

Colonization by *Pseudomonas aeruginosa* and *Staphylococcus aureus* of Antral Biopsy Specimens from Gastritis Patients Uninfected with *Helicobacter Pylori*

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Purpose: Roles and incidence of some microorganisms that transiently or permanently colonize the human stomach are still unknown despite advances in gastroenterology. We aimed to examine the incidence of four microorganisms, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, in the antral biopsy specimens of patients with gastroduodenal conditions.

Patients and Methods: Patients (67 females, 33 males; mean age = 49.5 years) were initially examined and diagnosed by a gastroenterologist at the Mehrad Hospital, Tehran, Iran. We enrolled those who underwent the upper gastrointestinal endoscopy because of gastroduodenal conditions. Two antral biopsy samples were taken by endoscopy; the first sample was used for the “rapid urease test” to confirm *H. pylori*. The second was used for DNA extraction and PCR analyses with specific, corresponding primer sets to establish the presence of the four microorganisms. Our study was approved by the Ethics Committee at the Tarbiat Modares University, Tehran.

Results: Based on pathology and endoscopy findings, we divided the patients into three groups: 62 presented with gastritis, 18 with duodenal ulcer, and 20 gastric ulcer. The number of patients with *P. aeruginosa* but without *H. pylori* significantly differed from the number of those co-infected with both microorganisms ($P = 0.03$). Additionally, a similar significance was found between the incidence of *S. aureus* in patients without *H. pylori* and those with both infections ($P = 0.04$). Our results indicated that a significant number of patients with gastritis were colonized with *P. aeruginosa* or *S. aureus* without being co-infected with *H. pylori* ($P < 0.001$). Interestingly, the incidence of colonization by *P. aeruginosa* of patients without *H. pylori* (45/49, 91.8%) was higher than that by *S. aureus* (28/49, 57%).

Conclusion: The number of patients without *H. pylori* but with *P. aeruginosa* or with *S. aureus* infection significantly differed from that with both infections, respectively. Our study thus shows that patients without *H. pylori* infection are prone to be colonized by *P. aeruginosa* or *S. aureus*, indicating that targeted antibiotic regimens are necessary for clinically treating them.

Keywords: gastritis, *Helicobacter pylori*, microbiota, PCR, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

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Introduction

The cumulative epidemiological knowledge about *Helicobacter pylori* as a pathogenic gastric colonizer of humans has profoundly changed the general

mindset in the field.^{1,2} Surprisingly, only small proportions of infected subjects, mainly in the developing countries, end up with severe gastroduodenal diseases such as gastric cancer. Thus, field investigators have started studying other bacterial species that could potentially permanently or transiently colonize the human stomach and potentially cause gastroduodenal diseases.^{3,4} *Prevotella*, *Neisseria*, *Staphylococcus*, and *Streptococcus* have been reportedly found in the stomach; however, some of these bacteria were found also in the esophagus with less-studied clinical relevance.^{5–7} Evidence suggests that colonization by some species, for example, *Streptococcus salivarius*, which have urease activity, generates a less acidic environment, thus favoring *H. pylori* colonization.⁸ Hansson et al have described that the production of acid in the antrum is increased mainly after gastritis whereas the risk of gastric cancer is slightly reduced.⁹ With the exception of *H. pylori*, the clinical relevance of other bacteria in gastroduodenal disorders has not been fully understood. Like the patchy gastric distribution of *H. pylori* that changes with changing acidic output in some patients,¹⁰ other bacterial species also may transiently colonize the human stomach likely with unmanifested clinical outcomes. To unravel non-*Helicobacter* species, Andersson et al investigated six patients (three with and three without *H. pylori* (non-*pylori*) infection) using barcoded pyrosequencing; surprisingly, they found diverse bacterial populations in subjects without *H. pylori*.¹¹ Pyrosequencing is a synthesis-based sequencing method that involves measuring deoxynucleotide incorporation to primer-guided complementary DNA sequences by measuring the release of pyrophosphate.¹² Llorca et al showed that the presence or absence of *H. pylori* infection in pediatric subjects could change the composition of the gastrointestinal microbial communities.¹³ Schulz et al reported that colonization by *H. pylori* affects the composition of the bacterial communities in the oral cavity.¹⁴ Many researchers so far have focused on specific roles of *H. pylori* in initiating or exacerbating some gastroduodenal disorders; not many have directly investigated the coexistence of the certain microorganisms in the human stomach in the absence or presence of *H. pylori*.¹⁵ Some researchers have studied pediatric populations or colonization of the oral cavity,^{16–18} thus, our understanding of gastric microbiota in adults is limited.

Here, we sought to determine the prevalence of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* in the antral biopsy samples

of patients positive or negative for *H. pylori*. These microorganisms frequently colonize the human stomach.^{11,19–21} Iran has a high prevalence of *H. pylori* infection (40–90%)^{22,23} and provides a novel population sample for conducting such a study.

Patients and Methods

Study Design, Sample Collection, and Ethics

Biopsy samples were collected at the gastroenterology unit of Mehrad Hospital, Tehran from January 2018 to March 2019. Patients were candidates for the upper gastrointestinal endoscopy because of gastroduodenal complaints, including epigastric pain. Participation in the study was voluntary, and subjects were informed that they could disenroll whenever they wished. The participants signed the informed consent forms, and the study protocols were reviewed and approved by the Clinical Research Ethics Committee of Tarbiat Modares University, Tehran (IR.MODARES.REC.1397.244). Our study was conducted in accordance with the Declaration of Helsinki.²⁴ The exclusion criteria included being under 18 or older than 80 years of age, having autoimmune diseases, history of abdominal surgery 3 months before endoscopy, history of antibiotic treatment against *H. pylori*, active gastric bleeding, taking antacid medications 2 weeks before endoscopy, and pregnancy. Gastroscopic endoscopy was performed by an expert gastroenterologist, and the reports were subsequently sent to our laboratory within 2 to 3 weeks. All clinical documents were kept confidential. In brief, two antral biopsy specimens were taken; the first one was used for rapid urease test (RUT) testing, and the second specimen was kept in sterile 1.5-mL Eppendorf tubes containing the thioglycolate broth (Merck, Germany) at 4°C for transporting to the laboratory for subsequent microbiological testing. Microbiology included genetically identifying and confirming the four bacterial species, that is, *H. pylori*, *S. epidermidis*, *S. aureus*, and *P. aeruginosa*.

DNA Extraction, PCR, and RUT

Genomic DNA was extracted using a commercial kit (QIAGEN, Germany) according to the manufacturer's instructions. To confirm *H. pylori* infection in the biopsy samples, we used the *glmM*-specific primer sets described previously.²⁵ For the three other microorganisms, DNA was amplified using their corresponding universal primer sets as

Table 1 Primer Sequences and PCR Conditions

Microorganisms	Primer	Sequences	Conditions Including °C and Time (s)	PCR Product	Reference
<i>S. aureus</i>	<i>nuc</i>	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	95 for 60 59 for 45 72 for 50 32 cycles	279 bp	(26)
<i>S. epidermidis</i>	<i>aap</i>	ATGGGCAAACGTAGACAAGG GCTTTCGCTTCATGGCTACT	95 for 60s 59 for 45s 72 for 50s 36 cycles	155 bp	This study
<i>P. aeruginosa</i>	<i>PA431C</i>	CTGGGTCGAAAGGTGGTTGTTATC GCGGCTGGTGCGGCTGAGTC	94 for 60s 57 for 30s 72 for 56 35 cycles	232 bp	(27)
<i>H. pylori</i>	<i>glmM</i>	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	94 for 60s 58 for 30s 72 for 60s 34 cycles	294 bp	(25)

Table 2 Distribution of the Three Microorganisms Among Subjects Positive or Negative for *H. Pylori*

Patient Characteristics and Sample Colonization	With <i>H. pylori</i>	Without <i>H. pylori</i>	P value
n = 100	51%	49%	$P > 0.05$
<i>S. aureus</i>	18 (35.2%)	28 (57%)	$P = 0.04$
<i>S. epidermidis</i>	20 (39.2%)	24 (48.9%)	$P = 0.42$
<i>P. aeruginosa</i>	38 (74%)	45 (91.8%)	$P = 0.03$

detailed in Table 1. We designed and confirmed by sequencing the primers for amplifying *aap* (Gene ID: 2828035), which is specific for a genetic region of *S. epidermidis*. We used *PA431C* (GenBank accession No. NP_253925) amplification to confirm the presence of *P. aeruginosa*. In parallel, we used *nuc* (thermonuclease gene specific for *S. aureus*) to

confirm the presence of *S. aureus* in the samples.^{26,27} PCR amplification was done in 20 µL using a T100 PCR machine (Bio-Rad, Berkeley, California). Optimal PCR conditions for each primer set are summarized in Table 1.

PCR products were checked by gel electrophoresis in 1.5% (w/v) agarose (Sina-clon, Tehran, Iran) in Tris/borate/EDTA (TBE) buffer. Gels were stained with GelRed® and subjected to UV transillumination (Biometra, Germany) and imaging. We included previously diagnosed clinical samples as positive controls, and negative controls by excluding the DNA template to ensure experimental quality.

RUT was used to confirm *H. pylori* biochemically. We included the samples positive for both *glmM* PCR and RUT in our first group (*H. pylori* positive). For the second group, we included only the samples that were negative for both RUT and *glmM* PCR.

**Figure 1** Gel electrophoresis for *PA431C* gene to determine the prevalence of *P. aeruginosa*. The band size was 232 bp. Samples 5–8 were positive, whereas 3 and 4 were negative clinical samples.

Statistical Analyses

The IBM SPSS 18.0 software was used for statistical analyses. The status of each subject as positive or negative for *H. pylori* was independently determined and reported using the Student's *t*-test. A *p*-value of <0.05 was deemed to be statistically significant.

Results

We examined the antral biopsy specimens from 100 patients. Patients included 67 females and 33 males, averaging 49.5 years of age. Patients with positive RUT and positive *glmM* PCR were categorized as definitely positive for *H. pylori*. Only two patients had negative RUT but positive *glmM*; these patients were excluded from the study. Altogether, 49 patients were negative and 51 were positive for *H. pylori* (35 F, 16 M). We repeated 10% of the PCR experiments in a double-blinded approach to double-check the consistency and integrity of our experiments and results. Unsurprisingly, the reported findings from the two independent operators were consistent.

Table 2 shows the prevalence of the three different microorganisms among the two groups, positive or negative for *H. pylori*. Of the total biopsy samples, 46% were positive for *S. aureus*, 44% positive for *S. epidermidis*, and 83% positive for *P. aeruginosa* (Table 2 and Figures 1 and 2). The concurrent colonization by Gram-negative and Gram-positive bacteria was found in 13% of samples.

The incidence of the three microorganisms among the three investigated gastroduodenal diseases with positivity

for *H. pylori* is presented in Table 3. Here, all patients were divided into three groups according to their pathology reports and clinical diagnoses established by a clinician: 62 had gastritis, 18 duodenal ulcer, and 20 gastric ulcer. We found a significant difference between subjects without *H. pylori* or with *H. pylori* who were also colonized with *P. aeruginosa* ($P = 0.03$). Similarly, colonization with *S. aureus* was significantly different between patients with or without *H. pylori* infection ($P = 0.04$).

We calculated the incidence of each microorganism among the three groups of patients. We found that patients with gastritis were colonized significantly more with *P. aeruginosa* regardless of being coinfecting with *H. pylori* ($P < 0.001$). Interestingly, colonization of *P. aeruginosa* in patients without *H. pylori* was more prevalent than in those with *H. pylori*, that is 38/49 (77%) versus 26/51 (50%), respectively (Table 2).

Discussion

Historically, the low gastric pH has been thought to be a physiological barrier against colonization by microorganisms.^{28,29} Moreover, colonization by Gram-negative bacteria in the upper gastrointestinal tract has reportedly been uncommon.^{30–32} To assess the prevalence and likely clinical relevance of the three microorganisms—*P. aeruginosa*, *S. aureus*, and *S. epidermidis*—in the context of gastritis, gastric ulcer, and duodenal ulcer, we studied 100 Iranian patients with or without *H. pylori* infection. The patients were diagnosed according to

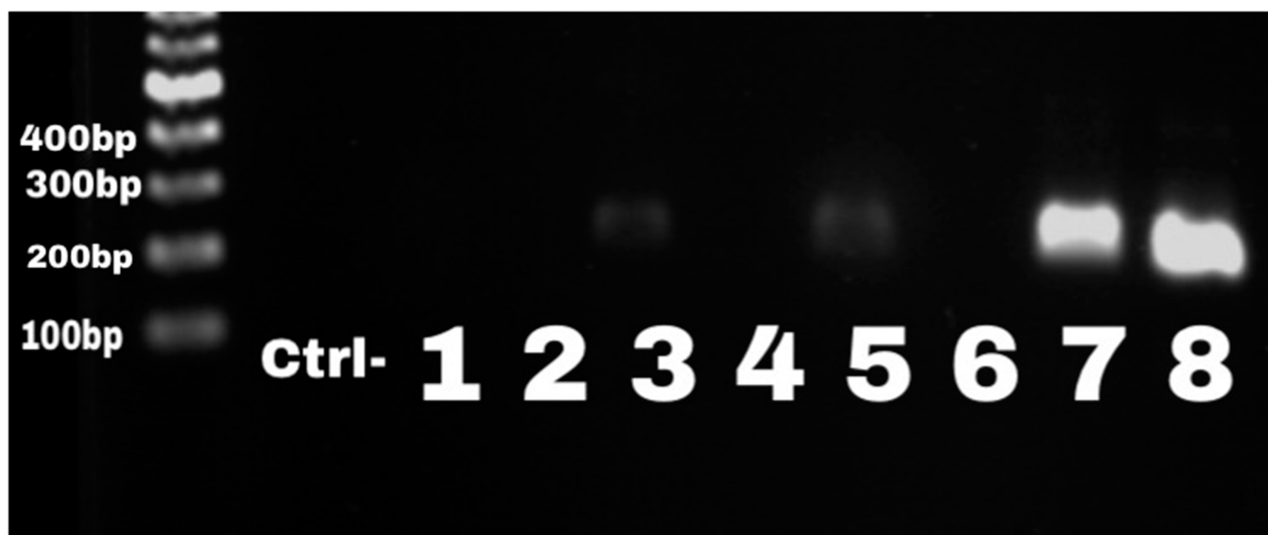


Figure 2 Gel electrophoresis for *nuc* gene to determine the prevalence of *S. aureus*. The band size was 279 bp; sample 8 was the positive control. Samples 3, 5, and 7 were considered as positive clinical samples.

Table 3 Incidence of the Three Microorganisms Among Patients with the Three Digestive Conditions

<i>H. pylori</i> Status Disease Groups	<i>H. pylori</i> Positive (n = 51)			<i>H. pylori</i> Negative (n = 49)		
	<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>S. aureus</i>
Gastritis (n = 62)	26	5	4	38	8	14
Duodenal ulcer (n = 18)	5	7	11	2	10	8
Gastric ulcer (n = 20)	7	8	3	5	6	6

pathological and endoscopic findings. We found that colonization by *P. aeruginosa* in the antral biopsies of patients with or without *H. pylori* was high, 74% and 91.8%, respectively. Because the incidence of *P. aeruginosa* in patients without *H. pylori* was higher 38/49 (77%) than that in patients with *H. pylori*, we speculated that this higher incidence may be associated with *pylori*-negative gastritis patients. As presented in Table 3, patients with gastritis were more significantly colonized with *P. aeruginosa* ($P < 0.001$) than patients with *S. epidermidis* or *S. aureus* ($P > 0.05$). As far as we know, such a finding has not been reported previously. Present literature mostly reports on the microbial communities in the gastrointestinal tract of pediatric patients,^{33–36} whereas our subjects were older adults. The population studied here represent those of a particular socioeconomic status and a different ethnical group and genetic pool. Several studies have shown that patients with or without *H. pylori* infection are colonized with discrepant *Enterobacteriaceae* populations.^{37–41} As we found, subjects without *H. pylori* infection were colonized more significantly with the three investigated microorganisms compared to those with *H. pylori* co-infection ($P < 0.05$; Table 2). Our findings do not agree with that by Benavides et al, reporting a significant link between colonization by *Prevotella*, *Clostridium*, and the presence of *H. pylori* infection.⁴²

Association of gastritis with *H. pylori* infection has been well documented.^{43–45} However, gastritis due to non-*pylori* bacteria and its prognosis to develop into severe gastroduodenal disorders are not well studied.⁴⁶ We, thus, present that the roles of *P. aeruginosa* or *S. aureus* in initiating local acute mucosal gastritis will be an interesting future area of gastroenterology research. Our study was limited by the small population size, hampering the conclusions on the impact of *H. pylori* on other potential gastric co-infections. A longitudinal study with a larger population size is necessary to elucidate various aspects of the implications of our study.

Non-Pylori Gastritis: Unknown Clinical Relevance

Presently, the predominant clinical understanding of *H. pylori* is that this microorganism majorly causes gastritis, and that successful eradication of *H. pylori* leads to the elimination of problematic gastroduodenal diseases and their complications.⁴⁷ Haber et al reported that up to 90% of non-*pylori* gastritis may be explained by colonization with other microbiological agents;⁴⁸ for example, autoimmune gastritis and long-term administration of proton-pump inhibitors (PPIs).⁴⁹ In our study, we documented whether patients had a history of PPI medication and excluded those with any PPI treatment done 2 months before the endoscopy. We tend to conclude that easy access to PPI treatment in Iran and other developing countries should be an acceptable factor for non-*pylori* gastritis cases. We did not have any history of autoimmune gastritis for patients who participated in our study. To date, the infectious cause of non-*pylori* gastritis has been underestimated as evidenced by the lack of relevant studies. Our findings showed that high incidence of *P. aeruginosa* among the non-*pylori* gastritis cases (77.5%) should be a starting point for future studies. Importantly, our findings also highlight that the clinical prognoses of non-*pylori* gastritis are unknown. For instance, can the patients progress to develop pan-gastritis or gastric cancer later in life? Is there a risk of peptic ulcer in such patients? We hope that molecular and future long-term clinical studies will resolve such questions for patients with non-*pylori* gastritis.

Conclusion

We found a relatively high incidence of *S. epidermidis*, *P. aeruginosa*, and *S. aureus* in non-*pylori* gastritis patients, a previously unexpected observation. The novelty of our findings reflects the significant colonization of *P. aeruginosa* among patients suffering from gastritis. An initial endoscopy without positive RUT results or positive microbiological or genetic *H. pylori* results should trigger

the search for other causative agents. We conclude that clinicians should consider eradication of this multidrug-resistant Gram-negative bacterium (*P. aeruginosa*) before prescribing antibiotics against *H. pylori*.

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Disclosure

The authors report no conflicts of interest in this work.

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