Pre-formed urease activity of *Helicobacter pylori* as determined by a viable cell count technique—clinical implications

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Summary. The pre-formed urease activity of three NCTC reference strains and five clinical isolates of *Helicobacter pylori* was determined at room temperature (21°C) and 37°C by a viable cell count technique with a conventional urea slope test (Christensen's agar) as well as the commercial CLO-test. The urease activity of two gastroduodenal commensals, *Proteus mirabilis* and *Klebsiella pneumoniae*, was also tested. *H. pylori* strains produced positive reactions with viable cell counts of 10^6 – 10^8 cfu within 30 min and with counts of 10^3 – 10^6 cfu within 2 h. For some strains, smaller numbers of organisms were needed with the CLO-test than with the conventional test, and incubation of the CLO-test strips at 37°C slightly decreased the number of organisms required for positive results. *P. mirabilis* produced a positive result on urea slopes with an initial inoculum of 10^7 – 10^8 cfu at 2 h, but no positive reaction occurred for *K. pneumoniae* at 12 h, even with an initial inoculum of 10^{11} cfu. However, both *P. mirabilis* and *K. pneumoniae* gave a positive result after incubation for 24 h with initial inocula of $< 10^1$ cfu and 10^3 – 10^4 cfu respectively. Incubation at 37°C significantly reduced the inoculum size of these organisms required for a positive result after incubation for 4 h when tested with the slopes, but not with the CLO-test. These findings indicate that *H. pylori* possesses much greater pre-formed urease activity than *P. mirabilis* and *K. pneumoniae*. False negative results for clinical detection of *H. pylori* in gastroduodenal biopsies may be due to small numbers of organisms, especially after treatment with antimicrobial agents, and false positive results may arise from gastroduodenal commensals or contaminants.

Introduction

Helicobacter pylori, formerly *Campylobacter pylori*, a common pathogen of gastroduodenal diseases, possesses an unusual characteristic of rapid urea hydrolysis, forming carbon dioxide and ammonia.¹ Several invasive and non-invasive methods for detecting *H. pylori* infection in gastric biopsy specimens have been developed based on this characteristic. McNulty and Wise² developed a urease test, which has been modified and evaluated by many other investigators.^{3–11} Later, urea breath tests with ^{13}C - or ^{14}C -labelled urea were established^{12–13} and the $^{15}\text{NH}_4^+$ excretion test was introduced.¹⁴ However, false results occur with these tests, and their practical use is controversial.^{6, 15} The aim of this study was to determine the pre-formed urease activity of *H. pylori*, as well as *Proteus mirabilis* and *Klebsiella pneumoniae*, by a viable cell count technique, and to evaluate the CLO-test by comparing it with the conventional slope urease test.

Materials and methods

Bacterial strains

The *H. pylori* strains studied were three reference strains—NCTC11637, NCTC11638 and NCTC11639 (National Collection of Type Cultures, Public Health Laboratory Service, 61 Colindale Avenue, London)—and five clinical isolates—HP92181, HP92587, HP92766, HP921052 and HP93280—from gastric antra of dyspeptic patients. Clinical isolates of *P. mirabilis* (PM617 and PM627) and *K. pneumoniae* (KP620 and KP637) were also used. *H. pylori* strains were subcultured on chocolate agar plates (Columbia Agar Base, Lab M, Bury, with horse blood 7%). The plates were incubated in gas jars at 37°C for 3 days immediately after flushing with CO_2 . *P. mirabilis* and *K. pneumoniae* isolates were subcultured on MacConkey agar plates and incubated at 37°C for 18 h before use. Colonies were identified by Gram's stain, urease, catalase and oxidase tests and the API 20E assay (API System, S. A. La Balme Les Grottes, France).

Fig. 1. Urease activity of *H. pylori* strains as determined by viable cell count: —○— NCTC11637, —●— NCTC11639, —△— HP921052, —▲— HP92766, —□— HP92587.

Table. Urease activity of *K. pneumoniae* as determined by a viable count technique with the conventional slope test and the CLO-test

Incubation time (h)	Number of organisms of strain KP637 required to give a colour change			
	Slope test		CLO-test	
	21°C	37°C	21°C	37°C
0.5	— (—)*	— (—)	—	—
2	— (—)	2.5×10^9 (—)	2.5×10^9	2.5×10^9
4	— (—)	2.5×10^9 (—)	2.5×10^9	2.5×10^9
6	— (—)	2.5×10^9 (—)	2.5×10^9	2.5×10^9
8	— (—)	2.5×10^7 (1.4×10^7)	2.5×10^9	2.5×10^9
12	— (—)	2.5×10^7 (1.4×10^7)	2.5×10^9	2.5×10^9
24	2.5×10^4 (1.4×10^5)	2.5 (1.4)	2.5×10^9	2.5×10^9

* Numbers in parentheses are for strain KP620.
—, Negative test result.

Urease test

The bacterial cultures of *H. pylori*, *P. mirabilis* and *K. pneumoniae* (strains NCTC 11637, NCTC11639, HP92587, HP92766, HP921052, PM617, PM627, KP620 and KP637) were suspended in 5 ml of sterile distilled water to yield a turbidity of McFarland Standard No. 2–3. The suspensions were then diluted in 100-fold steps to 10^{10} . One hundred μ l of each dilution was placed on paired urea slopes (Christensen's agar) and spread on chocolate agar plates or MacConkey agar plates. One pair of slopes was incubated at room temperature (21°C) and the remainder at 37°C. Slopes were examined after 30 min, 2, 4, 6, 8, 12 and 24 h and tests were recorded as positive when the yellow colour changed to pink in the area of the inoculum. Viable counts were made from the plate cultures after incubation for 5 days for *H. pylori* and 18 h for *P. mirabilis* and *K. pneumoniae*.

Comparison of CLO-test with conventional test

The reference strains, NCTC11638 and NCTC11639, and clinical isolates of *H. pylori*, *P. mirabilis* and *K. pneumoniae* HP92181, HP93280, PM617 and KP637, were used in the comparative tests. After suspension and dilution as above, 100 μ l of each dilution were inoculated on to the agar areas of paired CLO-test strips (Delta West Ltd, Western Australia) and urea slopes. The strips were incubated at room temperature and 37°C with the slopes and read at intervals as described above.

Results

Urease activity of *H. pylori*, *P. mirabilis* and *K. pneumoniae*

H. pylori strains produced a positive result on urea slopes with viable bacterial counts of 10^6 – 10^7 cfu after

Fig. 2. Effect of temperature on urease activity of *P. mirabilis* as determined by viable cell count: —○— PM617 at 21°C, —●— PM617 at 37°C, —△— PM627 at 21°C, —▲— PM627 at 37°C.

Fig. 3. Comparison of the CLO-test with the conventional urease test for *H. pylori*: —○— NCTC11638 in slope test, —●— NCTC11638 in CLO, —△— HP92181, —▲— HP93280, —□— NCTC11639 in slope test, —■— NCTC11639 in CLO. There was no difference between the two tests for strains HP92181 and 93280.

incubation for 30 min at room temperature, but the required inoculum decreased to 10^3 – 10^6 cfu when incubation was prolonged to 2 h (figs. 1, 3 and 5). After this time, the required inoculum remained unchanged (fig. 1). For *P. mirabilis* with an inoculum of 10^7 – 10^8 cfu, no colour change was observed before incubation for 2 h at room temperature (fig. 2). *K. pneumoniae* possessed even less pre-formed urease and did not produce a positive result with an initial inoculum of 10^{11} cfu after 12 h at room temperature (table). However, both *P. mirabilis* and *K. pneumoniae* gave positive results at 24 h with initial inocula of

$< 10^1$ cfu and 10^3 – 10^4 cfu, respectively (fig. 2 and table).

Comparison of CLO-test with conventional test

Smaller inocula of two of four *H. pylori* strains produced positive results with the modified CLO-test (fig. 3), but this was not so for *P. mirabilis* and *K. pneumoniae* (fig. 4 and table).

Incubation at 37°C made no difference for *H. pylori* with the conventional urease test (data not shown), but two of the four strains required a lower inoculum

Fig. 4. Comparison of the CLO-test with the conventional urease test for *P. mirabilis* PM617: —○— at 21°C and —△— at 37°C in the slope test, —□— with the CLO-test. There was no difference between the results of the CLO test at the two temperatures.

Fig. 5. Effect of incubation temperature on urease activity of *H. pylori* in the CLO-test: —○— NCTC11638 at 21°C, —●— NCTC11638 at 37°C, —□— HP92181 at 21°C, —■— HP92181 at 37°C, —△— NCTC11639, —▲— HP93280. There was no difference between the results obtained with strains NCTC11639 and HP93280.

with the CLO-test (fig. 5). However, for *P. mirabilis* and *K. pneumoniae*, incubation at 37°C significantly reduced the inoculum required for a positive result after incubation for 4 h with the conventional urease test, but not with the CLO-test (figs. 2, 4 and table).

Discussion

The present study showed that at least 10^3 bacterial cells of *H. pylori* were required for a positive urease reaction in the CLO-test (fig. 5). However, most strains gave a positive result with an inoculum of $< 10^6$ cfu.

The human gastric mucosa may become colonised with various bacterial species,¹⁶ including urease-

positive bacteria other than *H. pylori*.¹⁵ It is suggested that the urease of *H. pylori* can be distinguished from that produced by *Proteus* and *Klebsiella* spp. by its high substrate affinity and rapid hydrolysis of urea.¹⁷ In this study, the pre-formed urease activity of *H. pylori*, which usually acted within 2 h in the conventional urease medium, was found to be more than 100 times greater than that of *P. mirabilis* and 10000 times more than that of *K. pneumoniae* in terms of initial inoculum requirement.

The observations in the present study provide further evidence for the high specificity (86–100%) and sensitivity (59–100%) of urease tests in detecting *H. pylori* infection in gastric biopsy specimens.^{2-4, 8-11}

It is unlikely that *P. mirabilis* and *K. pneumoniae* would reach the critical concentration of 10^7 – 10^{10} cfu in gastroduodenal specimens. In patients infected with Enterobacteriaceae, the colonising concentration in gastric mucosa was only 2×10^2 – 2×10^4 cfu per gastric specimen.^{16, 18} However, Khulusi *et al.* have shown that, in untreated patients with duodenal ulcer or gastritis, the density of *H. pylori* infection in the gastric antrum and body is as high as $(1.3-6.9) \times 10^8$ cfu/g of tissue.¹⁹ After antimicrobial therapy, *H. pylori* can be cultured from antral biopsies at concentrations of 10^5 cfu/g of mucosa.²⁰ Since gastroduodenal biopsy specimens average c. 0.02 g (authors' observation), a concentration of *H. pylori* in gastric mucosa is at least 2.7×10^6 organisms/gastric specimen for most infected, but untreated patients, and for treated patients, the concentration can still reach 2×10^8 organisms/specimen.

The urease tests must be performed carefully and the results interpreted cautiously. Firstly, although most *H. pylori* strains produce positive results at 2 h, an incubation time of > 4 h, and up to 24 h, may be required for a few strains (fig. 5). To avoid overgrowth of urease-positive gastric commensals or contaminants, bacteriostatic or bactericidal agents should be included in the reaction media if prolonged incubation is expected. This principle has been applied to the

CLO-test. Sensitivity and specificity may also be improved by accelerating the action of pre-formed urease of *H. pylori*, e.g., by changing the concentration of the substrate or the indicator or both, or by changing the indicator. Incubation at 37°C slightly increases the sensitivity for some strains (fig. 5), but it will also increase the possibility of the false positive results due to gastric commensals or contaminants that grow rapidly at 37°C (figs. 2, 4, and table). False negative results may occur in some situations. For example, the number of organisms in gastroduodenal biopsies will be reduced significantly (even if not fully eradicated) by antimicrobial agents or *H. pylori*-suppressing drugs. Finally, since the distribution of *H. pylori* in gastric mucosa is patchy, false negative results may occur when the biopsy specimen contains small numbers of organisms.

The urease test is simple, easy, rapid and cheap. It has high specificity and sensitivity for detecting *H. pylori* in the gastric mucosa, and is suitable for primary diagnosis in patients undergoing endoscopy. However, other methods that are not based on urease activity may be needed for some patients, especially those who have been treated already with antimicrobial agents.

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