Mixed infections of *Helicobacter pylori*: tissue tropism and histological significance

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Abstract

Mixed infections with *Helicobacter pylori* facilitate interstrain gene transfer and the maintenance of genetic diversity for adaptation to the gastric environment, but whether mixed infections with histological significance and tissue tropism occur in the human stomach is still unclear. *Helicobacter pylori* was isolated from the antrum and the corpus of 30 dyspeptic patients. Four to eight colonies were randomly collected from each site. The genetic diversity of each isolate was evaluated by comparing random amplified polymorphic DNA banding patterns. The prevalence of mixed infections was 23.3% (7/30), and different dominant strains were isolated from the antrum and the corpus specimens. In the 23 patients infected with a single strain, the acute inflammation (AI) score, chronic inflammation (CI) score, atrophy (AT) score and lymphoid follicle (LF) score of the antrum were usually higher than those of the corpus (p ≤0.002). However, in the seven patients with mixed infections, the CI, *H. pylori* density (HPD), AT and LF scores of the antrum and the corpus were similar (p >0.05). Moreover, the patients with mixed infections had marginally higher CI and HPD scores than those with single-strain infection (p 0.062 and p 0.095, respectively) in the corpus and had a significantly higher rate of appearance of intestinal metaplasia (IM) in the antrum (p 0.005). These data show that *H. pylori* tissue tropism was found in the human stomach, and suggest that mixed infections could change the histological features in the antrum and in the corpus, and that they could be associated with the appearance of IM in the antrum.

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Introduction

Chronic infection with *Helicobacter pylori* in the human stomach can cause gastritis and peptic ulcers, and is a risk factor for gastric cancer [1–4]. *H. pylori* is one of the most genetically diverse bacterial species. Genetic recombination is one of the mechanisms that increases genetic diversity in this species, as demonstrated by Kersulyte *et al.* [5]. Their study showed that genetic recombination occurs between two strains during natural mixed infection. Suerbaum *et al.* [6] reported that frequent recombination in *H. pylori* suggests that mixed infections with different strains could occur

repeatedly. In addition, a study with several different mouse strains demonstrated that, in mixed infections, *H. pylori* exhibits tissue tropism [7].

Mixed infections could facilitate interstrain gene transfer and genetic diversity, allowing adaptation to the environment of the human stomach [5,6,8]. However, the prevalence of mixed *H. pylori* infections in the clinical setting seems to be variable, ranging from 0% to 85% in different reports [9–15]. The diverse prevalence rates of mixed *H. pylori* infections could possibly be accounted for by the diverse methods used to sample isolates from the stomach or by the sensitivity of the strain typing method used in different studies [16]. Therefore, because of the difference in the methods used to evaluate the prevalence and distribution of mixed *H. pylori* infections topographically, the exact impact of mixed infections on disease outcome requires further validation.

In this prospective study, topographic gastric samples were collected from dyspeptic patients, and the genetic diversity was compared by analysing the profiles obtained

using random amplified polymorphic DNA (RAPD) PCR with colonies from the antrum and the corpus biopsy specimens. In addition, once the presence of mixed infection was established, it was determined whether it could be associated with particular histological features of stomach tissue.

Materials and Methods

Patients, bacterial isolation and culture

In total, 71 dyspeptic patients who had undergone panendoscopy to obtain gastric biopsy specimens gave informed consent to participate in the study. Among them, 30 patients had a positive culture from both the antrum and the corpus, one patient harboured *H. pylori* in the corpus only, and the other 40 were either *H. pylori*-negative (33 patients) or culture-negative (seven patients). The demographic characteristics, including age, female/male ratio, and proportion of patients with peptic ulcer, were not significantly different between the group of 30 patients with successful culture and that of the 41 remaining patients (51.2 vs. 48.1 years, p 0.423, 14 : 16 vs. 22 : 19, p 0.561, and 53.3% (16/30) vs. 31.7% (13/41), p 0.09, respectively). None of these patients had a previous history of *H. pylori* eradication. The endoscopic diagnosis of the 30 patients with successful culture included 12 patients with duodenal ulcer, four patients with gastric ulcer, and 14 with gastritis. For each patient, three individual endoscopic gastric biopsy samples were obtained from both the antrum and the corpus. Two biopsy samples were taken for bacterial culture, and the remaining sample was used for staining with haematoxylin and eosin, as well as with modified Giemsa stains, to evaluate the *H. pylori*-related histological features and to grade severity, using the updated Sydney system [17], and *H. pylori* density (HPD) [18]. The histological features included an acute inflammatory (AI) score (range: 0–3), chronic inflammation (CI) score (range: 0–3), atrophy (AT) score (range: 0–3), and intestinal metaplasia (IM) score (range: 0–3). HPD scores ranged from 0 to 5.

Biopsy specimens for culture were placed in Brucella broth (BBL; Microbiology Systems, Cockeysville, MD, USA) and kept on ice for transport to the laboratory. Biopsy specimens from the antrum and the corpus were separately homogenized and cultured on both selective and non-selective agar plates at 37°C in micro-aerophilic conditions for 3–5 days. The selective agar plates were Brucella agar containing horse serum (10%) (GIBCO BRL, Grand Island, NY, USA), trimethoprim (2.5 g/L) (Sigma, St Louis, MO, USA) and vancomycin (5.0 g/L) (Sigma), and the non-selective agar plates were CDC anaerobic blood agar plates (BBL; Microbiology Systems). Four to eight colonies from among the antrum and the corpus specimens were randomly picked from the primary culture plates. *H. pylori* isolates were identified as positive for urease, catalase and oxidase activities and specifically by PCR detection of the *vacA* gene. All colonies were stored at −70°C in brain–heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA) containing 30% glycerol until testing. *H. pylori* strains were cultured on CDC plates for fewer than eight passages to decrease the possibility of genetic variation, and their genomic DNA was extracted.

DNA extraction and RAPD PCR

Genomic DNA of *H. pylori* was extracted using the QIAamp DNA mini kit (Qiagen, Chatsworth, CA, USA). In this study, which aimed to evaluate the strain differentiation, two primers were selected for the RAPD PCR. They were D14216 (5′-NNNAACAGCTATGACCATG-3′) and D8635 (5′-GAGCGGCCAAAGGGAGCAGAC-3′), as designed by Akopyanz et al. [19]. The PCR mixtures were made in a volume of 50 μL containing c. 100 ng of DNA, 20 pmol of primer, 0.15 mM each deoxynucleoside triphosphate, reaction buffer with MgCl$_2$, and 1 U of *Taq* DNA polymerase (New England Biolabs, Beverly, MA, USA). PCR was carried out in a Perkin-Elmer 2720 thermal cycler (Perkin-Elmer; Applied Biosystems, Foster City, CA, USA) through four cycles of low-stringency amplification and 30 cycles of high-stringency amplification. The cycling programme was as described previously [19]. The PCR products (20 μL) were electrophoretically separated in agarose (1%) gels.

Definition of mixed infections using RAPD PCR

For each patient, the genomic diversity among the several colonies of *H. pylori* isolates from the antrum and the corpus was analysed by RAPD PCR. Moreover, the genomic diversity of the colonies isolated from the antrum was compared among these colonies and with those of the isolates from the corpus. Highly homogeneous RAPD banding patterns in the isolates of one patient were defined as single-strain infection. Bacterial infection with more than one RAPD banding pattern, found in isolates from either the antrum or the corpus, or both, was defined as mixed infection.

The sequences of the virulence genes

Sequences of three gene fragments of *flaA*, *flaB*, *vacA* and the 5′-terminal region of *cagA* are unique among *H. pylori* strains [6,8]. The 5′-terminal region of four virulence genes and the 3′-terminal region of *cagA* were amplified by using the following primers: *flaA*, flaA-F (5′-ACTCAAAACGCGCTTAAAAC-3′) and flaA-R (5′-TTCTGCTAACACGCCAATC-3′; *flaB*, flaB-F (5′-CAATATCGCCGCTTTAACTTC-3′) and

flaB-R (5′-TTCGCTTAACCGCTCCAATC-3′); *vacA*, vacA-1 (5′-ATGGAAATACAACAAACACAC-3′) and vacA-2 (5′-CT CCAGAACCCACACGATT-3′); *cagA*, cagA-3 (5′-GATAAC AGGCAAGCTTTTGAGG-3′) and cagA-4 (5′-CTGCAAAA GATTGTTTGGCAGA-3′); and the 3′-terminal region of *cagA*, cagA-12 (5′-ACCCTAGTCGGTAATGGGTTA-3′) and cagA-13 (5′-GTAATTGTCTAGTTTCGC-3′). The PCR reaction was carried on through 30 cycles consisting of 94°C for 1 min, 48°C (*flaA* and *flaB*) or 50°C (*vacA* and the 5′- and 3′-terminal regions of *cagA*) for 1 min and 72°C for 1 min in a Perkin-Elmer 2720 thermal cycler (Perkin-Elmer, Applied Biosystems). The automated sequencing of PCR products was performed by the Mission Biotech Company (Taiwan).

Statistics

Statistical analysis was performed by using the Wilcoxon signed ranks test, the Mann–Whitney *U*-test or the chi-square test as appropriate. Differences were considered significant at p-values <0.05.

Results

DNA fingerprinting of *H. pylori* isolates by RAPD PCR

The observation of an identical RAPD banding pattern of DNA from the same colony in separate reactions confirmed the high reproducibility of the technique (data not shown). There were different RAPD banding patterns showing a high degree of variation among the isolates collected from the different hosts using primers D14216 and D8635 (Fig. 1a,b, respectively). There were 226 and 223 single colonies of *H. pylori* collected from the antrum and the corpus, respectively, of the 30 enrolled patients. With use of the first primer (D14216), as shown in Table 1, at least two different RAPD banding patterns were found in the same individual in five patients (cases 5, 9, 13, 15 and 18). Two additional patients (cases 8 and 23) were confirmed as having mixed infections when the second primer (D8635) was used. In addition, the 5′-terminal regions of *flaA*, *flaB* and *vacA* and the 5′-terminal and 3′-terminal regions of *cagA* in one strain of each RAPD genotype, except in strains from mixed infections with the RAPD banding patterns L1 and L2, were determined. The sequence diversity in the five gene fragments was revealed by differences of >1% in each RAPD genotype of the isolates from each of the mixed infections. Accordingly, the rate of mixed infections of the 30 enrolled patients with *H. pylori* infection was 23.3% (7/30), including four patients with gastritis, two with duodenal ulcer and one with gastric ulcer. The demographic characteristics, including age, female/male ratio and the proportion of patients with
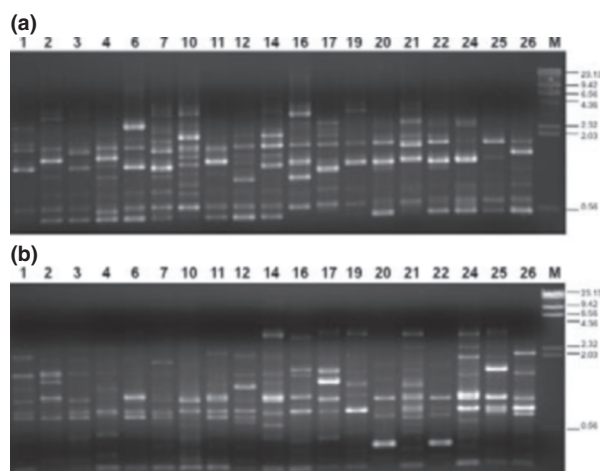

FIG. 1. Random amplified polymorphic DNA (RAPD) banding patterns of isolates from the different patients. RAPD banding patterns were obtained using the D14216 (a) and D8635 (b) primers. Cardinal numbers indicate the different patients' isolates, and M is *Hind*III-digested λ DNA as a molecular size standard (kb).

TABLE 1. Random amplified polymorphic DNA banding patterns of *Helicobacter pylori* isolates from seven patients with mixed infections

Patient	Genotype	No. of isolates	
		Antrum	Corpus
Mixed infections identified using the first primer (*n* = 5)			
5	A1	8	0
	A2	0	8
9	B1	6	0
	B2	0	7
13	C1	8	1
	C2	0	3
	C3	0	4
15	D1	8	2
	D2	0	6
18	E1	1	7
	E2	5	0
	E3	2	1
Mixed infections identified using the second primer (*n* = 7)			
5	F1	8	0
	F2	0	8
8	G1	7	3
	G2	0	4
9	H1	6	0
	H2	0	7
13	I1	8	1
	I2	0	3
	I3	0	4
15	J1	8	2
	J2	0	6
18	K1	1	7
	K2	5	0
	K3	2	1
23	L1	5	7
	L2	3	1

peptic ulcer, were not significantly different between the 23 patients with a single-strain infection and the seven patients with a mixed infection (49.9 vs. 55.4 years, p 0.478, 12 : 11
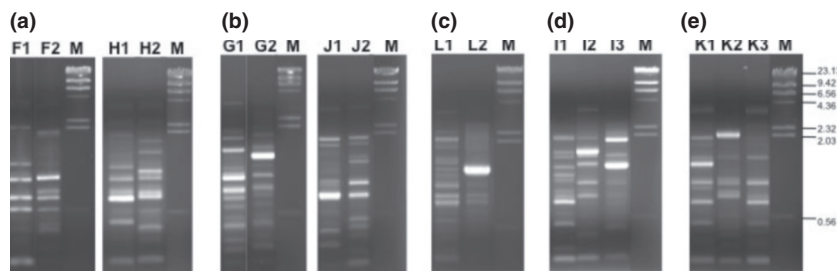
FIG. 2. Each random amplified polymorphic DNA (RAPD) genotype from each of seven patients with mixed infections. RAPD banding patterns of the isolates from seven patients (cases 5, 9, 8, 15, 23, 13 and 18) are shown. Capital letters (F, G, H, I, J, K and L) indicate the genotypes that were found in different patients, 1–3 were different genotypes found in the same patient, and M is *Hind*III-digested λ DNA as a molecular size standard (kb).

vs. 2 : 5, p 0.273, and 56.5% (13/23) vs. 42.9% (3/7), p 0.191, respectively).

In Table 1, it can be seen that five (71.4%) of the seven patients with mixed infections had two different RAPD banding patterns among the isolates collected from both the antrum and the corpus. Among the patients with strains yielding two different RAPD banding patterns, there were two patients (cases 5 and 9) with uniformly different patterns between the antrum and the corpus (Fig. 2a, Table 1). In cases 8, 15 and 23, there were also two different RAPD banding patterns in the *H. pylori* isolates, but isolates with such diverse RAPD banding patterns were located only at the corpus in the former two cases (Fig. 2b, Table 1) and at both the antrum and the corpus in the last case (Fig. 2c, Table 1). Besides the five patients infected with the strains of *H. pylori* with two different RAPD banding patterns, there were only two patients (28.6%) who had three different *H. pylori* isolates (cases 13 and 18). In case 13, three diverse RAPD banding patterns were found in the isolates collected from the corpus, but not in those from the antrum (Fig. 2d, Table 1). In contrast, case 18 had diverse isolates from the antrum and the corpus (Fig. 2e, Table 1). Despite the diverse patterns, there was a predominant isolate at each site of infection within the same host (Table 1).

Differences in histological features observed in cases of single-strain and mixed infections

Table 2 presents the differences in histological features between the antrum and the corpus within the same infected host for the patients with a single-strain infection and those with mixed infections. In the 23 patients with single-strain infection, the AI, CI, AT and lymphoid follicle (LF) scores of the antrum indicated predominantly greater severity of the pathological signs than the scores of the corpus (p ≤0.002; Wilcoxon signed ranks test). The *H. pylori* density in the antrum was marginally higher than that in the corpus

TABLE 2. The association of single and mixed infections with histological features

Parameter	Single infection (*n* = 23)		Mixed infections (*n* = 7)	
	Z-score	p-Value	Z-score	p-Value
AI score (C−A)	−3.5	0.000*	−1.732	0.083
CI score (C−A)	−3.153	0.002*	−1.000	0.317
HPD score (C−A)	−1.929	0.054	−0.138	0.890
AT score (C−A)	−3.622	0.000*	−1.518	0.129
LF score (C−A)	−3.149	0.002*	−0.552	0.581
IM score (C−A)	−1.342	0.180	−1.890	0.059

The Z-score was calculated from the parameter (e.g. AI score, CI score) of the corpus subtracted from that of the antrum (C−A). If the Z-score had a minus value and p <0.05, this meant that the parameter of the antrum indicated greater severity of the alteration than that of the corpus.
AI, acute inflammation; CI, chronic inflammation; HPD, *H. pylori* density; AT, atrophy; LF, lymphoid follicle; IM, intestinal metaplasia.
*Significant difference (p <0.05, Wilcoxon signed ranks test).

(p 0.054). However, for the seven patients with mixed infections, the CI, HPD, AT and LF scores were similar in the antrum and the corpus (p >0.05; Wilcoxon signed ranks test). Only for the histological features measured as AI score and IM was there a marginally significant difference between the antrum and the corpus (AI score, p 0.083; IM, p 0.059; Wilcoxon signed ranks test).

It was further investigated whether there were significant differences in any histological feature between the patients with single-strain and mixed infections (Table 3). There were no significant differences between the two groups of patients with respect to AI, AT and LF scores for the antrum or the corpus. Moreover, the CI and HPD scores in the corpus were marginally higher in patients with mixed infection than in those with a single-strain infection (p 0.062 and p 0.095, respectively). In addition, the patients with mixed, as opposed to single, infections had a significantly higher rate of IM in the antrum (57.1% vs. 8.7%, p 0.005; chi-square test).

TABLE 3. Comparison of histological features between patients with single-strain and mixed infections

Parameter	Single-strain infection (n = 23)	Mixed infections (n = 7)	p-Value
	Mean score (SD) or %		
Antrum			
AI score (0–3)	1.96 (0.47)	2.14 (0.38)	0.300
CI score (1–3)	3.00 (0.00)	3.00 (0.00)	1.000
HPD score (1–5)	3.65 (1.43)	4.00 (0.58)	0.978
AT score (%)	69.6	57.1	0.542
LF score (%)	60.9	57.1	0.860
IM score (%)	8.7	57.1	0.005*
Corpus			
AI score (0–3)	0.78 (1.00)	1.29 (1.25)	0.284
CI score (1–3)	2.17 (0.89)	2.86 (0.38)	0.062
HPD score (1–5)	3.13 (1.25)	4.00 (1.41)	0.095
AT score (%)	4.3	14.3	0.356
LF score (%)	21.7	28.6	0.708
IM score (%)	0	0	

The differences between the AI, CI and HPD scores in the two groups were assessed using the Mann–Whitney *U*-test. The differences between the proportions with AT, LF and IM were assessed using the chi-square test.
AI, acute inflammation; CI, chronic inflammation; HPD, *H. pylori* density; AT, atrophy; LF, lymphoid follicle; IM, intestinal metaplasia.
*p <0.05.

Discussion

H. pylori strains exhibit a high degree of heterogeneity [8,20–22]. One obvious possibility is that mixed infections provide an opportunity to increase interstrain recombination [23], which generates genetic diversity to help bacterial adaptation to the new or changing environment of the human stomach [5,6,8]. Understanding the prevalence of mixed infections in a population becomes more important because they sustain a reservoir of bacterial diversity. Although several studies have described the prevalence rate of mixed *H. pylori* infections in the human stomach and of genetic recombination under those conditions [5,6,8–15], the effect of mixed infections on gastric histology is a topic worthy of further investigation. In the present study, the prevalence of mixed infections was 23.3%, and there were different predominant isolates found in the antrum and the corpus. In single-strain infections, histology revealed more severe pathological features in the antrum, whereas in mixed infections, the difference in histological features between the antrum and the corpus was small.

The prevalence of mixed infections according to previous reports is variable [10–15], and the discrepancies could be due to different methods of sampling of gastric biopsies, bacterial isolation, and DNA analysis [16]. The authors of these reports suggest that at least biopsy specimens from both the antrum and the corpus need to be included. Additionally, isolating and analysing single colonies from each biopsy would increase the rate of detection of different genotypes. In this study, four to eight single colonies from the antrum and the corpus, respectively, from 30 patients (in total, 449 single colonies) were isolated, and seven patients were identified as having mixed infections. This study is unique, in that large numbers of colonies were used to define the prevalence of mixed infections. Moreover, the detection of the genetic diversity of isolates from patients with single-strain and mixed infections was highly consistent using two different primers, except in the case of two patients identified as having a mixed infection using only the second primer (D8635) (Table 1). This indicates that the second primer provides higher sensitivity for the differentiation of different genotypes.

Akada *et al.* [7] used mouse-adapted *Helicobacter* strains, SS1 and X47, to co-inoculate several different mouse strains. SS1 was found to be more abundant than X47 in the antrum, whereas X47 was found more frequently than SS1 in the corpus. This implies that mouse-adapted *H. pylori* strains exhibit tissue tropism in stomach colonization. These authors proposed that mixed infections in humans may also occur, with strains occupying different gastric niches. In the present study, the isolates from seven patients with mixed infections had diverse RAPD banding patterns. Despite the diversity of the patterns, there was a predominant RAPD banding pattern in isolates from each site of the stomach within the same host (Table 1, Fig. 2). The present results further support the hypothesis that *H. pylori* strains prefer different niches of the human stomach [7]. Marshall *et al.* [24] and Hua *et al.* [25] also showed that a single strain predominated in isolates from the antrum, but they did not assay the isolates from the corpus. In contrast, Jorgensen *et al.* [14] reported that the majority of patients with mixed infections had a single predominant strain found in both the antrum and the corpus. The discrepancy between these studies and the present study could be explained by the complex interaction between bacteria and host, or by the differences in the patient populations. The distinct anatomical and physiological characteristics of the antrum and the corpus might select for the different strains, which express suitable factors to adapt to particular niches in the stomach. As chronic infection with *H. pylori* strains is the result of a series of bacterial competitions for persistent colonization, the point after *H. pylori* infection when biopsy specimens are taken could be an important factor affecting the detection of isolates. Hua *et al.* [25] assessed the mixed growth of two *H. pylori* strains *in vitro* and found that one strain could be suppressed by the other.

The histological features of single-strain infection were dominant in the antrum, but the histological features of mixed infections were similar in the antrum and the corpus (Table 2). Table 3 shows that CI and HPD scores in the

corpus are marginally higher in mixed infections than in single-strain infections. This indicates that in mixed infections there is more abundant bacterial colonization of the corpus, leading to higher CI scores, thus decreasing the histological differences observed between the antrum and the corpus.

Moreover, mixed infection is significantly related to the appearance of IM in the antrum (p 0.005; chi-square test). It could be that mixed infections are due to strains of differing virulence, which cause histological alterations of varying degrees or changes in the physiology of the stomach that influence clinical outcome [26]. For instance, Murata-Kamiya *et al.* [27] demonstrated that CagA is involved in intestinal transdifferentiation of gastric epithelial cells through interaction with E-cadherin and deregulation of the β-catenin signal.

In summary, this study demonstrates that 23.3% of *H. pylori*-infected patients have mixed infections and that bacteria show tissue tropism in the human stomach. Furthermore, mixed infections change the histological difference between the antrum and the corpus and are associated with the appearance of IM in the antrum. Therefore, the presence of mixed infections due to *H. pylori* strains within a host has important clinical consequences. The reasons for the tissue tropism observed in *H. pylori* and the effect of mixed infections on histological features deserve further investigation.

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Transparency Declaration

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