

Urease-positive bacteria in the stomach induce a false-positive reaction in a urea breath test for diagnosis of *Helicobacter pylori* infection

Takako Osaki,¹ Katsuhiko Mabe,² Tomoko Hanawa¹ and Shigeru Kamiya¹

¹Department of Infectious Diseases, Kyorin University School of Medicine, Mitaka, Tokyo, Japan

²Department of Internal Medicine, Yamagata Prefectural Central Hospital, Yamagata, Japan

Correspondence

Shigeru Kamiya
skamiya@kyorin-u.ac.jp

This study investigated the influence of urease-positive non-*Helicobacter pylori* bacteria on the results of a urea breath test (UBT) to evaluate the diagnostic utility of a UBT using film-coated [¹³C]urea tablets. The UBT was performed in 102 patients treated with a proton pump inhibitor and antibiotics for the eradication of *H. pylori*. Urease-producing bacteria other than *H. pylori* were isolated and identified from the oral cavity and stomach. In 4/102 patients, the UBT gave false-positive results. These false-positive results were found to be caused by the presence of urease-positive bacteria in the oral cavity and stomach. Five bacterial species with urease activity (*Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Staphylococcus aureus*) were subsequently isolated from the oral cavity and/or stomach. As there was no correlation between the *in vitro* urease activity of urease-positive non-*H. pylori* bacteria and the UBT value, and all of the patients with a false-positive UBT result were suffering from atrophic gastritis, it is possible that the false-positive results in the UBT were a result of colonization of urease-positive bacteria and gastric hypochlorhydric conditions. Thus, for the diagnosis of *H. pylori* infection using a UBT, the influence of stomach bacteria must be considered when interpreting the results.

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INTRODUCTION

Invasive and non-invasive diagnostic methods are used to detect *Helicobacter pylori* infection. Invasive methods using endoscopically obtained gastric biopsy specimens primarily include histopathological examination, a rapid urease test (RUT) and isolation of *H. pylori* by culture. Non-invasive methods include an anti-*H. pylori* antibody test, a [¹³C]urea breath test (UBT) and a stool antigen test (Kusters *et al.*, 2006). In patients treated with proton pump inhibitors and antibiotics for the eradication of *H. pylori*, the diagnosis needs to focus on an increased frequency of false-negative reactions of the RUT due to marked decreases in the *H. pylori* population.

A UBT is a useful method for diagnosis of *H. pylori*, particularly in patients treated with eradication therapy (Metz *et al.*, 1998; Savarino *et al.*, 1999; Riepl *et al.*, 2000; Wu *et al.*, 2006). As bacteria other than *H. pylori* possess urease activity and colonize the oral cavity and gastric mucosa, false-positive reactions can occur with the UBT. Mouth washing prior to a standard UBT is therefore recommended (Lee *et al.*, 2001). Moreover, a novel film-coated tablet-based UBT has recently been developed. Use of the film-coated [¹³C]urea tablets decreases the

false-positivity rate and yields greater accuracy when compared with a standard UBT (Ohara *et al.*, 2004), as the urea in the tablets cannot be catalysed by urease-positive bacteria in the oral cavity. However, false-positive results with this novel UBT are still observed due to urease-producing bacteria other than *H. pylori* in the stomach.

In the present study, we evaluated the diagnostic utility of this UBT using film-coated [¹³C]urea tablets. Furthermore, we isolated and identified urease-producing bacteria other than *H. pylori* from the oral cavity and stomach, and investigated the influence of urease-positive non-*H. pylori* bacteria on the results of the UBT.

METHODS

Patients. Between April 2003 and March 2004, 102 patients (83 men and 19 women; aged 14–80 years) at Yamagata Prefectural Central Hospital, Japan, who were diagnosed as having peptic ulcer diseases with *H. pylori* infection were analysed. These patients were recruited randomly and sequentially to form a cohort of at least 100 cases. Patients were treated with 60 mg lansoprazole or 40 mg omeprazole daily and two antibiotics (400 or 800 mg clarithromycin and 1500 mg amoxicillin) for 1 week to eradicate *H. pylori* at Yamagata Prefectural Central Hospital.

Evaluation of *H. pylori* status. From 4 to 6 weeks after the end of the eradication therapy, patients were evaluated for *H. pylori* status by UBT using film-coated [¹³C]urea tablets (UBIT; Otsuka

Abbreviations: RUT, rapid urease test; UBT, urea breath test.

Pharmaceutical). Breath samples were collected at 0, 5 and 20 min after administration of a UBT tablet, and δ - ^{13}C (UBT value) was measured by infrared spectrometry using a UBiT-IR300 (Otsuka Pharmaceutical). The cut-off value for the UBT was 2.5‰ at 20 min (Ohara *et al.*, 2004). When UBT values were <2.5‰ or \geq 2.5‰, test results were evaluated as negative and positive, respectively. However, UBT values that were \geq 2.5‰ but <10‰ were defined as borderline. UBT values at 5 and 20 min were considered markers for urease-positive non-*H. pylori* bacteria in the oral cavity and stomach, respectively. Furthermore, patients were examined by gastroendoscopic testing, and three gastric biopsy specimens were obtained from the greater curvature of the upper body and antrum of the stomach. One biopsy specimen was used for an RUT (Helico-check; Otsuka Pharmaceutical) and one specimen was inoculated onto *H. pylori*-selective medium (MBHM plate; Nikken Seibutsu) and cultured at 37 °C under microaerobic conditions (Osaki *et al.*, 2002), whilst another biopsy specimen and throat swab from the patient were transported at 4 °C in transport media (Carrybrea transportation medium; Eiken-Kizai) to Kyorin University, Japan. Patients with either high UBT values (\geq 2.5‰) or a positive RUT were re-examined by a second UBT and gastroendoscopic testing 4–8 weeks after the first UBT. In both UBTs, exactly the same protocol was used without proton pump inhibitor and antibiotic administration.

With regard to *H. pylori* infection status, positive results in at least two of the performed tests were considered to indicate positive *H. pylori* infection (failed eradication). Negative results in the above three tests were considered to indicate negative *H. pylori* infection (successful eradication). When the UBT value was \geq 2.5‰ and <10‰ (borderline), false positivity for *H. pylori* infection was suspected and the culture, RUT and UBT were repeated.

Isolation and identification of urease-producing bacteria.

Gastric biopsy specimens and throat swabs were inoculated onto brain heart infusion agar supplemented with 7% sheep blood (BHI/blood) plates without selective supplements and chocolate agar plates containing 7% heated sheep blood with bacitracin (5 U ml⁻¹), followed by cultivation at 37 °C under aerobic conditions. For the identification of urease-producing colonies, the urease activity of all bacteria that appeared on both media was examined using test paper, as described below. At 1 or 2 days after incubation, colonies were covered with sterile filter paper treated with urea/phenol red solution containing 50 g urea l⁻¹, 0.8 g NaH₂PO₄ l⁻¹, 0.4 g citrate l⁻¹ and 0.1 g phenol red l⁻¹. This filter paper exhibits a red colour in the presence of urease-producing bacterial colonies. Urease-producing bacteria were then subcultured and identified using the API system (bioMérieux): API 20E strips were used for identification of Gram-negative and cytochrome oxidase-negative rods and API Staph strips

for identification of Gram-positive and catalase-positive cocci. All urease-positive bacteria were stored at -30 °C until further testing.

After thawing of the stored strains, urease-positive isolates were subcultured on BHI/blood agar at 37 °C for 18 h. A bacterial suspension in PBS was sonicated (Sonifer 250; Branson Ultrasonics) for 1 min at 20 kHz and stored at -30 °C until use. Protein concentrations in sonicated samples were measured using a protein assay dye reagent (Bio-Rad Laboratories), according to the manufacturer's instructions.

Quantification of the *in vitro* urease activity of urease-producing bacterial isolates. For measurement of *in vitro* urease activity, samples and urea/phenol red solution (pH 7.4) were incubated at 37 °C. Colour change was measured at A₅₅₀ at each time point. Based on a standard curve obtained from an experiment using urease from jack beans (Wako Chemical), the urease titre was determined as U (mg protein)⁻¹.

The urease activity of urease-producing bacteria, including *H. pylori*, was quantified at various pH values to examine the effect of pH on urease activity. The rate of urea conversion into ammonia was measured by mixing a urea solution (final concentration 250 mM) with urease-producing bacteria suspended in 0.1 M citrate buffer with the pH adjusted to 1, 3 or 5 by the addition of HCl. The amount of ammonia produced was quantified using an ammonia test (Wako Chemical), as described previously (Kamiya *et al.*, 1993). As a positive control in the urease assay, *H. pylori* strains TK1029 and TK1402 were used.

RESULTS AND DISCUSSION

Evaluation of *H. pylori* status in patients undergoing eradication therapy

All 102 patients completed the eradication protocol. Based on the results of the UBT using film-coated [¹³C]urea tablets, 90 patients with low UBT values (<2.5‰) were considered *H. pylori*-negative and seven patients with a high UBT value (10‰ or higher) that were RUT-positive and culture-positive were considered *H. pylori*-positive (Table 1). Five patients showed borderline results in the UBT. As one of these five patients was RUT-positive, this patient was diagnosed as *H. pylori*-positive. The remaining four patients were RUT-negative and culture-negative in the second tests, so were considered to be *H. pylori*-

Table 1. *H. pylori* status in patients treated by eradication therapy

A total of 102 cases (83 males, 19 females; mean age 54.6 ± 12.8 years; range 19–80 years) were examined.

UBT*	RUT	Culture	No. of cases (%)	<i>H. pylori</i> status
+	-	-	4 (3.9)	Negative†
+	+	-	1 (1.0)	Positive
+	+	+	7 (6.9)	Positive
-	-	-	90 (88.2)	Negative

*The cut-off value for the UBT was 2.5‰ at 20 min.

†Four cases that were UBT-positive, culture-negative and RUT-negative in two sequential gastroendoscopic examination tests were judged to be false-positive UBT results.

negative (3/4 patients showed borderline results in the second UBT). The final eradication rate was 92.2% (94/102 patients). The four cases (all male) that were UBT-positive, culture-negative and RUT-negative on two sequential gastroendoscopic examinations were judged to be false positives in the UBT. In these four patients, UBT values at 20 min were significantly higher than those at 5 min, suggesting the influence of urease-producing bacteria in the stomach rather than in the oral cavity. Three patients (Table 2; cases 1, 2 and 4) with a false-positive UBT result were aged over 70 years and suffered from atrophic gastritis (all cases), gastric ulcer (cases 1 and 4) and gastric cancer (case 2). These patients also had prostatic hypertrophy. Two patients (cases 2 and 3) were smokers and one patient (case 2) had high levels of alcohol usage. The remaining patient (Table 2; case 3) was aged 41 and suffered from duodenal stenosis with ulcer scar formation. The mean age (66.3 ± 16.9 years old) of the false-positive patients was higher than that of all the patients (54.6 ± 12.8 years old). There was no correlation between smoking habit, alcohol usage, medication and UBT results (data not shown).

Thus the UBT is a highly accurate test, with a sensitivity and specificity of more than 90% (Lerang *et al.*, 1998; Savarino *et al.*, 1999; Gomez *et al.*, 2002; Perri *et al.* 2002). Ohara *et al.* (2004) reported that a UBT using film-coated [^{13}C]urea tablets resulted in better accuracy compared with a standard UBT in Japanese patients. The present study confirms that a UBT using film-coated [^{13}C]urea tablets has a low false-positivity rate (3.9%; 4/102 patients) among patients in whom eradication therapy was indicated. In a clinical trial evaluating a standard UBT using the same protocol as the present study, we found the false-positivity rate to be 7.5% (22/294 cases; data not shown), which is significantly higher than the false-positivity rate in the UBT using film-coated [^{13}C]urea tablets. However, even when a film-coated, tablet-based UBT is used, false-positive results are seen in some patients, particularly in those with suppression of gastric acid secretion, such as elderly patients over 70 years old with atrophic gastritis after eradication therapy.

Isolation of urease-producing bacteria from stomach biopsy specimens and throat swabs

In the UBT false-positive cases, urease-producing bacteria were isolated from the stomach and/or oral cavity. The isolates were identified as Gram-negative *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae* and *Enterobacter cloacae* and Gram-positive *Staphylococcus aureus* (Table 2). In three out of four cases, members of the *Enterobacteriaceae* were detected. In one false-positive case (case 4), a very high UBT value (152.2‰) was seen on the second UBT, and *S. aureus* was isolated from the oral cavity, but no urease-producing bacteria were isolated from the stomach. However, in case 4, urease-positive *S. aureus* was isolated only from the oral cavity, and the UBT value in the second test in case 4 was the highest among the four false-positive UBT cases. In this patient, a third UBT was performed, which gave a value of 0.3‰. It is possible that the second UBT was not performed correctly and included some technical errors. Although the cause of the high UBT values in the second test in this case was unclear, the patient had deformation of the stomach with stenosis in the pylorus to the duodenal bulb, and it is also possible that this gastric deformation affected the delivery of the film-coated [^{13}C]urea tablet.

Urease-positive non-*H. pylori* micro-organisms reportedly cause false-positive results in UBTs (Michaud *et al.*, 1998; Gurbuz *et al.*, 2005; Brandi *et al.*, 2006). Urease-positive micro-organisms are detected in the oral cavity, gastrointestinal tract, urogenital tract and skin. In humans, these microbial species include *Actinomyces*, *Clostridium*, *Corynebacterium*, members of the *Enterobacteriaceae* (*Citrobacter*, *Enterobacter*, *Klebsiella*, *Morganella*, *Proteus* and *Providencia*), *Haemophilus*, *Mycobacterium*, *Staphylococcus*, *Streptococcus*, *Ureaplasma* and *Yersinia* (Moblely & Hausinger, 1989; Murray *et al.*, 2003). Michaud *et al.* (1998) reported that colonization and overgrowth of urease-producing bacteria other than *H. pylori* induce false-positive UBT results. In a patient treated with H_2 receptor antagonists for long periods, a false-positive result was induced by urease-producing *P. mirabilis*, whilst in a patient undergoing surgery for

Table 2. Urease-producing bacteria in patients with false-positive UBT results

Case	First test				Second test			
	$\Delta^{13}\text{C}$ value (‰)		Isolates from stomach	Isolates from oral cavity*	$\Delta^{13}\text{C}$ value (‰)		Isolates from stomach	Isolates from oral cavity*
	5 min	20 min			5 min	20 min		
1	1.1	3.1	<i>P. mirabilis</i>	<i>P. mirabilis</i>	54	14.6	<i>P. mirabilis</i>	<i>P. mirabilis</i> , <i>C. freundii</i>
2	0.7	2.9	ND	<i>K. pneumoniae</i>	0.6	0	<i>K. pneumoniae</i>	<i>K. pneumoniae</i> , <i>E. cloacae</i>
3	0.1	4.2	<i>K. pneumoniae</i>	ND	2.2	4.5	<i>S. aureus</i>	ND
4†	1.8	5.9	ND	ND	0.3	152.2	ND	<i>S. aureus</i>

ND, None detected.

*The oral urease-positive non-*H. pylori* bacteria were isolated from swabs obtained from within the oral cavity.

†For the case 4 patient, a third UBT was performed, which gave a value of 0.3‰ at 20 min.

gastroschisis at birth and showing decreased gastric movement, urease-positive *Micrococcus* in the gastric mucosa induced a false-positive result in a UBT. Brandi *et al.* (2006) recently reported that ten strains of urease-positive non-*H. pylori* bacteria (five genera: *Streptococcus*, *Staphylococcus*, *Gardnerella*, *Lactococcus* and *Enterococcus*), among which *Staphylococcus capitis* subsp. *urealyticus* showed the strongest urease activity, were isolated from the stomachs of six hypochlorhydric patients. These urease-positive non-*H. pylori* bacteria were thought to be responsible for false-positive results in the UBT. Gurbuz *et al.* (2005) detected yeast-like micro-organisms in the gastric mucosa of all patients with false-positive results in a UBT. A higher proportion of false-positive results (24 %; 8/34 cases) in the UBT may be due to the presence of the yeast-like micro-organisms, although *in vitro* urease activity was not examined in that study.

A total of 59 and 12 urease-producing bacterial strains were isolated from negative and false-positive UBT cases, respectively. The *in vitro* urease activity of 39 and 8 urease-positive bacteria isolated from negative and false-positive UBT cases, respectively, was quantified (Table 3). There were no significant differences in urease activity between UBT-negative and UBT-false-positive case-derived isolates. A total of 20 and 51 urease-producing bacterial strains were isolated from gastric biopsy specimens and throat swabs, respectively. When the urease activity of urease-producing bacteria isolated from stomach biopsies ($n=10$) and throat swabs ($n=37$) was compared, no significant differences were observed between false-positive and negative cases (Fig. 1). Correlations between UBT values and *in vitro* urease activities of urease-positive bacteria other than *H. pylori* isolated from the stomach and oral cavity were then examined. No significant correlations between *in vitro* urease activity and UBT values were observed (Fig. 2).

Effects of pH on urease activity of isolates

In order to examine the effects of pH on the urease activity of urease-producing bacteria (except for *E. cloacae* isolated

from false-positive UBT case 2), ammonia production was measured at pH values of 1, 3 and 5 (Table 4). The urease activity of *H. pylori* was found to have a wide optimal pH range. Among other urease-producing bacteria, the urease activity of *P. mirabilis* was highest, followed by that of *S. aureus*. Unlike *H. pylori*, the urease activity of these bacteria was markedly inhibited under acidic conditions (pH 1).

In the present study, 12 strains from five species (*P. mirabilis*, *C. freundii*, *K. pneumoniae*, *E. cloacae* and *S. aureus*) were isolated from the oral cavity and/or stomach of four false-positive UBT cases. The *in vitro* urease activity of these non-*H. pylori* bacteria was weaker than that of *H. pylori*. There were no significant differences in the urease activity of urease-positive non-*H. pylori* bacteria between false-positive and negative UBT cases. In addition, no significant correlations between UBT value and *in vitro* urease activity were observed among urease-positive non-*H. pylori* strains. These results suggest that the *in vitro* urease activities of urease-positive non-*H. pylori* bacteria are not related to the false-positive UBT results. As the number of urease-positive non-*H. pylori* bacteria was not determined in the gastric biopsy specimens in the present study, further quantitative studies of such urease-positive bacteria are needed.

The urease activity of non-*H. pylori* bacteria was markedly inhibited at strongly acidic pH values. Husebye *et al.* (1992) reported that fasting hypochlorhydria with Gram-positive gastric flora was prevalent in healthy old people. In the present study, false-positive results on UBT were seen in three patients (cases 1, 2 and 4) over 70 years of age. As gastric acid secretion is often suppressed due to the progression of mucosal atrophy in elderly patients (all four patients with false-positive UBT suffered from atrophic gastritis in the present study), it is likely that urease-positive non-*H. pylori* Gram-positive bacteria, such as staphylococci, were responsible for the false-positive results in the UBT. Urita *et al.* (2006) reported that colonic bacterial ureolysis influences the results of UBTs; a duodenal UBT in which 20 ml water containing 100 mg [13 C]urea was sprayed into the duodenum gave positive results in 6/143 *H. pylori*-negative patients. It is also

Table 3. *In vitro* urease activity of bacterial isolates in UBT negative and false-positive cases

Urease activity (U mg ⁻¹)	Isolates from negative UBT patients*	Isolates from false-positive UBT patients†
>1	15 (4)	1 (1)
0.1–1	9 (0)	1 (0)
0.01–0.99	6 (0)	5 (3)
0.001–0.0099	9 (2)	1 (0)

*A total of 59 strains were isolated as urease-positive bacteria from negative UBT patients, but urease activity was only quantified for 39 strains due to unsuccessful subculture of the remaining 20 strains. The number of bacterial strains isolated from the stomach is shown in parentheses.

†A total of 12 strains were isolated as urease-positive bacteria from false-positive UBT patients, but urease activity was only quantified for eight strains due to unsuccessful subculture of the remaining four strains. The number of bacterial strains isolated from the stomach is shown in parentheses.

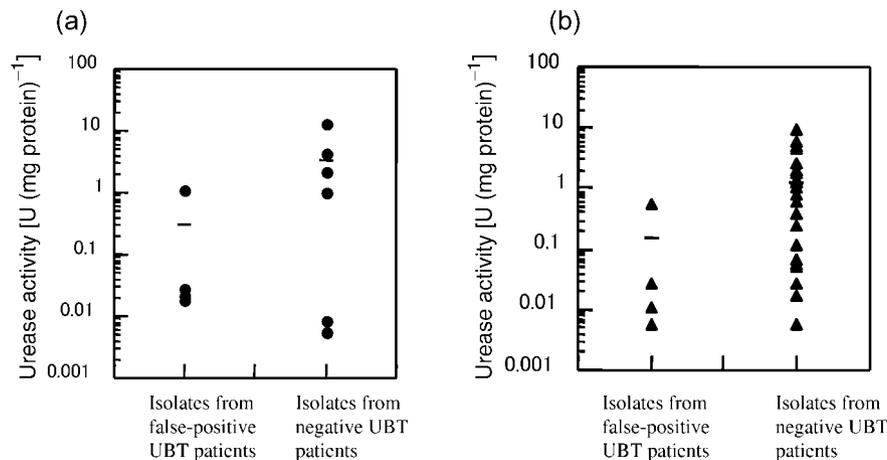


Fig. 1. *In vitro* urease activity of urease-positive bacteria other than *H. pylori* isolated from the stomach (a) and oral cavity (b) in false-positive and negative UBT patients. The oral urease-positive non-*H. pylori* bacteria were isolated from swabs obtained from within the oral cavity. The *in vitro* urease activity of *P. mirabilis* isolated from the oral cavity (first and second UBT) and stomach (second UBT) in case 1 and *K. pneumoniae* isolated from the oral cavity (second UBT) in case 2 was not tested due to unsuccessful subculturing. In total, 71 strains were isolated as urease-positive bacteria from negative and false-positive UBT patients, but urease activity was only quantified for 47 strains due to unsuccessful subculture of the remaining 24 strains.

possible that the gastric urease-positive non-*H. pylori* bacteria detected in our study originated from the duodenum or small intestine under hypochlorhydric conditions. Similarly, urease-positive non-*H. pylori* bacteria in the oral cavity might be responsible for the false-positive reactions in the UBT after their translocation from the oral cavity to the gastric mucosa in hypochlorhydria.

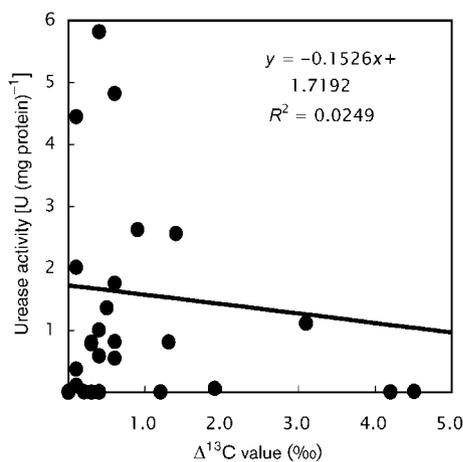


Fig. 2. Correlation between UBT value and *in vitro* urease activity of urease-positive bacteria other than *H. pylori* isolated from the stomach and oral cavity. In total, 47 strains of urease-positive bacteria isolated from the oral cavity (37 strains: four and 33 from false-positive and negative cases, respectively) and stomach (ten strains: four and six from false-positive and negative cases, respectively) were evaluated for *in vitro* urease activity, as described in Methods.

Implications of the experimental data

Film-coated [^{13}C]urea tablets were used for the UBT in this study. It is likely that non-*H. pylori* urease-positive bacteria in the stomach rather than in the oral cavity caused the false-positive reactions, as UBT values at 20 min were significantly higher than those at 5 min.

In one of five patients showing borderline results in the UBT (4.2%) (Table 1), urease-positive non-*H. pylori* bacteria were not detected and an RUT using Helico-check was positive. This patient was diagnosed as *H. pylori*-positive in the present study. An RUT can be evaluated within 2 h because of the strong urease activity of *H. pylori*. In this study, the *in vitro* urease activity detected using an ammonia test for *H. pylori* strains was significantly higher than that of other urease-positive non-*H. pylori* strains (Table 4). In addition, Vaira *et al.* (1988) reported that non-*H. pylori* urease-producing bacteria at a concentration of 10^7 c.f.u. ml^{-1} did not give a positive result in an RUT with at least a 1 h incubation period at either room temperature or 50 °C. However, there is a possibility that a false-positive reaction in the RUT was induced in the patient.

As administration of gastric acid inhibitors or antibiotics changes the gastric environment, diagnosis of *H. pylori* infection in patients treated with these drugs needs to be considered carefully. It is difficult to assess the influence of urease-producing bacteria other than *H. pylori* in the stomach in the results of a UBT. Hypochlorhydric conditions in the gastric mucosa of elderly patients accelerate colonization and overgrowth of non-*H. pylori* urease-positive bacteria. As it seems that the false-positive results in UBTs are dependent on colonization of urease-positive non-*H. pylori* bacteria and hypochlorhydric

Table 4. Urease activity of various isolated bacteria under acidic conditions

The urease activity of *P. mirabilis* isolated from the oral cavity (first UBT and second UBT) and stomach (second UBT) in case 1, and *K. pneumoniae* and *E. cloacae* isolated from the oral cavity (second UBT) in case 2, was not tested due to unsuccessful subculturing.

Strain	Urease activity (NH ₃ production min ⁻¹)*		
	pH 1	pH 3	pH 5
<i>S. aureus</i> 1†	166.4 (40.1)	751.8 (181.0)	415.5 (100)
<i>S. aureus</i> 2‡	76.4 (60.4)	110.0 (87.1)	126.4 (100)
<i>P. mirabilis</i>	125.5 (10.1)	1173.6 (94.2)	1246.4 (100)
<i>K. pneumoniae</i>	30.9 (43.0)	84.5 (117.7)	71.8 (100)
<i>C. freundii</i>	15.5 (60.7)	29.1 (114.3)	25.5 (100)
<i>H. pylori</i> TK1402	1496.4 (92.5)	1507.3 (93.1)	1618.2 (100)
<i>H. pylori</i> TK1029	1842.7 (93.7)	1955.5 (99.4)	1967.3 (100)

*The relative urease production (%) is shown in parentheses.

†Isolated from case 3.

‡Isolated from case 4.

conditions in the gastric mucosa, attention should focus on the influence of stomach bacteria.

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