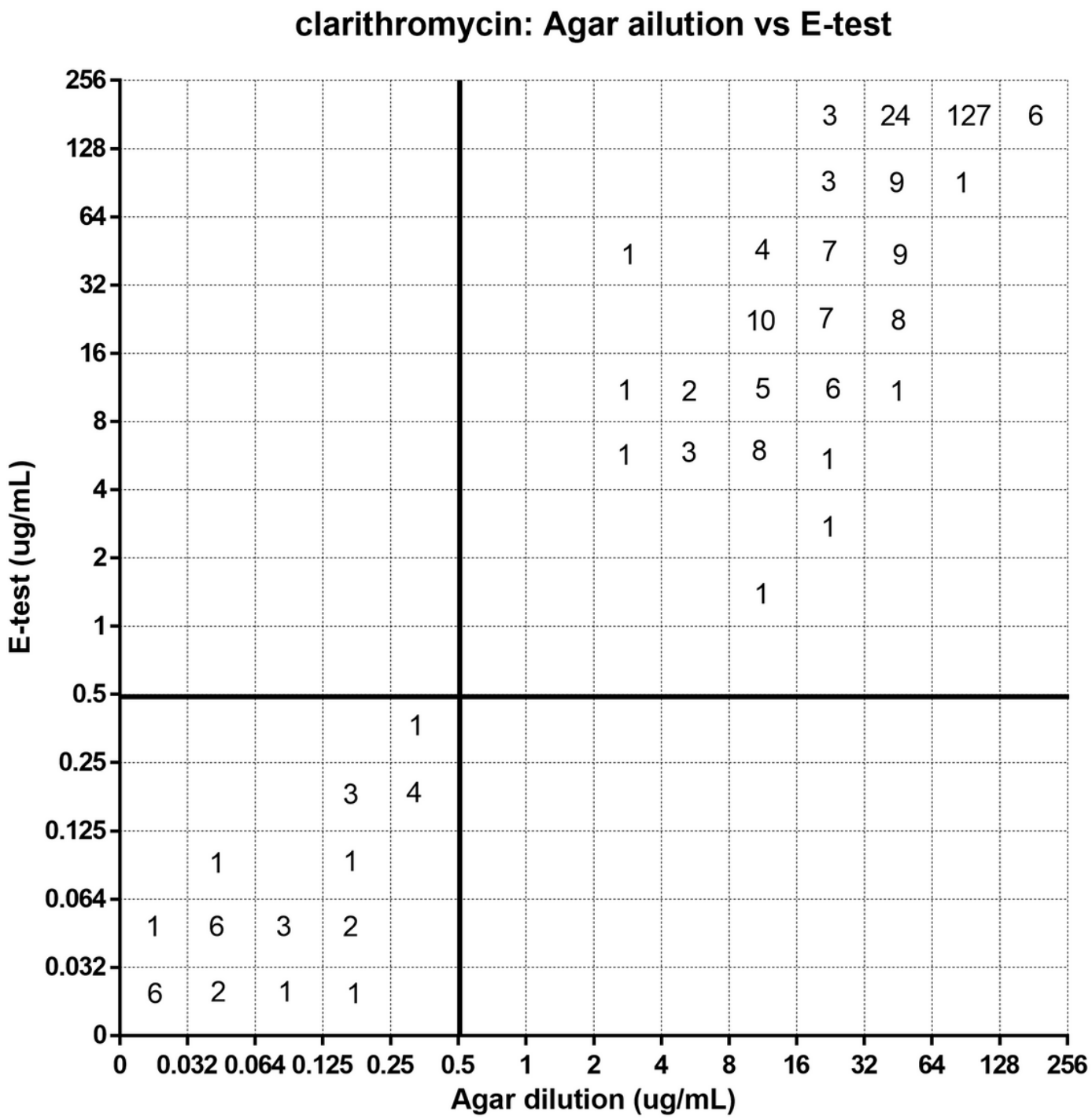

Figure 1

error-rate bounded analysis of Clarithromycin MICs tested by Agar dilution and E-test

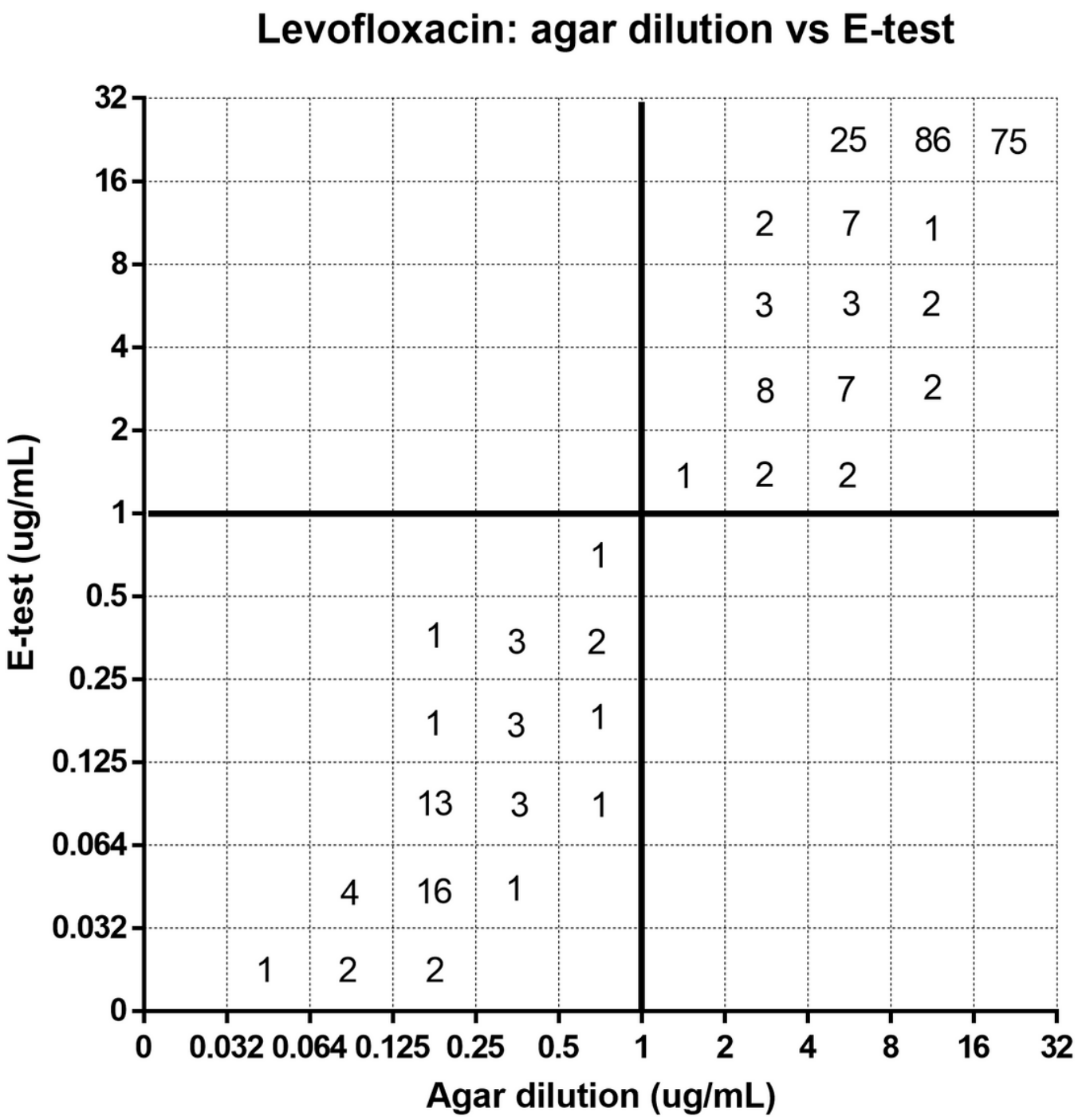

Figure 2

error-rate bounded analysis of Levofloxacin MICs tested by Agar dilution and E-test

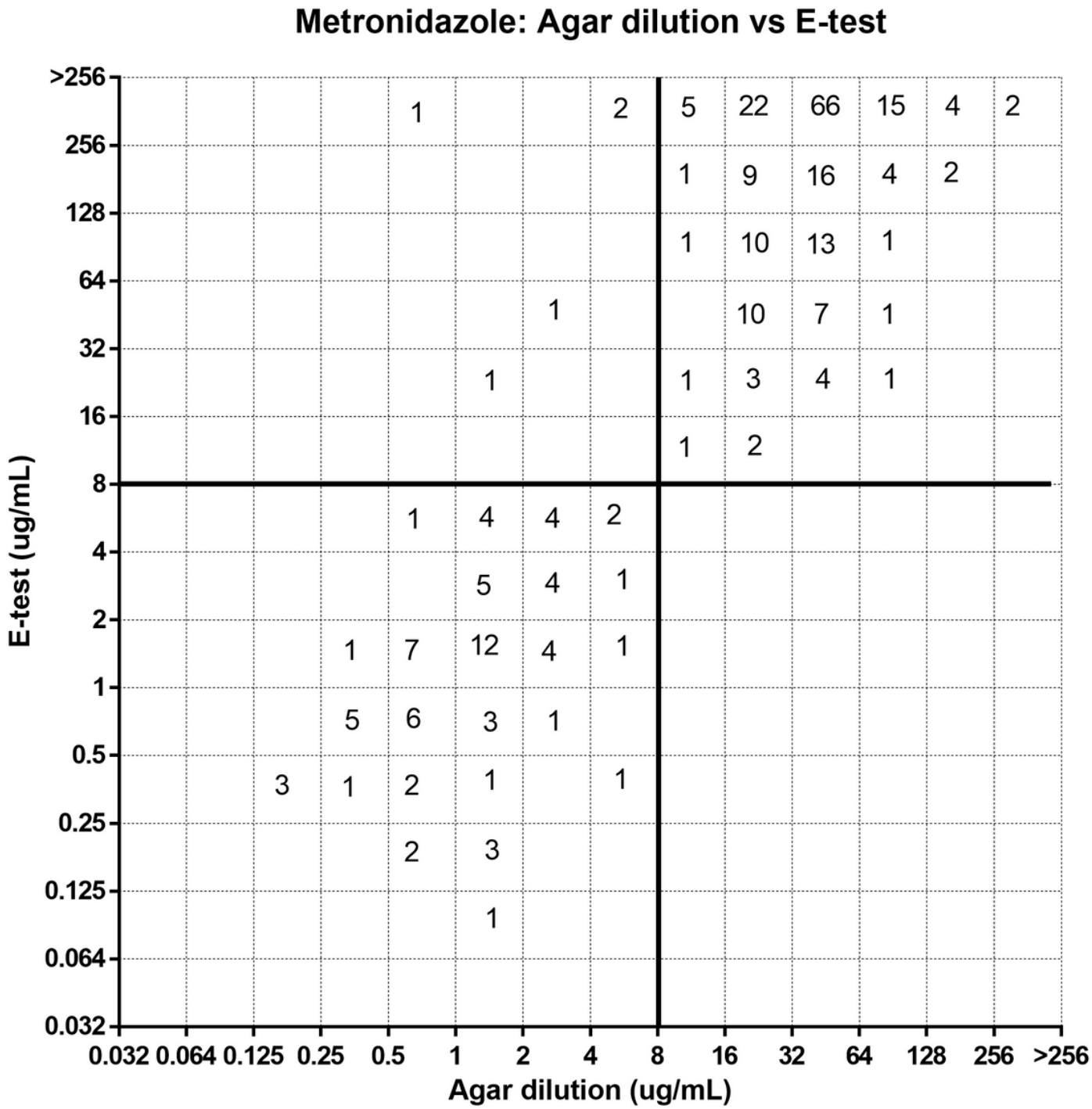

Figure 3

error-rate bounded analysis of Metronidazole MICs tested by Agar dilution and E-test

Supplementary Files

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- table1.pdf
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