¹³C-urea breath test for diagnosis of experimental *Helicobacter pylori* infection in barrier born pigs

K Meyer-Rosberg, S Gustavsson

Abstract

Previous studies with *Helicobacter pylori* infected barrier born pigs indicate that the infection has a patchy distribution, resulting in false negative culture results on endoscopic biopsy specimens. This study aimed to adapt the ¹³C-urea breath test as used in humans to diagnose *H pylori* infection in barrier born pigs. The breath test was also performed after bismuth as a single treatment and after triple therapy (bismuth, ampicillin, metronidazole). In control pigs the median excess of ¹³CO₂ in expired air was 2.2 (range 0-12 n=22) ppm. The infected pigs (n=4) showed consistently high values (median 23 range 14-43) when examined on four occasions (n=16) four to 10 weeks after inoculation. Biopsy specimens for culture had lower sensitivity than the breath test. No reduction in excess ¹³CO₂ was seen after three days' single bismuth treatment, but after two weeks' triple therapy the breath test results had returned to normal. This suppression was temporary only, however, as the breath test was positive again four weeks after stopping treatment. In conclusion, the ¹³C-urea breath test is a simple and reliable test for determining *H pylori* infection and monitoring treatment effects in barrier born pigs. Because the test can be performed in awake pigs anaesthesia and gastroscopy are unnecessary.

(Gut 1993; 34: 594-598)

Department of
Pharmaceutical
Pharmacology, Uppsala
University, Sweden
K Meyer-Rosberg

Department of Surgery,
Uppsala University
Hospital, Sweden
S Gustavsson

Correspondence to:
Dr K Meyer-Rosberg,
Department of Pharmaceutical
Pharmacology, Uppsala
University, Sweden.

Accepted for publication
22 September 1992

Helicobacter pylori infection of the human gastric mucosa is now recognised as the cause of type B (non-auto immune) gastritis¹ and an important factor in duodenal ulceration and possibly gastric ulcer.²⁻⁷ The correlation between the clearance of bacteria and reduced duodenal ulceration relapse frequency⁸ has led to the search for an anti-*H pylori* treatment to replace or complement acid inhibitory drugs. This task has been slowed by the lack of adequate animal models of *H pylori* infection.^{9,10}

In vitro models in which there is adhesion of *H pylori* to the gastric mucosal cells¹¹ and also to mucosal biopsy tissues^{12,13} have been developed. Although these are useful for mechanistic studies of adhesion, a functional in vivo model is needed for detailed studies of the pathogenic mechanisms of the infection as well as for determination of the efficacy of new anti-*H pylori* compounds. The only animals reported to be susceptible to *H pylori* infection are nude mice,¹⁴ gnotobiotic beagle dogs, and pigs,¹⁵⁻¹⁷ as well as barrier born pigs.¹⁸ We have previously reported that it is possible to maintain barrier born pigs infected with *H pylori* for at least six months with a developed superficial gastritis.¹⁹ This model has great potential for therapeutic studies as each animal is its own control, and the pattern of infection can be monitored longitudinally for a substantial time. In our former study we performed a detailed mapping at necropsy. We noted patchy distribution of the infection within the gastric mucosa,¹⁹ which can result in false negative results when a limited number of random biopsy samples are obtained for culture. Since one of the aims of this model is to elucidate the efficacy of different treatments on *H pylori* infection, the risk of getting false negative results has to be minimised.

The principle of a breath test that would explore most of the stomach was attractive in our pig model as it would reduce the risk of sampling error inherent in the gastroscopy/biopsy technique. The normal abundance of ¹³C in human breath is approximately 11 000 ppm ¹³C, and the ratio of ¹³C/¹²C can be measured by mass spectrometry. In humans infected with *H pylori* the ratio of ¹³C/¹²C in breath increases 10-30 minutes after ingestion of ¹³C-urea because of the split of ¹³C-urea into ¹³CO₂ and ammonia. The ¹³C-urea breath test has been used in epidemiological studies to determine *H pylori* status and to monitor the effect of different treatments.²⁰⁻²³ A recent report also indicated a correlation between the degree of gastritis and the response to the ¹³C-urea breath test.²⁴ This study aimed to adapt the clinically used ¹³C-urea breath test for use in barrier born pigs with an established *H pylori* infection, before and after treatment with bismuth alone and triple therapy.

Figure 1: Anaesthetic mask and plastic bag used to collect breath samples from awake pigs.

Methods

ANIMALS

Barrier born pigs (specific pathogen free) of Swedish native breed were used in this study. The pigs are free from pathogens such as *Mycoplasma* spp, *Bordetella* spp, *Haemophilus* spp, *Treponema* spp, *Campylobacter* spp, *Brucella* spp, *Leptospira* spp, *Pasteurella multocida*, pathogenic *Escherichia coli*, *Salmonella* spp, *Clostridium perfringens*, and *Mycobacterium* spp. These pigs may harbour *Corynebacterium pyogenes*, beta haemolytic streptococci, and coagulase positive staphylococci. The piglets were kept in an aseptic environment together with the sow and given autoclaved food (Sugfor, Lantmännen, Uppsala) until the start of the experiment. At 6–7 weeks of age (body weight 12–15 kg) the pigs were moved to a normal sty and were given food that had not been autoclaved throughout the experiment. The study was approved by the Institutional Review Board for Animal Research.

INOCULATION PROCEDURE

Acid secretion was inhibited before the first inoculation by oral omeprazole, 40 mg daily for three days. The pigs were then inoculated with a suspension of 5 ml *H. pylori* (10^9 colony forming units/ml) on three occasions within one week. *H. pylori* was grown on blood agar plates in a microaerophilic atmosphere for two days before inoculation and was suspended in 0.9% NaCl. The strain, obtained from a 43 year old patient with a long history of peptic ulcer, was the same as that used in our previous studies.^{18,19} The pigs were given general anaesthesia and the bacterial suspension was then injected into the stomach via a gastroscope.

^{13}C -UREA BREATH TEST

As in humans, we collected breath from the pigs before administration of ^{13}C -urea to determine the ratio of natural existing ^{13}C in breath (here referred to as baseline). After the peroral administration of ^{13}C -urea, labelled CO_2 released by hydrolysis was collected serially from exhaled

breath. Since the amount of naturally occurring ^{13}C is increased by certain plant metabolites,²⁵ pigs were fasted overnight before the test.

The breath test was repeated in all four *H. pylori* infected pigs 3, 4, 6, 8, 9, 10, and 14 weeks after the last inoculation, with biopsy specimens taken in parallel. The breath test was also performed on uninfected pigs in order to evaluate its reliability to determine, for example, the presence of other urease producing bacteria in the pig stomach that could result in false positive results. The control study included 13 pigs of 6–8 weeks of age, and all but four were examined twice. The breath test was performed in six pigs both without anaesthesia on day one, and with anaesthesia the following day.

The breath test was initially performed on anaesthetised animals who had been intubated endotracheally in order to ensure correct sampling of exhaled air. The pigs were anaesthetised with intramuscular azaperon (Stresnil), 1 ml/20 kg in combination with intraperitoneal metomidate (Hypnodil), 4 ml/20 kg, with sedation maintained by small intravenous injection of metomidate. The pigs were breathing spontaneously and exhaled breath was collected before and after the administration of ^{13}C -urea (Cambridge Isotope Laboratory, USA). Two mg/kg ^{13}C -urea was dissolved in 50 ml water and administered into the stomach at gastroscopy.

Exhaled breath was collected through a tube into a plastic bag (Scantec AB, Box 238, Partille, Sweden) over one minute and samples were taken out by a syringe, into 20 ml vacutainers (Beckton Dickinson, USA). Samples were taken 20, 40, and 60 minutes after urea administration in anaesthetised pigs, and after 30 and 60 minutes only when the pigs were conscious. Tubes were sent for analysis of ^{13}C -labelled CO_2 by mass spectrometry (BSIA Limited, Middlesex, UK). The ratio of $^{13}\text{C}/^{12}\text{C}$ in exhaled CO_2 caused by the split of ^{13}C -urea into ammonia and CO_2 by *H. pylori* produced urease was determined. The ratio was expressed as ppm excess of ^{13}C over baseline, and in humans a test result exceeding 5.0 ppm is considered positive. As a further development the breath test was performed in conscious pigs without anaesthesia 6, 8, 9, and 14 weeks after inoculation. This was possible by the development of an anaesthetic mask that covered the pig snout (Fig 1). As a result of practical difficulties the one minute collection of breath was too long in the conscious pigs, but since they were breathing more deeply without anaesthetics five to 10 breaths were enough to fill the plastic bag.

BIOPSY SPECIMENS FOR CULTURE

The pigs were anaesthetised and biopsy samples were taken (Olympus GIF Type K2). After each completed gastroscopy, the endoscopic equipment, including the forceps, was thoroughly rinsed in tap water and wiped off in order to prevent cross contamination. Three to four specimens were taken from the antral area in each pig and immediately ground in 1 ml saline in a mortar. 100 μl were spread directly on selective blood agar plates (Skirrow's antibiotic

Figure 2: Excess $^{13}\text{CO}_2$ (ppm) over baseline (baseline = before administration of ^{13}C -urea). Each point represents the mean (SD) value from four pigs on one occasion, (◆) uninfected control pigs; (●) infected pigs 6 weeks after inoculation.

supplement), which were incubated in a micro-aerophilic atmosphere for four days before colony counting. No biopsy specimens were obtained eight and nine weeks after inoculation because of equipment failure. At the time of necroscopy, however, 14 weeks after *H. pylori* inoculation, five antral biopsy specimens were obtained from each pig for culture.

TREATMENT REGIMENS

Six weeks after inoculation, two pigs received three days' treatment with 500 mg bismuth $\times$ 2 (DeNol) dissolved in water. The breath test was performed one day after this treatment. Eight weeks after inoculation the same pigs received triple therapy for 14 days. Triple therapy included bismuth 500 mg $\times$ 2 (as above), pivampicillin (suspension) 700 mg $\times$ 2, and metronidazole 1200 mg $\times$ 2 (tablets dissolved in water), and the breath test was performed seven and 14 days after the start of treatment, as well as four weeks after the end (14 weeks after inoculation). The two pigs receiving triple therapy were moved to a separate room. Four weeks after stopping treatment the final breath test was performed and the pigs were slaughtered the day after final breath test.

HISTOLOGY

At necroscopy the stomachs were immediately taken out, cut opened through the greater curvature, and gently rinsed in tap water. Tissue samples from the antrum, corpus, and cardia were fixed in 4% buffered formaline and were embedded in paraffin. Five μ m sections were exposed to FITC-labelled rabbit polyclonal antibodies (kindly provided by Dr Dan Danielsson, Örebro, Sweden).²⁶

Results

¹³C-UREA BREATH TEST

An example of the change in ¹³C over baseline values in the exhaled breath from uninfected and infected pigs 20, 40, and 60 minutes after urea administration is presented in Figure 2. In this example, values from the infected pigs are obtained six weeks after inoculation of *H. pylori*. As can be seen there is a time dependent increase

Figure 3: Excess ¹³CO₂ (ppm) over baseline (baseline = before administration of ¹³C-urea). Each line represents one pig. (●) infected pigs 10 weeks after inoculation; (■) infected pigs 10 weeks after inoculation and one day after completing 14 days' triple therapy; (▲) same pigs as (■) 4 weeks after stopping triple therapy (14 weeks after inoculation).

in the amount of ¹³CO₂ in the infected pigs that is not seen in the controls. Values from all experiments and all sampling times are listed in Table I. In the uninfected pigs examined with and without anaesthesia, a lower ¹³CO₂ was seen when animals were examined without anaesthesia ($p < 0.05$). In the infected pigs the change in ¹³C values over baseline were mean (SD), 8 (4), 28 (12), 35 (6), 22 (8) and 26 ppm 3, 4, 6, 8, and 10 weeks after inoculation of *H. pylori*, with no difference between anaesthetised and awake pigs. After bismuth single therapy, no difference could be seen between treated and untreated pigs for three days. After seven and 14 days' triple therapy, however (nine and 10 weeks after inoculation respectively) the breath test reverted back to baseline (1.0 and 2.7 respectively), and the values in untreated pigs remained at 23.4 and 28.7 over baseline (Fig 3) at 60 minutes. The final breath test on the pigs taken four weeks after stopping triple therapy (14 weeks after inoculation) resulted in an increase again of 8.3 and 11.9 (Fig 3), indicating a temporary suppression only of the urease activity.

TABLE I Delta ¹³C (mean (SD)) in expiration air before and after inoculation (ppm)

Weeks after inoculation	Minutes after ¹³ C-urea				No
	20	30	40	60	
0*	2.5 (1.2)	ND	3.4 (1.2)	5.4 (2.3)	10
0†	ND	3.2 (1.1)	ND	2.5 (1.1)**	6
0*	ND	4.1 (3.8)	ND	5.0 (2.1)	6
3*	3.0 (1.7)	ND	5.6 (4.2)	7.8 (4.5)	4
4*	11.4 (2.8)	ND	22.6 (6.3)	28.3 (5.6)	4
6*	14.0 (7.5)	ND	25.6 (5.8)	35.2 (5.6)	4
8†	ND	19.5 (15.0)	ND	22.0 (8.0)	4
9†‡	ND	0.8	ND	1.8	2
10†	ND	11.7	ND	26.1	2
10†§	ND	1.0	ND	1.6	2

*Anaesthetised pigs; †awake pigs; ‡after 7 days' triple therapy; §after 14 days' triple therapy.

ND = not done.

** $p < 0.05$.

BIOPSY CULTURE

Results obtained from the ¹³C-urea breath test did not correlate with culture results on all sampling occasions (Table II). Three weeks after inoculation, all four pigs were positive by culture but only three were positive as measured by the ¹³C-urea breath test. After an additional week, all four pigs were positive as determined both by culture and breath test and we considered the infection to be established. Six weeks after inoculation, however, all pigs were still positive by the urease test, but only one by culture.

TABLE II Culture results from antral biopsy specimens and FITC stained sections at necroscopy

Pig	Culture result at week			At necroscopy week 14	FITC positive in		
	3	4	6		Antrum	Corpus	Cardia
1	+	+	-	+	+	+	-
2	+	+	-	-	+	-	+
3	+	+	-	-	+	-	+
4	+	+	+	-	-	-	+

HISTOLOGY

At the time of necroscopy, *H pylori* was observed in all four pigs included in the study by the FITC-labelled polyclonal antibodies. *H pylori* were seen as straight and curved rods in both the cardia and antrum in pigs 2 and 3, in cardia only in pig 4, and in corpus and antrum of pig 1 (Table II).

Discussion

This is the first report of the successful adaptation of the ¹³C-urea breath test to use in pigs with an established *H pylori* infection of the gastric mucosa. The technique of determining *H pylori* infection in experimental animal studies has, to date, attracted little attention. Usually *H pylori* status is determined by conventional methods such as culture and histological examination of gastric tissue at necroscopy.¹⁴⁻¹⁷ In the barrier born pigs we have been using repeated gastroscopies with biopsy of antral mucosa for culture. The infection in the barrier born pig has a patchy distribution, however, within a localised area of gastric mucosa.¹⁹ Therefore, there is an obvious risk of obtaining false negative results because of sampling error if gastroscopic biopsy tissue is the only method of determining the infection in this model.

As seen in this study, culture of mucosal specimens obtained at gastroscopy does not seem to be the most accurate method of determining the persistence of infection. According to our data, the accuracy of *H pylori* detection in the barrier born pig is higher if both the breath test and histology are used. When monitoring new treatment regimens the importance of obtaining reliable results has to be emphasised. This study, as well as our previous studies, indicates that the risk of obtaining false negative culture results increases with time, possibly because of a redistribution of the infection. In the clinical setting the diagnosis of *H pylori* is usually established by histological examination or culture of gastric biopsy specimens, or both, although non-invasive tests such as serology and urea breath test have been valuable in epidemiological studies as well as in treatment studies.²⁰⁻²⁵

Pilot studies in pigs from the slaughterhouse confirmed that the gastric flora of the native breed pig does not contain any strongly urease producing bacteria, and because of this we decided to apply the ¹³C-urea breath test to barrier born pigs. In the early phase of this study the labelled urea was injected into the stomach at gastroscopy during anaesthesia and expired air was collected via an endotracheal tube. With small modifications to the technique, however, including oral administration of urea and collection of expired air through a specially designed

anaesthetic mask, the breath test could equally well be performed in unsedated awake pigs. Why awake pigs had a lower ¹³CO₂ value than anaesthetised uninfected pigs is not known. The fact that awake pigs move around during the experiment combined with peroral administration through the oesophagus, which might lead to a more uniform distribution of ¹³C-urea in the stomach are possible explanations. Furthermore, awake pigs are more stressed which may cause hyperventilation and thus affect the result of the breath test. This was not seen, however, in the infected pigs, and the matter will be elucidated further in our future studies. In future research, this in vivo model will be useful for monitoring therapy effects of promising agents selected from larger screening methods using in vitro models.^{12 13}

In spite of the small number of infected pigs included in this study, the results were consistent, in that positive results in all four pigs were obtained on several occasions. Triple therapy was followed by a complete return to normal of the breath test. The reactivation of the urease activity as a result of recrudescence of *H pylori* infection four weeks after stopping treatment is a further parallel to the situation in humans. In addition, similar results were obtained when breath tests were taken on several occasions over a longer period of time.

Why all pigs were culture positive three weeks after inoculation but only weakly positive on the breath test is not known. A positive breath test did not occur until four weeks after inoculation, suggesting that establishment of the infection takes time. One hypothesis is that the infection starts in the antrum, from which the biopsy specimens are taken, but in a density that is too low to be detected in the breath test. The infection then spreads to other areas of the stomach and becomes easier to detect with the ¹³C-urea breath test than by single biopsy specimens. Since the area of the stomach grows very fast in pigs during the first months, the positive areas are difficult to detect by randomly selected biopsies.

Conclusion

The consistently high levels of ¹³C in expired air in *H pylori* infected pigs show that the ¹³C-urea breath test is a simple and reliable test to determine *H pylori* infection in barrier born pigs. In addition, the ability to repeat the test on the same animal over a longer period of time is an advantage when monitoring the efficacy of future new anti-*H pylori* treatments.

We express our gratitude to Professor Thomas Berglindh for valuable contributions to this report.

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