Determination of clearance of *Helicobacter pylori* through bacterial density, Nairobi, Kenya.

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SUMMARY

Background: Eradication of *Helicobacter pylori* (*H. pylori)* is a challenge to physicians and gastroenterologists worldwide and has led to change of drug regimens and their durations to ensure cure. *H. pylori* density has been considered as a factor which may influence eradication failure.

Objective: To establish whether bacterial density found during diagnosis is a determinant of clearance of *H. pylori* after treatment.

Design: A retrospective study was carried out to establish clearance of *H. pylori* through stool antigen testing on those patients who were positive for *H. pylori* on rapid urease test, and had bacterial density reported on histology.

Setting: Centre for Clinical Research (CCR), Kenya Medical Research Institute (KEMRI).

Methods: Biopsy samples taken on endoscopy were used for rapid urease testing and histology. Giemsa stain was used on histology to identify *H. pylori*. At the end of triple therapy treatment, stool antigen testing was done using the enzyme linked immunosorbent assay (ELISA) method to detect the presence or absence of *H. pylori*.

Results: 150 patients were positive for *H. pylori* on rapid urease testing. 42/150 (28%) patients had results for histology after completion of the study. Those who reported no *H. pylori* on histology were 24.4%, slight *H. pylori* 41.5%, moderate *H. pylori* 14.6%, numerous *H. pylori* 19.5%. This was compared to clearance of *H. pylori* on stool antigen testing after treatment. Those with no *H. pylori* on histology had a 70% cure rate after treatment; mild *H. pylori* - a 47% cure rate; moderate *H. pylori* on - 100% cure rate and those with numerous *H. pylori* - a 50% cure rate. There was no statistical significance noted in all categories ($P>0.05$). There was no relation of patients positive for *H. pylori* with bacterial density noted on histology and clearance of *H. pylori* at the end of treatment.

Conclusion: *H. pylori* bacterial density cannot be used as an indication for clearance of *H. pylori* after treatment. One may need a larger sample or other diagnostic test to determine bacterial density and clearance of *H. pylori*.

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Introduction

Helicobacter pylori (*H. pylori*) is major cause of gastritis and peptic ulcers worldwide. *H. pylori* is a sheathed monoflagellated Gram-negative rod. It is microaerophilic, catalase negative and produces urease that enables it to survive in the hostile acidic environment of the stomach. Transmission in humans is usually oral-to-oral, or fecal-to-oral route [1]. The organism is non-invasive but stimulates chronic gastritis by provoking a local inflammatory response in the underlying stomach epithelium due to release of cytokines e.g. *VacA, cagA, BabA2*, phospholipases and porins.

In most people, *H. pylori* causes antral gastritis associated with depletion of somatostatin from D cells in the stomach and gastrin release from G cells stimulating acid production by parietal cells. This in some people leads to duodenal ulceration [2].

Patients present with recurrent epigastric pain related to food and episodic in occurrence. Other symptoms may include burping, bloating, nausea and vomiting. Complications of *H. pylori* include progression to gastric cancer [3, 4]. *H. pylori* has also been associated with iron deficiency anemia and idiopathic thrombocytopenic purpura [5].

Diagnostic tests for *H. pylori* can be divided into non-invasive and invasive test or as non-endoscopic respectively. Non-endoscopic methods include blood antibody detection tests, urea breath tests (UBT) and stool antigen tests. UBT and stool antigen tests denote active *H. pylori* infection unlike blood antibody tests.

Stool antigen testing has emerged as a rapid non-endoscopic method of *H. pylori* detection [6]. In the stool antigen test, simple sandwich enzyme linked immunosorbent assay (ELISA) is used to detect presence of *H. pylori* antigen shed in faeces. Studies have reported sensitivities and specificities similar to those of UBT (>90%). It is highly sensitive and specific in detection of *H. pylori* in patients with dyspepsia and in those who have completed and *H. pylori* eradication regimen [6]. A study showed stool antigen detection performed 4 weeks after completion of an *H. pylori* eradication regimen had a sensitivity of 90%, specificity 95%, negative value 98% [7]. False positives however have been noted especially with the monoclonal stool antigen test [8]. Sensitivity of stool antigen reduces to 69% after two to three days at room temperature [5].

Endoscopic methods used in detecting *H. pylori* include histology, urease activity or culture. Histology was noted to have a sensitivity and specificity of 95-98% [9, 10]. However the process takes several days and false negatives may occur occasionally.

The rapid urease test assesses the biopsy sample for urease activity, and it is highly sensitive and specific with rates of above 90% and 99-100% respectively[11]. Rapid urease tests (RUT) are cheap and quick tests in terms of getting results. However, RUTs can give false negative results especially in patients receiving PPIs or high dose H2 receptor antagonists which decrease *H. pylori* density.

Microbiologic culture is the theoretical gold standard and it defines antibiotic sensitivity [12]. It is however slow and laborious and needs specific and expensive equipment. Sensitivity is about 80-90%, lower than that of histological testing of 90-95% [13]. Performance of culture is useful for the determination of antibiotic resistance, especially in patients who continue to be

positive for *H. pylori* after an initial treatment regimen [6].

Treatment of *H. pylori* has been a challenge from the time it was discovered until present. *H. pylori* eradication is however the cornerstone of therapy for peptic ulcers especially in symptomatic patients. Options for therapy for *H. pylori* include a PPI and two antibiotics [14]. PPIs include esomeprazole, lansoprazole, pantoprazole etc. Antibiotics most commonly used include amoxicillin, metronidazole, or clarithromycin used in combination. A stomach protecting agent e.g. bismuth or misoprostol may be added to this combination. Compliance, side effects and metronidazole resistance influence the success of therapy [2].

Choice of alternative regimens should be based on initial treatment regimen, especially with replacement of first-line antibiotic with a second-line antibiotic, or antibiotic which has been shown to be sensitive on culture [15].

Successful eradication should be confirmed with UBT or an endoscopy-based test if endoscopy is clinically indicated, for example, to confirm ulcer healing after treatment. Stool antigen test is the alternative if UBT is not available [14]. Further attempts at eradication are indicated in patients with confirmed peptic ulcers. If the patient was being treated for uninvestigated dyspepsia with unlikelihood of ulcer, further eradication therapy is unclear [16].

For those who are still colonized after two treatments, the choice lies between a third attempt with quadruple therapy (bismuth, PPI and two antibiotics) or long-term maintenance therapy with acid suppression. Continuous maintenance treatment should not be necessary after successful *H. pylori* eradication. For the minority who require maintenance treatment the lowest effective dose should be used [2].

Resistance to antibiotics and poor adherence is important as it leads to treatment failure [17,18]. Clarithromycin resistance is not as common as metronidazole. Primary amoxicillin resistance is very rare [18]. Successful eradication of *H. pylori* is affected more by the presence of resistance to clarithromycin than to metronidazole [19]. In studies, dual therapy with the combination of PPI and clarithromycin showed higher resistance than with triple therapy using PPI and clarithromycin and amoxicillin, suggesting these regimens containing amoxicillin may prevent selection of secondary clarithromycin resistance [20]. The prevalence of clarithromycin resistant *H. pylori* is low but appears to be increasing. Point mutations in the 23s rRNA gene are responsible for the resistance. Because of this eradication rates may be reduced and treatment failure rates may rise [21].

Host or bacterial factors affect *H. pylori* density in the gastric mucosa e .g age, smoking, alcohol, degree of gastric inflammation, use of PPIs [22, 23, 24]. Some studies have shown *H. pylori* density being a factor in eradication failure while others have shown the contrary [25, 26]. Determining clearance of *H. pylori* is therefore important after treatment.

Proton pump inhibitors are known to reduce *H. pylori* bacterial density through a direct anti-*H. pylori* effect [27, 28]. Bacterial density has been measured in association with certain diagnostic tests for example UBT [29], to test their effectiveness but not to indicate clearance post-treatment. *H. pylori* has been noted to survive in the presence of high levels of antibody and this is poorly understood in its pathogenesis. It is also

remarkable that individuals cured of infection by antibiotic therapy after decades of colonization are susceptible to re-infection, although at a slightly lower rate [30].

Materials and Methods

Setting: The study was conducted at the endoscopy centre at the Centre for Clinical Research-KEMRI, Nairobi, Kenya. Nairobi is the referral city for most patients who are sent for endoscopy: it also has a high population density.

Patients referred to the endoscopy centre, were seen by the gastroenterologist who took 4 gastric biopsies – 2 from the antrum and 2 from the body of the stomach, for histology and rapid urease testing. Those for histology were kept in 10% formal saline to fix the specimens; the other 2 were used for rapid urease testing.

H. pylori treatment that is triple therapy with esomeprazole, amoxicillin and clarithromycin was given for a minimum of one week. Patients were asked to come after 4 weeks of treatment at the appointed time and asked to give a stool sample for stool antigen testing using the ELISA method, which was transported immediately to the immunology lab – Kenyatta National Hospital.

Rapid Urease testing

Biopsy specimens were tested for urease production. The rapid urease test kit (Esokit HP Test, Cambridge Life Sciences) consisted of a twin well cartridge, containing urea, phenol red and buffer salts in tablet form and an ampule of buffer. The cartridge lid was opened and each well was filled to a marked line with buffer. The lid was then closed and the tablet dissolved by gentle shaking. The biopsies taken at endoscopy were put in the wells. The lid was closed and the kit labeled with the patient's details – name, age, sex, study number and date.

If the urease enzyme of *H. pylori* was present in biopsy specimens, the rise in pH associated with the hydrolysis of urea caused a color change from yellow to pink/red. The color change usually occurred within 30 min to 24 h from insertion of biopsy specimen depending on *H. pylori* bacteria density. The kit was stored at room temperatures (20 –22°C) for 24 to 48 h after which the color changed and the biopsies were read and discarded. Patients were asked to come the following day for rapid urease results and allocation of treatment.

Histology procedure of biopsy specimens

Biopsy specimens were fixed in 10% formal saline. These specimens were sent to an experienced histopathologist unaware of the patients *H. pylori* status, to determine histopathological morphology and presence of *H. pylori*. To do this, specimens were stained separately with Haematoxylin-eosin stain to identify tissues, Giemsa stain to look for comma or S-shaped bacilli organisms which would indicate a positive result for *H. pylori* and observed under a microscope under high magnification. Blue rods were expected to be seen if *H. pylori* was present [31]. The version of the visual analogue scale in the updated Sydney system was used to grade the density of *H. pylori* (4 grades: normal, no bacteria; mild, focal few bacteria; moderate, more bacteria in several areas; and marked, abundance of bacteria in most glands) [32]. If the density varied, the highest grade of density in the specimens was selected.

Stool antigen testing procedure

Stool samples were collected in plastic polypot containers, carried in a cooler if necessary, transported to the lab and stored at −20°C to −80°C, until tested. Those with watery stools were excluded. The monoclonal stool antigen test (Meridian Bioscience – Premier Platinum HpSA PLUS) uses the enzyme linked immunosorbent assay technique (ELISA) to detect *H. pylori* antigen. This test involved the following: - A mixture of murine monoclonal antibodies specific for *H. pylori* was already fixed to the microwells of a microtitration plate which came with the stool antigen kit. 100 uL of diluted stool was placed in microwells. Soluble microbial antigen (in stool) bound to this antibody. Washing was done to remove excess stool antigen. Antibody conjugated to an enzyme was added (a mixture of murine monoclonal antibodies specific for *H. pylori* conjugated to horseradish peroxidase). Conjugated antibody bound to the antibody-antigen complex. Washing was done to remove excess conjugated antigen. A chromogenic substrate joined to an indicator (Premier substrate solution - buffered solution containing urea peroxide and tetramethylbenzidine) was added. The enzyme in the conjugated antibody hydrolyzed the substrate, producing a color reaction. Color was read visually and/or spectrophotometrically. Positive results on visual reading showed a definite yellow color, and spectrophotometrically was ≥0.100 at dual wavelength. Negative results on visual reading showed a colorless to faint yellow color, and spectrophotometrically was < 0.100 at dual wavelength. This was done using a built-in control, which came with the kit, for quality control.

The infection was considered to be successfully eradicated if results were negative. Those who were positive after treatment were given treatment for another week and stool antigen test was repeated after 4 weeks.

Ethical Considerations

The study was performed according to good clinical practices, good laboratory practices and followed the Declaration of Helsinki. Permission to carry out the study was obtained from the graduate school - JKUAT, KEMRI Scientific and Ethics Review Committees. Patients were enrolled into the study only after voluntary informed consent. Subject confidentiality was kept and data coded and kept safely. The study did not involve use of vertebrate animals. Laboratory tests were done at the Immunology lab at Kenyatta National Hospital.

Data analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) statistical package 11. Analysis of data was done using Fishers exact test and chi-square test. The p value of < 0.05 was considered significant. The data was presented in form of tables.

Results

Out of 112 patients who completed follow up, 41 had complete results of histology and stool antigen testing after treatment. Of these 23/41(56.1%) were female and 18/41(43.9%) were male, with a mean age of 31 years.

Results of the number of people positive or negative for *H. pylori* on histology and stool antigen testing were as presented in the table below. Histology results were classified in the following categories: Negative for *H. pylori*=0, Mild *H. pylori*=+, Moderate *H. pylori*=++, Numerous *H. pylori*=+++.

Table 1: Stool and Histology results

Variables	n=41	%
Stool interpretation		
Positive	16	39.0
Negative	25	61.0
Histology results		
P0	10	24.4
P+	17	41.5
P++	6	14.6
P+++	8	19.5

Stool antigen test results were then compared to results found on histology and the results were as shown in Table 2.

Table 2: Stool interpretation in relation to histology results

	Positive (n=16)		Negative (n=25)			95% CI		
Variables	N	%	N	%	OR	Lower	Upper	p value
Histology results								
P0	3	30.0	7	70.0	Reference			
P+	9	52.9	8	47.1	2.63	0.50	13.73	0.253
P++	0	0.0	6	100.0	UD	UD	UD	0.999
P+++	4	50.0	4	50.0	2.33	0.34	16.18	0.391

UD–Undefined

There was no significant difference between histology and stool antigen testing of *H. pylori.*

Limitations

The study population was small. The study did not include other factors contributing to eradication failure e. g. smoking, and alcohol. Bacterial culture and drug sensitivity testing to assess antibiotic resistance was not performed. Therefore we could not demonstrate the influence of drug resistance on eradication therapy by our results. Culture and susceptibility testing of *H. pylori* are time-consuming and expensive and therefore rarely performed in most developing countries unless for purposes of research.

Discussion

H. pylori is a known etiological factor in gastrointestinal disease. It causes gastric inflammation, ulceration and cancer through different mechanisms, including the production of virulence factors by the bacteria itself, host inflammatory responses and associated environmental factors. [33, 34, 35].

H. pylori management involves diagnosis, treatment and confirmation of cure. Eradication *of H. pylori* is important in the reduction of gastritis and duodenal ulcers. A PPI and two antibiotics (triple therapy) is still first line in the treatment of *H. pylori* [14]. The rapid urease test and histology are able to identify *H. pylori* both quantitatively and qualitatively. In our study we used histology to define bacterial density quantitatively using the Updated Sydney system of grading of *H. pylori*. *H. pylori* infection in the stomach may be patchy but studies have shown more *H. pylori* to be found in the antrum than in the corpus. This may also be influenced by type of gastritis. Biopsy specimens for histology were taken from both the antrum and corpus in our study [36, 37].

Stool antigen testing is recommended in diagnosis of *H. pylori* post-treatment, one of the reasons being it is easy to use and non-invasive compared to doing repeat endoscopy [5]. Its superior efficacy in detection of *H. pylori* pretreatment is established with sensitivity of 93% [38]. Studies supporting use of stool antigen testing post-treatment are several with one showing a sensitivity of 95.6 % compared to 94.7% UBT sensitivity [39, 7]. In another study however *H. pylori* showed low validity post treatment with an overall sensitivity of 73% [38]. According to the Maastricht guidelines, though stool antigen testing is less accurate than UBT, it is still recommended post-treatment where UBT is unavailable, and good to use when doing multiple tests. It should be done at least four weeks after treatment. [5].

Bacterial density has been proposed as a factor that may affect eradication failure of *H. pylori* treatment. In some studies eradication failure has been related with *H. pylori* density and age, with both corpus and total *H. pylori* density being negative predictive factors for the rate of *H. pylori* eradication [25]. Association of pretreatment *H. pylori* density with the eradication rate and ulcer healing rate was found to be significant in one study [40]. Increased bacterial density especially in the antral area lowers somatostatin, leading to increased levels of gastrin and acid production leading to duodenal ulceration [37]. Several reports reveal that patients with higher intragastric *H. pylori* load had reduced eradication rates. This association was found in both bismuth and PPI-based triple therapies [41- 44]. Patient compliance and antibiotic resistance are regarded as key factors affecting the outcome of treatment [41, 44]. We therefore aimed to establish if *H. pylori* density would be a factor predicting eradication of treatment in our setting, which would be used as a basis of prolonging duration

of treatment or giving better regimens to ensure effective eradication. [41].

The study showed no association between eradication of *H. pylori* and bacterial density as has been noted in some studies [26]. We only studied *H. pylori* bacterial density as a factor which may predict eradication failure, however many other factors may influence eradication failure e.g. smoking, alcohol, and antibiotic resistance. This may have had a bearing on our results. In terms of antibiotic resistance, *H. pylori* may contribute in its intracellular settling so that it needs a long duration of treatment as in the case of brucellosis. Also the intracellular location of *H. pylori* could contribute to its difficulty in eradication. [45, 46]. Duration of antibiotic therapy for those recruited in this study was a minimum of two weeks, with no statistical significance in those given triple therapy for one week or two weeks.

Conclusion

In conclusion, our findings did not show any significant association between bacterial density of *H. pylori* in the stomach and success of eradication therapy. Further studies need to be done for comparison with a larger number of subjects and which would compare rapid urease testing and histology in terms of *H. pylori* quantities and type of gastritis to predict eradication failure.

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