Bacterial killing in gastric juice – effect of pH and pepsin on *Escherichia coli* and *Helicobacter pylori*

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The susceptibility of *Escherichia coli* and *Helicobacter pylori* to pH and the effect of pepsin-mediated proteolysis were investigated. This was to establish the relative importance of their bacterial killing properties in gastric juice. Solutions in the pH range 1.5–7.4 with or without pig pepsin A were used, together with seven gastric juice samples obtained from patients undergoing routine gastric collection. *Escherichia coli* C690 (a capsulate strain), *E. coli* K-12 (a rough mutant) and *Helicobacter pylori* E5 were selected as the test organisms. Suspensions of bacteria (1×10^6 *E. coli* ml⁻¹ and 1×10^8 *H. pylori* ml⁻¹) were pre-incubated with test solutions at 37 °C for up to 2 h, and then cultured to establish the effect on subsequent growth. Survival of bacteria was diminished at pHs of less than 3.5, whereas killing required a pH of less than 2.5. Pre-incubation with pig pepsin at 0.5, 1.0 and 2.0 mg ml⁻¹ at pH 3.5 reduced viable counts by 100 % for *E. coli* 690 and *E. coli* K-12 after 100 min incubation. With *H. pylori*, the viable counts decreased to 50 % of the control after 20 min incubation in 1 mg pepsin ml⁻¹ at pH 2.5, 3.0 and 3.5. The gastric juices showed bactericidal activity at pH 3.5, and the rate of killing was juice dependent, with complete death of *E. coli* 690 occurring between 5 and 40 min post-incubation. Thus, killing of *E. coli* and *H. pylori* occurs optimally at pHs of less than 2.5. At pH 3.5, little effect is observed, whereas addition of pepsin alone or in gastric juice causes a marked increase in bacterial susceptibility, suggesting an important role for proteolysis in the killing of bacteria.

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INTRODUCTION

The bactericidal effects of gastric juice are well established; however, little is known of the role of components other than hydrochloric acid. As early as 1939, it was proposed that several constituents of gastric juice might contribute to its bactericidal activity (Garrod, 1939). Since these first observations were made, it has generally been considered that gastric acid is the major if not the only antibacterial factor in gastric juice (Gianelli *et al.*, 1972). However, most studies have shown that killing of bacteria requires a pH of less than 2.0, a value which is rarely maintained for any length of time, especially during food intake, which is when most bacteria enter the stomach. In a recent review it was argued that the acid barrier is of fundamental importance in the inactivation of micro-organisms and for fighting off infection, with conditions such as hypochlorhydria or achlorhydria showing increased risk of infection (Martinsen *et al.*, 2005). Yet the role of the individual components of gastric juice is still unclear, and in particular the importance of proteolysis in bacterial killing remains to be elucidated. The key role of proteolysis in the digestion of bacteria by neutrophils has recently been confirmed to be the final pivotal act in bacterial killing (Ahluwalia *et al.*, 2004). The aim of the study therefore was to compare the effects of acid, pepsin and human gastric juice on bacterial survival to

understand the relative importance of each component. The intestinal commensal and potential pathogen *Escherichia coli* and the gastric pathogen *Helicobacter pylori* were chosen as representative bacteria for this study.

METHODS

Reagents and enzymes. All reagents were obtained as Aristar grade from Sigma, unless otherwise stated. Solutions were prepared in pure double-deionized water (UHQ, Elga). Eagle's medium (Invitrogen) and PBS were adjusted to the desired pHs of 1.5–6.0 and 7.4, respectively, by the addition of 1 M HCl or 1 M NaOH, and pig pepsin A (Sigma, P 7012) was added to a final concentration of 0.5–2.0 mg ml⁻¹.

Human gastric juice samples. Individual human gastric juice samples ($n=7$) were used (obtained in accordance with the ethical committee of The Royal Liverpool and Fazakerley University Hospitals, Liverpool, UK): three were from patients undergoing routine gastric drainage, and four were collections after pentagastrin stimulation (none of the patients was receiving antibiotics at the time of collection). Mucous material was removed by centrifugation at 4000 r.p.m. for 10 min at 4 °C. Samples were tested for obvious bacterial contamination, and no growth was observed on blood agar plates under aerobic conditions at 37 °C. The initial pH of the gastric juice was measured and then adjusted with 2 M HCl or 2 M NaOH to pH 3.5. The pepsin activities of the juices (in mg ml⁻¹)

were measured by A_{280} of the TCA-soluble protein fragments, using bovine haemoglobin as substrate at pH 2.0 and pig pepsin as standard (Roberts & Taylor, 1978).

Strains and culture conditions. P-fimbriate and capsulate *E. coli* C690, *H. pylori* E5 and the other bacteria were clinical isolates and were stored at -70°C on Protect Beads (Technical Service Consultants) until required. *E. coli* K-12 is a standard laboratory strain used for genetic manipulations and has a rough LPS surface. On thawing, bacteria were cultured at 37°C under aerobic conditions on blood agar for *E. coli* or under microaerophilic conditions for 3 days for *H. pylori*.

Determination of the effects of pH, pepsin and human gastric juice. Separate bacterial suspensions were made by emulsifying colonies from day 3 (*H. pylori*) or overnight cultures (other bacteria) in ~ 10 ml PBS, after which OD_{540} was measured and viable counts (c.f.u. ml^{-1}) interpolated from a standard curve. The suspensions were further diluted with PBS to give the concentrations of 1×10^6 c.f.u. ml^{-1} for *E. coli* and 1×10^8 c.f.u. ml^{-1} for *H. pylori*. Inoculum (0.5 ml) was pre-incubated with 4.5 ml solution containing the test substances described above, and a 10 μl sample was then taken at 0, 5, 10 and 20 min, and then every 20 min up to 2 h, and diluted with 9.99 ml PBS, pH 7.4. Neutralized bacterial suspension (200 μl) was plated in triplicate on blood agar plates for *E. coli* or chocolate blood agar for *H. pylori* (Table 2). Viable counts were determined after culture at 37°C for 15 h under aerobic conditions for *E. coli* (and other bacteria tested, see Table 2), and 3 days under microaerophilic conditions in a gas jar with hydrogen and carbon dioxide for *H. pylori*. Solutions of pH 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 were also prepared with or without 5 mM urea (final concentration) and were preincubated with *H. pylori* for various times, as indicated above, before further dilution in PBS and testing for any effects on growth.

Statistical analysis. Statistical analysis was carried using the unpaired Student *t* test, and a significant difference was established as $P < 0.05$.

RESULTS

Acid tolerance

Acid-tolerance experiments were carried out in both Eagle's medium and PBS using *E. coli* C690 and *H. pylori* E5 (Figs 1 and 2). At the same pH, the effects of acid were dependent on the suspension media used: at pH 2.5, *E. coli* was able to survive for up to 80 min in Eagle's medium, but was killed within 5 min in PBS (Fig. 1). At pH 3.0 in PBS, *E. coli* was relatively sensitive, with up to 70 % loss of viability after 60 min and complete loss after 100 min, whereas with pretreatment of *E. coli* C690 in Eagle's medium at pH 3.0, only a slight fall in viable counts was observed over 120 min.

The number of *H. pylori* colonies showed a sharp drop within the first 20 min of incubation at pH 2.5, and after 120 min, most bacteria were killed; however, *H. pylori* was able to survive at pH 3.5 (Fig. 2 and Table 1). Incubation of *H. pylori* in Eagle's medium provided considerable protection, with at least 40 % bacterial survival after 120 min at pH 2.5. The protective effect of 5 mM urea (Table 2) was significant with respect to the controls, but only relatively small numbers ($< 5\%$ of the total) of bacteria survived after

Fig. 1. Effect of preincubation at different pHs and in different media on subsequent growth of *E. coli* 690. $\blacklozenge$, pH 2.5; $\blacksquare$, pH 3.0; $\blacktriangle$, pH 4.0; asterisks, pH 7.4. (a) Dilution with Eagle's medium before culture; (b) dilution with PBS before culture.

60 min incubation at pH 3.5 and below, whereas at pH 4.0, greater numbers of bacteria survived.

The effect of preincubation for 2 h at various pHs, i.e. 2.0, 3.0 and 4.0, on other micro-organisms, e.g. *Klebsiella* spp., *Salmonella* spp., *Shigella flexneri*, *Proteus* spp., *Enterobacter* spp., *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Candida albicans*, showed that all the micro-organisms tested did not survive pretreatment at pH 1.0 or 2.0, whereas at pH 3.0 only *Staph. epidermidis*, *Staph. aureus* and *Shig. flexneri* did not survive; with the other micro-organisms, some survival of up to 10 % of control growth at pH 7.0 was observed. At pH 4.0, all bacteria tested survived. Interestingly, at pH 7.4, survival of *H. pylori* was markedly decreased.

Effect of pig pepsin

When bacteria were incubated with 0.5, 1.0 or 2.0 mg pig pepsin ml⁻¹ in PBS, all strains showed sensitivity to proteolysis, with a marked decrease in survival at pH 3.5 (Fig. 3). Both *E. coli* strains were susceptible to pepsin at pH 3.5, with 100 % loss of viability after 100 min exposure (Fig. 3). For *H. pylori* at pH 2.5 without pig pepsin (Fig. 2), there was a sharp drop in numbers of viable bacteria within

Fig. 2. Effect of pH and pepsin on the survival of *H. pylori*. (a) ◆, pH 2.5 control; ▲, 1 mg pepsin ml⁻¹, pH 2.5. (b) ◆, pH 3.0 control; ▲, 1 mg pepsin ml⁻¹, pH 3.0. (c) ◆, pH 3.5 control; ▲, 1 mg pepsin ml⁻¹, pH 3.5.

the first 20 min, although some cells (~10%) were able to survive for up to 120 min (the end of the study period). At pH 2.5 in the presence of 1 mg pepsin ml⁻¹, most *H. pylori* cells did not survive after 40 min (Table 1 and Fig. 3). Similarly, after incubation at pH 3.0 with 1 mg pepsin ml⁻¹, there was complete killing of bacteria after 100 min, and at pH 3.5 a marked decrease in the numbers of surviving bacteria compared with the medium at pH 3.5, with up to 50% reduction in the survival of *H. pylori*.

Pre-incubation in Eagle's medium gave protection to *H. pylori*, shown by reduced sensitivities to pepsin (Table 1).

Bactericidal properties of human gastric juice

The effects of the gastric juice samples adjusted to pH 3.5 are shown in Fig. 4, and indicate a significant increase ($P < 0.05$) in bactericidal activity compared with pH 3.5 controls, suggesting increased killing as a result of proteolysis.

DISCUSSION

These studies have confirmed that in solutions below pH 2.5 there is a marked killing effect on *E. coli* 690, *E. coli* K-12, *H. pylori* and various other micro-organisms, with lesser effects at pH 3.0 and little or no effect above pH 3.5. Other studies (Gianelli *et al.*, 1972 and Borriello *et al.*, 1985) have also shown that the killing of bacteria (*Serratia marcescens*) is related to pH, with the time required to kill more than 90% of bacteria at pH 2.0 being less than 30 min, at least 60 min at pH 3.0, and with no effect at pH 4.0. Earlier studies have also concluded that there is no bactericidal effect between pH 4.0 and 7.0 (Waterman & Small, 1998). The bactericidal effect of acid in the stomach is therefore dependent on the maintenance of a low gastric pH for at least 20–30 min. However, the buffering effect of food (the ingestion of which is also the time of bacterial intake) means that the gastric pH is much higher, at pH 3.0–4.5. Also, bacteria can be protected from low pH by binding to food constituents (Rosina, 1982), which was also shown in this study by the protective effect of the nutrient-rich Eagle's medium.

Bacteria may, however, develop a resistance to the effects of acid (Small *et al.*, 1994), therefore to overcome resistant strains and ensure that all foreign bacteria are killed, the pH should be 2.0 or less. *E. coli* becomes tolerant to low pH between 3.0 and 3.5 if it is grown in the exponential phase at a mildly acidic pH or if it has entered stationary phase (Arnold & Kaspar, 1995). The acid resistance of bacteria such as *E. coli* is related to increased buffering capacity within the bacteria and the enhanced production of certain membrane proteins (Booth, 1985). Also, the growth rate and phase of the cells determine the level of expression of the *rpoS*-controlled regulon, enabling bacteria to counteract a range of stresses (Garren *et al.*, 1997). The resistance can be passed through several generations, in particular during exponential-phase growth, but it is unlikely that normally acid-sensitive strains of *E. coli* acquire *rpoS* mutations (Small *et al.*, 1994).

Most studies have considered hydrochloric acid to be the only bactericidal agent in gastric juice (De Alwis, 1970), ignoring the possibility that digestive enzymes could be detrimental to growth. We suggest, however, that a combination of acid and pepsin is the most effective medium for killing bacteria. The results presented indicate that *H. pylori* is particularly sensitive to proteolysis-mediated

Table 1. Effect of acidity and pig pepsin on growth of *H. pylori*

++, Bacterial numbers were more than 200 c.f.u. ml⁻¹. Values show mean $\pm$ 1SD. After exposure to acid, values at pHs 2.0 and 2.5 were significantly reduced compared with those at pH 3.0 and above. After exposure to pig pepsin, values at comparable times were significantly reduced ($P < 0.05$) at pH 2.5 in PBS compared with Eagle's medium, PBS + 1 mg pepsin ml⁻¹ compared with PBS, and Eagle's medium + 1 mg pepsin ml⁻¹ compared with Eagle's medium.

Time (min)	After exposure to acid					After exposure to pig pepsin			
	pH					Exposure conditions			
	2.0	2.5	3.0	3.5	4.0	pH 2.5, PBS	1 mg pepsin ml ⁻¹ , pH 2.5, PBS	pH 2.5, Eagle's medium	1 mg pepsin ml ⁻¹ , pH 2.5, Eagle's medium
0	65 $\pm$ 1.41	++	++	++	++	++	++	++	++
10	0	162 $\pm$ 2.8	++	++	++	137 $\pm$ 17.7	77 $\pm$ 6.4	++	109 $\pm$ 8.5
20	0	26 $\pm$ 4.2	++	++	++	16 $\pm$ 0.7	2.5 $\pm$ 0.7	118 $\pm$ 2.1	14.5 $\pm$ 3.5
40	0	11 $\pm$ 8.5	++	++	++	2.50 $\pm$ 2.7	0.5 $\pm$ 0.7	94 $\pm$ 3.5	15.5 $\pm$ 6.4
60	0	7.5 $\pm$ 6.4	++	++	++	0.50 $\pm$ 0.7	0	60 $\pm$ 0.7	7.0 $\pm$ 1.4
80	0	4.0 $\pm$ 0.0	158 $\pm$ 12.0	++	++	0.50 $\pm$ 0.7	0	64 $\pm$ 5.7	3.5 $\pm$ 0.7
100	0	2.0 $\pm$ 0.0	117 $\pm$ 2.2	++	++	0	0	40 $\pm$ 2.8	3.0 $\pm$ 0.5
120	0	0.5 $\pm$ 0.8	62 $\pm$ 3.5	++	++	0	0	42.5 $\pm$ 4.9	0.50 $\pm$ 0.7

killing over a range of pH values; this is possibly related to areas of protein on the cell surface, e.g. membrane channels (Kim *et al.*, 2004). Nevertheless, *H. pylori* is still effective in colonizing the stomach mucosa, suggesting that *in vivo* only a small number of organisms are needed, particularly over relatively long periods of time. The strains present in the human stomach may also have become adapted to cope with the low pH and proteolytic conditions. The success of *H. pylori* in colonizing the stomach has also been related to the presence of a bacterial urease and the metabolism of urea, an effect which we have shown gives only slight protection. Nevertheless, *in vivo* studies have indicated that *H. pylori* colonizes the intercellular junctions between the epithelial cells where there is a plentiful supply of urea (Ferrero *et al.*, 1988). To explain *H. pylori* colonization of the stomach, as

little as 20 min might be sufficient for some bacterial cells to reach the mucosa, and survival may be more likely to occur at slightly higher pHs and in the achlorhydric state (Balan *et al.*, 1996).

Bacterial killing facilitated by pepsin was related to pH, with activity over the range pH 2.5–3.5, but with relatively little effect at pH 4.1, due to the lower rate of proteolysis at this pH. The concentration of pepsin present is also critical, as concentrations greater than 1.0 mg ml⁻¹ were much more effective than 0.5 mg ml⁻¹. It is noteworthy that the typical total pepsin concentration in gastric juice is between 0.5 and 1.0 mg ml⁻¹ (Balan *et al.*, 1996). The time pepsin takes to kill the bacteria *in vivo* is also important, as at 0.5 mg ml⁻¹ and pH 3.5 total kill is probably not achieved until 80 min,

Table 2. Effect of pH on the growth of *H. pylori* with or without 5 mM urea

Values shown are c.f.u. ml⁻¹, mean $\pm$ SD ($n=6$). –, Urea not added; +, 5 mM urea final concentration in preincubation.

Time (min)	pH											
	1.5		2.0		2.5		3.0		3.5		4.0	
	–	+	–	+	–	+	–	+	–	+	–	+
0	0	1.9 $\pm$ 0.1	0.3 $\pm$ 0.15	3.8 $\pm$ 1.0	2.8 $\pm$ 1.24	8.8 $\pm$ 2.2	9.3 $\pm$ 3.7	12.8 $\pm$ 3.7	81.8 $\pm$ 7.4	83.2 $\pm$ 8.0	106.9 $\pm$ 6.4	114.4 $\pm$ 8.8
5	0	1.3 $\pm$ 0.8	0	2.3 $\pm$ 1.4	0	8.1 $\pm$ 2.4	3.9 $\pm$ 2.2	9.1 $\pm$ 2.8	42.4 $\pm$ 3.7	53.3 $\pm$ 7.3	105.3 $\pm$ 2.4	104.8 $\pm$ 2.2
10	0	0.7 $\pm$ 0.3	0	2.2 $\pm$ 1.1	0	7.5 $\pm$ 1.5	1.2 $\pm$ 0.5	6.9 $\pm$ 2.2	20.3 $\pm$ 4.4	30.9 $\pm$ 6.8	84.8 $\pm$ 11.1	97.8 $\pm$ 4.9
20	0	0	0	0.5 $\pm$ 0.6	0	2.2 $\pm$ 0.8	0.2 $\pm$ 0.3	6.5 $\pm$ 1.1	9.7 $\pm$ 1.2	18.6 $\pm$ 1.9	18.0 $\pm$ 1.6	87.7 $\pm$ 5.9
40	0	0	0	0	0	0	0.05 $\pm$ 0.1	3.6 $\pm$ 1.9	3.1 $\pm$ 0.9	9.5 $\pm$ 1.2	8.8 $\pm$ 0.9	25.1 $\pm$ 1.5
60	0	0	0	0	0	0	0	1.8 $\pm$ 1.7	0.9 $\pm$ 0.2	4.3 $\pm$ 1.4	4.0 $\pm$ 0.6	17.7 $\pm$ 2.8
80	0	0	0	0	0	0	0	1.3 $\pm$ 1.5	0	1.7 $\pm$ 1.2	2.3 $\pm$ 0.2	7.8 $\pm$ 1.4
100	0	0	0	0	0	0	0	0.7 $\pm$ 0.9	0	0.6 $\pm$ 0.26	1.1 $\pm$ 0.1	3.5 $\pm$ 0.4
120	0	0	0	0	0	0	0	0.1 $\pm$ 0.1	0	0.2 $\pm$ 0.08	0.4 $\pm$ 0.1	1.2 $\pm$ 0.3

Fig. 3. Effect of preincubation at pH 3.5 with various concentrations of pepsin on the survival of *E. coli* 690 and K-12. con, no pepsin added; the numbers next to the strain names show the final concentrations of added pepsin (0.5, 1.0 and 2.0 mg ml⁻¹).

and by this time more than 60 % of the stomach contents has emptied, releasing the bacteria into the high pH (>7.0) of the duodenum and allowing further multiplication. At 1.0 mg pepsin ml⁻¹, total kill is achieved by 60 min, but this is still not quick enough to prevent viable cells from passing into the duodenum.

The experiments with human gastric juice confirmed that the significant antibacterial effect at pH 3.5 is probably mediated by the proteolytic activity of pepsin, in contrast to other studies which suggest that acid is the most important factor (Gray & Shiner, 1967; Martinsen *et al.*, 2005). Human gastric juice can also facilitate bacterial killing by the release of peptides from proteolytic breakdown (e.g. of lactoferrin) that are known to have powerful antimicrobial activity (Nibbering *et al.*, 2001; Ryley, 2001). Other studies have indicated that the acid tolerance at pH 3.0 of *E. coli* O157:H7 can be overcome by addition of lactate or ethanol (Jordan *et al.*, 1999), which is related to a fall in the bacterial cytoplasmic pH and the subsequent killing of *E. coli*. It is also possible that the presence of nitrite from foodstuffs may cause killing of bacteria (Xu *et al.*, 2001). However, all the gastric juices used were taken from patients after at least a 12 h fast and were shown not to contain nitrite or nitrate.

Pretreatment with trypsin at similar concentrations to those employed for pepsin, i.e. 1.0 mg ml⁻¹, but at pH 7.4, had

Fig. 4. Effect of different gastric juice samples adjusted to pH 3.5 on the survival of *E. coli* 690. The gastric juice samples (◆) ($n=7$) contained a mean $\pm$ 1SD pepsin concentration of 0.86 ± 0.2 mg ml⁻¹, and the control (■) ($n=4$) solutions at pH 3.5 did not contain enzyme. Error bars show 1SD. Results for the controls were at all times significantly lower ($P < 0.001$) than those of the gastric juices.

no effect on either *E. coli* C690 or *E. coli* K-12, similar to findings previously reported (De Alwis, 1970). Also, chymotrypsin had no effect on *E. coli* C690, although it decreased the survival of *E. coli* K-12. The latter strain is known to be susceptible to bile salts, and this is possibly related to the rough LPS outer layer (Niedhardt, 1987). Thus, if the structural LPS is important, then *E. coli* C690 would not be affected, because it is a smooth strain, with long LPS chains. However, both strains were sensitive to pepsin, suggesting that the nature of the surface carbohydrate does not affect the sensitivity to pepsin-mediated proteolysis.

In conclusion, we have shown that bacteria cannot survive in solutions of pH 2.0 or less, and that incubation in nutrient-rich Eagle's medium reduces the susceptibility to such low pH. The addition of pepsin increases the rate of killing of *E. coli* and *H. pylori* at pH 2.5, 3.0 and 3.5. The sensitivity of *E. coli* to pepsin is related to both pH and the concentration of enzyme used. Human gastric juice showed significant bacterial killing, related to effects caused by pepsin-mediated proteolysis.

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