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Multilocus sequence typing of *Ochrobactrum* spp. isolated from gastric niche

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KEYWORDS
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Ochrobactrum

Summary The human stomach is colonized by diverse bacterial species. The presence of non-*Helicobacter pylori* bacteria in urease-positive biopsies of individuals has been reported. Bacteria belonging to the *Ochrobactrum* genus have been documented in the human gastric niche. The co-occurrence of *Ochrobactrum* spp. with *H. pylori* was previously reported in an antral biopsy of a non-ulcer dyspeptic (NUD) subject from Northern India. There is no information on the genetic diversity of *Ochrobactrum* spp. isolated from the gastric niche in the stomach. We aimed to study the species distribution and diversity of *Ochrobactrum* spp. with and without *H. pylori* in urease-positive biopsies across three different geographical regions in India. Sixty-two *Ochrobactrum* isolates recovered from patients with an upper gastric disorder ($n = 218$) were subjected to molecular identification and multilocus sequence typing. *H. pylori* DNA was found in the majority of biopsies, which had a variable degree of *Ochrobactrum* spp present. Interestingly, some of the urease-positive biopsies only had Ochrobactrum without any *H. pylori* DNA. Based on

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phylogenetic analysis, the Ochrobactrum isolates were distributed into the *O. intermedium*, *O. anthropi* and *O. oryzae* groups. This indicates there are multiple species in the gastric niche irrespective of the presence or absence of *H. pylori*. Antibiotyping based on colistin and polymyxin B could differentiate between *O. intermedium* and *O. anthropi* without revealing the resistance-driven diversity. Considering the prevalence of multiple *Ochrobactrum* spp. in the human gastric niche, it is important to evaluate the commensal and/or pathogenic nature of non-*H. pylori* bacteria with respect to their geographical distribution, lifestyle and nutrition needs.

Introduction

The human stomach houses a diverse range of bacteria, including non-pathogenic and pathogenic strains, such as *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Streptococci*, and *Lactobacilli*. Recently, it has been postulated that the stomach houses 32 phylotypes from *Proteobacteria*, including 3 uncharacterized phylotypes [1]. Members of the *Ochrobactrum* genus are omnipresent; they have been isolated from various ecological niches, such as water, soil, plants, animals, and humans. Ochrobactrum is an opportunistic human pathogen that causes an infection in immunocompromised patients. We previously reported on the presence of *O. intermedium* and *H. pylori* in a subject from North-India who was diagnosed with non-ulcer dyspepsia (NUD) [2]. A unique observation was the presence of severe fibrosis in the lamina propria of the gastric mucosa according to histological examination of the gastric antral biopsy. It was unclear whether this fibrosis was caused either partially or completely by *O. intermedium* [2]. Comparative genomics of two isolated strains with *H. pylori* revealed the presence of numerous features, such as a secretion system, urease, and flagella, which might explain their concurrence in the gastric niche [3,4]. The present study was conducted to evaluate the prevalence and co-occurrence of *Ochrobactrum* in urease-positive biopsies from individuals in three geographical regions in India. Multilocus sequence analysis was performed to determine the population structure.

Methods

Patient details and sampling

To study the prevalence of *Ochrobactrum* sp. from Indian populations, we collected urease-positive biopsies from more than 218 patients. In brief, 158 were from Hyderabad (South India), 41 from Allahabad (North India) and 19 from Pune (West India); the biopsies were screened for *Ochrobactrum* and *H. pylori*. Gastroenterologists collected antral biopsies from patients after obtaining signed, pre-informed consents at respective centers. The institutional ethical committee of Hyderabad, Allahabad and Pune hospitals approved of the study prior to sample collection. However, a form (approved by Department of Biotechnology, Government of India, New Delhi) describing the guidelines was also circulated and used to acquire the patient details while obtaining consent. The exclusion criteria included patients who were taking non-steroidal anti-inflammatory drugs (NSAIDS), had undergone gastric resection, were pregnant, refused to give consent or were previously been treated for *H. pylori* infection. However, we cross-checked the negative samples by amplification to confirm the accuracy of the primary screening protocol, and there was no amplification. Antral gastric biopsy specimens were collected [22] during endoscopy from subjects presenting with symptoms of acidity, abdominal pain, epigastric pain, vomiting, heartburn, portal hypertension and upper gastrointestinal dyspepsia. A pediatric bronchoscope with a 5.5-mm diameter was used (Olympus America, NY). The specimens were collected for culture as well as microscopic and histologic examination. Among 138 individuals (including healthy subjects) in Hyderabad, 129 were males and 9 were females who ranged in age from 14 to 80 years and 20 to 75 years, respectively. Among 41 individuals (including healthy subjects) in Allahabad, 29 males and 11 females who ranged in age from 10 to 87 years and 18 to 71, respectively. Among 19 individuals (including healthy subjects) in Pune, 15 males and 4 females who ranged in age from 19 to 82 and 25 to 37 years, respectively. Primary diagnosis was performed according to three different criteria, including the patient history, rapid urease test (RUT) in urea solution, and histopathology. Secondary diagnosis was confirmed by the use of 16S rDNA primers specific for *H. pylori*.

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Histopathological examination

Histology was performed on all antral biopsies using hematoxylin-eosin (H&E) and Loeffler's methylene blue stains to visualize *H. pylori*. At the same time, specimens were histologically evaluated according to the Sydney system of classification [23]. In the histological interpretation, parameters such as inflammation, atrophy, activity, *H. pylori*, intestinal metaplasia and lymphoid aggregate/lymphoid follicles were considered. Molecular confirmation of *H. pylori* was performed using PCR analysis of the total biopsy DNA with *Helicobacter* species-specific primers as described previously [2].

Isolation of *Ochrobactrum* spp. from urease-positive biopsies

Urease-positive biopsies were processed to isolate *Ochrobactrum* using the same method as that for isolating *H. pylori* [24] in a microaerophilic atmosphere at 37 °C. For bacterial culture, each gastric biopsy was ground in Brucella broth (Difco, USA), and the ground tissue suspension was plated onto Columbia blood agar supplemented with 10% (v/v) defibrinated horse blood. Culture plates were observed after 36—42 h for bacterial colonies that resembled either *H. pylori* or non-*H. pylori* bacteria. The bacteria were subcultured and maintained on enriched media, such as Brucella Chocolate agar (Difco, USA) and MacConkey's agar (HiMedia, India), to determine their viability on simple, selective media. Bacterial colonies were analyzed using Gram staining and urease activity in urease broth.

Antimicrobial susceptibility testing

Susceptibility to various antimicrobials, including beta-lactams, cephalosporins, macrolides, quinolones, sulfonamides and aminoglycosides, was determined on Mueller-Hinton agar using standard commercial antibiotic octadiscs (Hi Media). Colistin (25 μg) and Polymyxin B (50 Units) were also tested to differentiate between *O. anthropi* and *O. intermedium*. Inhibition zones were calculated as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Screening of bla_{OCH} gene

The primers spanning the entire coding sequence of the *ampC* gene were selected to screen the bla_{OCH} gene of all of the isolates; (forward) 5′-GAGATGCGGTCGAACCAT-3′ and (reverse) 5′-CTGACTGAACTGCTGCCG-3′ [25]. PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions, and they were sequenced on both strands using PCR primers to initiate sequencing. The BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov) was used for database searches.

Allelic diversity analyses

An allelic profile was generated by comparing the DNA sequences of each of six MLST loci. Polymorphic sequences were assigned different allele numbers. Distinct allelic profiles were assigned with different sequence types (STs). Clustering of related STs was performed using eBURST Version 3 (7). Groups of STs consisted of at least five identical loci using eBURST v3. The founders of the groups were predicted with 1000 re-sampling for bootstrap analysis.

Recombination analyses

The Homoplasy [10] and Sawyer's [11] tests were performed with the START program, and *P* values of 1 indicated no recombination. Linkage Analysis (LIAN) Version 3.5 was used to calculate the standardized index of association (I^A_S) with 10,000 iterations by Monte Carlo based on the allelic profiles [8].

Results

Histological examination of the gastric biopsies for the presence of bacteria

In all populations, inflammation was observed in all biopsies, except for in asymptomatic individuals. There was no fibrosis of the lamina propria in asymptomatic individuals (for these subjects, we used the term 'healthy'). Conversely, antral atrophy was not detected in any of the patients. Similarly, intestinal metaplasia (IM) was absent in all biopsies except for those from asymptomatic individuals. Lymphoid aggregates and lymphoid follicles (LA/LF) were present in NUD patients, whereas LA/LF was variable in biopsy specimens from other gastric diseases. Although asymptomatic patients had *H. pylori*, histological data were normal in these patients' biopsies (data not shown). The prevalence of *H. pylori* and non-*H. pylori* (*Ochrobactrum* spp.) were only statistically significant for NUD cases ($P < 0.01$).

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Prevalence of *Ochrobactrum* spp. and *H. pylori*

Histological analysis using *H. pylori* antibodies coupled with direct biopsy PCR and *H. pylori*-specific primers for the 16S rRNA gene revealed that *H. pylori* DNA is present in the majority of biopsies except for those from DU. *Ochrobactrum* sp. were isolated from NUD individuals from South (29/75), North (5/13) and West (1/5) India. However, *Ochrobactrum* sp. were present in some biopsies that lacked *H. pylori*, such as in 3/75 NUD individuals from South India. Similarly, *Ochrobactrum* sp. were isolated from 5/38 DU individuals, 2/9 CDU individuals, 2/11 gastric ulcer individuals, 1/3 PPU individuals, 1/5 PU individuals and 1/6 GC individuals from Southern India.

Antibiotyping

All isolates were sensitive to six antibiotics, tetracycline (30 μg), trimethoprim-cotrimoxazole (1.25/23.75 μg), tobramycin (10 μg), tiprofloxacin (5 μg), ofloxacin (5 μg), and chloramphenicol (30 μg); they were resistant to 28 antibiotics, including penicillin G (10 Units), ampicillin (10 μg), cephalexin (30 μg), erythromycin (15 μg), gentamycin (10 μg), metronidazole (5 μg), carbenicillin (100 μg), trimethoprim (5 μg), norfloxacin (10 μg), imipenem (10 μg), kanamycin (30 μg), amikacin (30 μg) and augmentin (30 μg). The minimum inhibitory concentrations (MICs) of β-lactams for all *Ochrobactrum* strains showed that they were resistant to all β-lactams that were tested, except imipenem (data not shown). The resistance to β-lactams (bla_{OCH}) was also supported by PCR based sequence analysis. General susceptibility to aminoglycosides, fluoroquinolones, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol and fosfomycin was detected. Colistin (Polymyxin E) and Polymyxin B successfully differentiated *O. anthropi* strains from *O. intermedium*; all *O. intermedium* strains were resistant to both antibiotics, and *O. anthropi* was sensitive to them. Interestingly *O. tritici* had a variable response to colistin and polymyxin B. Antibiotyping suggests that a high level of antibiotic resistance in *Ochrobactrum* is a natural trait of the species [5].

Phylogenetic inter-relationship revealed by MLST

A total 45 unique STs were identified from 62 isolates that were tested; these were assigned into 7 lineages and 13 singleton STs using the START [6] and eBURST programs [7] (Table 1). The founders of the groups were predicted with 1000 re-sampling for bootstrap analysis. Fig. 1 shows the founder of group 2 (*i.e.*, ST39). The total number of alleles ranges from 5 (*aroC*) to 13 (*dnaK*), which is independent of the sequence length. The mean G + C content and mean genetic diversity were also determined for individual loci (Table 2). Linkage disequilibrium between alleles at the six gene loci was measured using the standardized index of association (I^A_S) with LIAN 3.5 with 10,000 iterations by Monte Carlo based on allelic profiles [8]. If there were more frequent recombination events than expected, the value of I^A_S was set at zero; if I^A_S was significantly different from zero, alleles were suggested to have a genetic linkage. The I^A_S of 45 STs were 0.1702 ($P \leq 1.00 \times 10^{-02}$), indicating linkage disequilibrium (or a low rate of recombination) between the alleles at the population level. The ratio between the numbers of synonymous (d_S) and nonsynonymous (d_N) substitutions was calculated using the method described by Nei and Gojobori [9]. This measures the type of selection occurring at each locus. If the d_S/d_N ratio is >1, nonsynonymus sites are under selective pressure (negative selection); <1 indicates positive selection, and $d_S/d_N = 1$ indicates neutrality. With the exception of the trpE gene, the housekeeping genes were under positive selection, which was favored to the next generation (Table 2). The population evolution rate was studied using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and the bottom-up hierarchical clustering method using the START program (6) (Fig. 2). Minimum spanning was generated using http://www.pubmlst.org to visualize possible evolutionary relationships between STs (Fig. 3). To detect the recombination events within the entire population; the Homoplasy index [10] and Sawyer's [11] tests were performed. The Homoplasy test discriminates between recurrent mutations and recombination. The numbers of informative sites for each loci were determined and the loci with >10 informative sites were selected for the homoplasy test. Statistically, there was no recombination event because the *P* value was greater than zero. Sawyer's test analysis for intragenic recombination was performed with the START program. The *P*-value ($P > 0$) of the sum of the squares of condensed fragments (SSCF) and maximum condensed fragment (MCF) in the Sawyer's test did not reveal any evidence of intragenic recombination (Table 2).

Discussion

With the discovery of *H. pylori* and other gastric Helicobacters, as well as subsequent insight

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Table 1 Characteristics of the allelic profile (STs).

Isolates	ST	*aroC*	*danK*	*gap*	*recA*	*rpoB*	*trpE*
21	1	1	16	33	31	16	20
35B	2	45	20	34	32	8	21
36	3	1	2	1	31	1	20
45A	4	1	16	33	32	8	20
45C	4	1	16	33	32	8	20
47	5	1	5	1	1	1	1
47A	6	1	4	1	1	1	1
59C	6	1	4	1	1	1	1
51	7	1	3	1	2	1	1
59A	8	1	2	33	1	1	1
59B	9	1	24	1	1	1	1
59D	9	1	24	1	1	1	1
59E	10	1	2	33	32	1	27
59H	11	1	2	1	32	1	2
59I	12	39	16	33	31	8	20
180A	12	39	16	33	31	8	20
183D	12	39	16	33	31	8	20
59L	13	1	1	1	1	1	1
180B	14	39	13	34	31	8	20
180C	15	39	13	33	31	8	20
180D	16	39	13	34	32	16	21
182C	17	39	13	34	31	8	21
182D	18	39	2	34	32	1	27
182E	19	39	8	1	31	8	27
182K	19	39	8	1	31	8	27
182F	20	39	17	34	31	8	21
182H	21	1	2	1	2	1	1
182L	22	1	2	1	3	1	1
182M	23	1	2	1	32	1	1
182P	24	1	13	34	26	8	20
183A	25	39	17	33	32	8	20
183B	26	39	2	34	32	16	27
183C	27	45	13	34	1	8	21
183E	28	39	2	34	1	8	27
183F	29	1	2	34	1	8	27
195A	30	39	16	33	36	8	20
195B	31	39	24	34	32	1	27
195D	32	1	24	34	37	1	27
195E	33	1	24	34	37	1	20
195F	34	45	13	34	31	8	21
211C	35	1	2	32	1	1	10
229C	36	1	2	1	2	1	6
229G	37	1	4	1	2	1	1
229O	38	39	24	33	31	1	27
M86	39	1	2	1	1	1	1
46A	39	1	2	1	1	1	1
40B	39	1	2	1	1	1	1
46B	39	1	2	1	1	1	1
46C	39	1	2	1	1	1	1
48	39	1	2	1	1	1	1
52A	39	1	2	1	1	1	1
59F	39	1	2	1	1	1	1
59K	39	1	2	1	1	1	1
59J	39	1	2	1	1	1	1
182B	39	1	2	1	1	1	1
182I	39	1	2	1	1	1	1
Och3	40	1	2	3	1	2	1
OchP	41	39	25	33	31	8	20
SP22D	42	1	15	34	1	10	29
SP22F	43	25	15	34	25	10	26
SP91A	44	7	15	38	9	11	26
SP91B	45	7	16	34	2	14	20

ST — sequence types.

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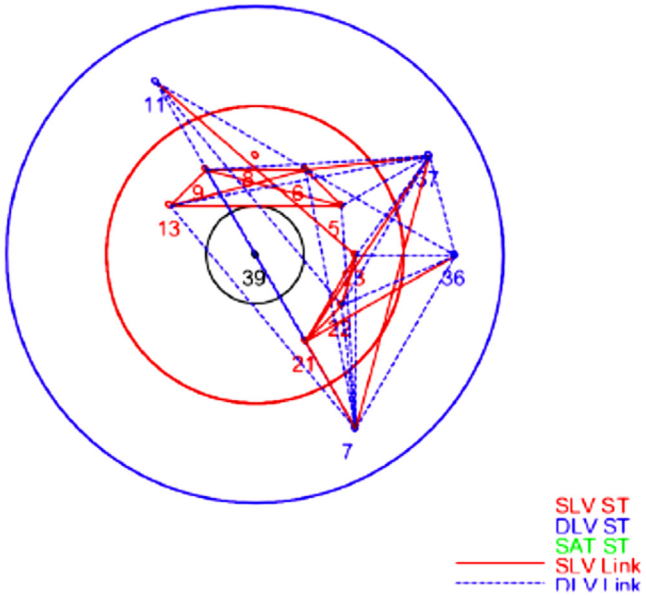

Figure 1 eBURST analysis of lineage 2 showing the link between genetic loci. The center shows the lineage founder.

into the mechanisms by which these organisms adapt to the gastric environment, it is plausible that a bacterial community has adapted to this human niche [12]. However, there is some information about the co-existence and specific role of bacteria other than *H. pylori* in the acidic lumen of the stomach [13]. Other *Ochrobactrum* species have also been associated with *H. pylori*, such as mild gastritis in squirrel monkeys from *O. anthropi* [14]. Importantly, both *H. pylori* and *O. intermedium* produce urease; thus, *H. pylori* detection using the urease test in the presence of *Ochrobactrum* may be confounded. Domínguez-Bello et al. reported that the rapid urease, catalase and oxidase tests are not reliable for identifying *H. pylori* because several other bacteria in the stomach are urease positive; hence, the use of the standard urease positive test for initial indication of *H. pylori* may be flawed [15]. *H. pylori* does not colonize the stomach, even after several weeks of oral implantation of specific pathogen-free animal models. However, it can establish successful colonization in germ-free animal models, suggesting that non-*H. pylori* bacteria affect the intragastric colonization of *H. pylori* and its activity in gastric diseases [16]. In a Malaysian study, the bacterial microbiota of *H. pylori* positive and negative gastric disease patients revealed that presence of *H. pylori* did not significantly modify the gut microbiota. One exception was *Streptococccci* in peptic ulcer disease. The authors also reported the first isolation of *Burkholderia pseudomallei* [17]. *H. pylori* infection evokes a strong immune response, which modifies the stomach pH [18]. With disease progression, there are alterations in the gastric microbiota based on gastric environment fluctuations, which has been shown during gastric cancer progression [17]. The geographically linked gastric microbial ecology and interactions between *H. pylori* and other stomach microbes, such as non-*H. pylori*

Table 2 Sequence analysis of seven loci.

Locus	No. of alleles	Genetic diversity (h)	d_S	d_N	d_S/d_N	% G+C	SSCF (*P*-value)	MCF (*P*-value)
aroC	5	0.6253	0.0894	0.2095	0.2688	62.15	533 (0.18)	8 (1.0)
dnaK	13	0.8475	0.1262	0.1749	0.5671	58.07	1604 (0.58)	10 (1.0)
Gap	6	0.7081	0.0116	0.0975	0.1146	60.61	5358 (0.21)	33 (1.0)
recA	10	0.8212	0.0179	0.119	0.3129	59.41	15,276 (0.82)	27 (1.0)
rpoB	7	0.6626	0.1704	0.238	0.3476	53.29	216 (1.0)	6 (1.0)
trpE	9	0.8141	0.4894	0.0648	14.2682	61.52	6240 (0.23)	22 (1.0)

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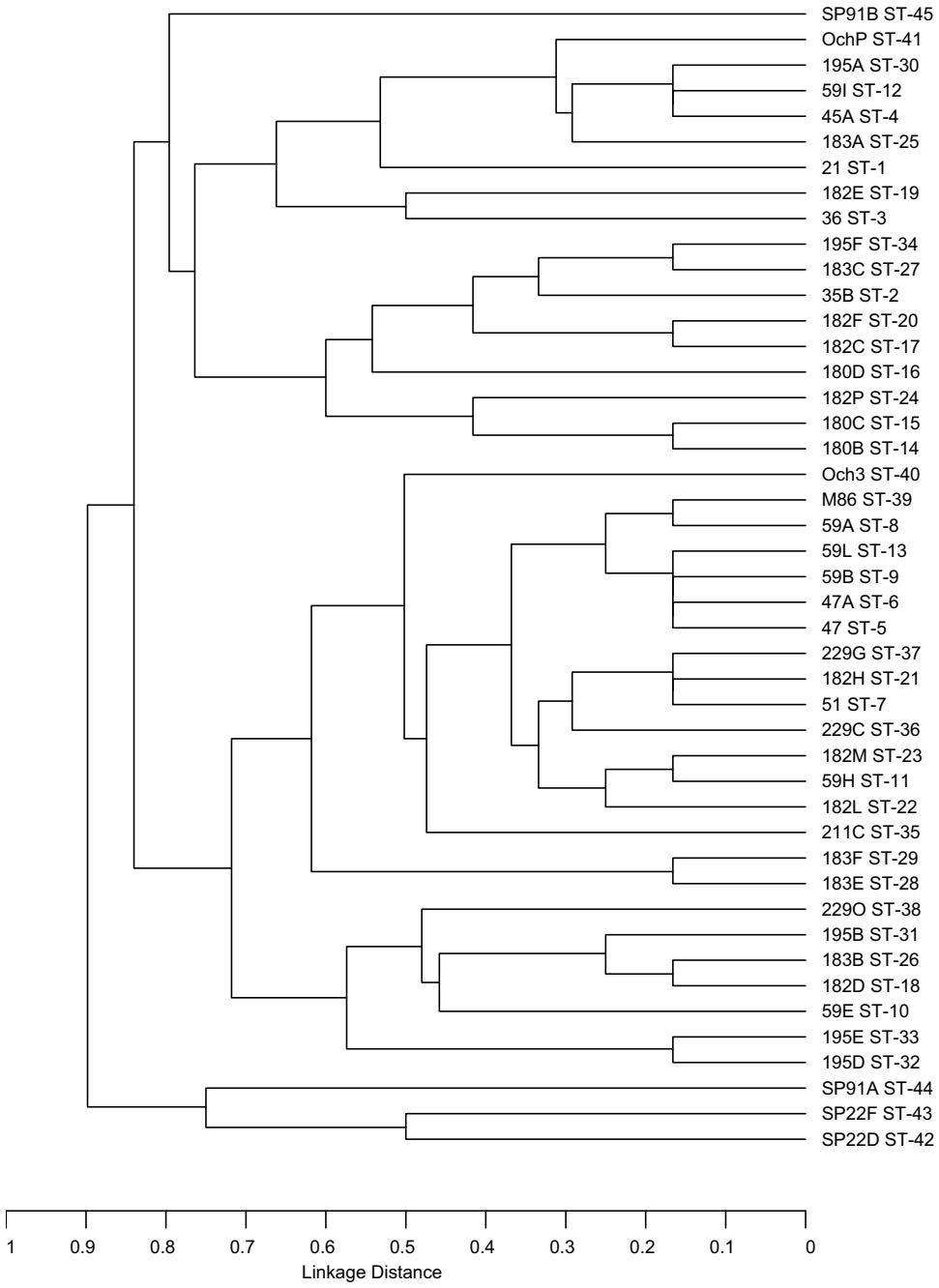

Figure 2 UPGMA Tree of 45 STs showing the linkage distance. The UPGMA Tree was constructed using the START program.

microbial populations that have positive or negative effects on inherent gastric microbiota, remain undefined [17,19].

Sanduleanu et al. showed that the co-occurrence of *H. pylori* and non-*H. pylori* bacteria is associated with a strikingly increased risk of atrophic gastritis and higher cytokine levels than in patients who lack these bacteria. This increase was also greater than in patients with *H. pylori* infection alone. These authors also showed that non-*H. pylori* bacteria could persist in the stomach as antigenic stimulators that enhance the immune response caused by *H. pylori* infection, and their co-infection could promote the development of atrophic gastritis [20]. Interestingly, genomic insights have revealed that *Ochrobactrum* share some features with the *H. pylori* genome, which might facilitate colonization and survival in the gastric environment [3,4].

The commensal nature of *Ochrobactrum* in the present study can be ruled out due to its high

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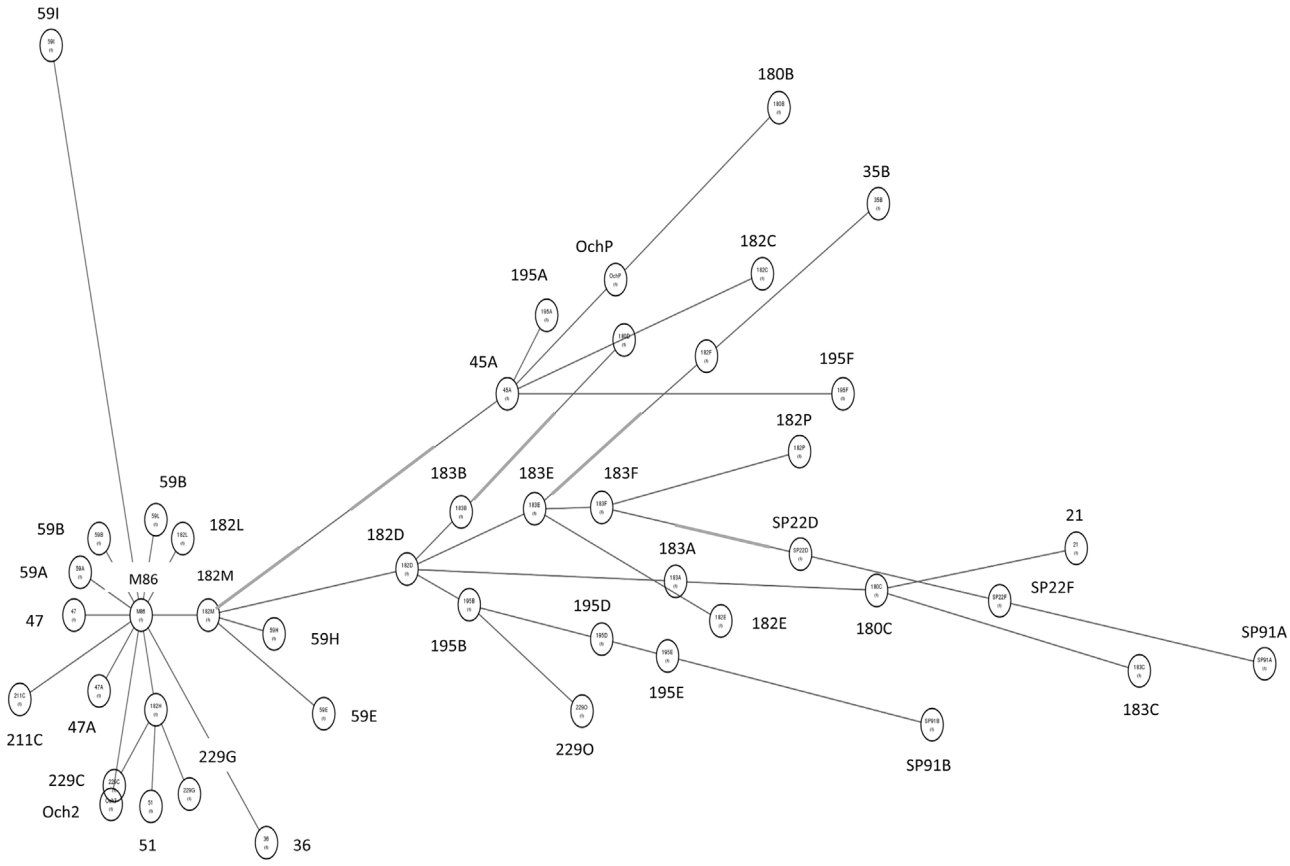

Figure 3 Minimum-Spanning (MS) tree for 45 STs of *O. intermedium* based on the MLST data. The tree is based on the allelic profiles. Each circle corresponds to a sequence type (ST).

prevalence, and it may be dense, as it could be isolated from many antral biopsies. Other possibilities could be that *Ochrobactrum* produce compounds that inhibit or suppress *H. pylori*. The production of compounds and inhibitory effects has not yet been reported. However, *Ochrobactrum* has mainly been observed in post-operative infections affecting immunocompromised individuals, indicating their multidrug resistant properties cause secondary infections. The pathogenic properties of the various *Ochrobactrum* strains need to be explored in animal models, such as gerbils. Earlier studies have reported on the technology-driven evolution of *Ochrobactrum* pathogenicity, and a hostile lifestyle appears to be indicative of the polyspecies prevalence in our study [10].

The present study highlighted that there were no recombination events in the isolated strains. In spite of this, more than one species has been reported. Since 2012, there has been no report of novel *Ochrobactrum* species (from clinical samples), indicating that fewer recombination events are contributing to new species delineation. The presence of polyspecies of *Ochrobactrum* is evident in this study, which merits further evaluation in terms of the etiology and interaction with *H. pylori* as well as the identification of other commensal bacteria in the gastric niche. Antibiotic resistance does not appear to be correlated with species diversity. It appears that *O. intermedium* is as commonly present in the human stomach as *H. pylori*, and the bacterial colonies are purely aerobic and faster growing than *H. pylori*. These additional bacteria may be either pathogenic or protective, conferring negative or positive effects, respectively.

This is the first comprehensive analysis of non-*H. pylori* bacteria, specifically in *Ochrobactrum* spp., to evaluate the population structure in a developing country. The prevalence and multispecies occurrence in the human stomach raises many questions for gastric microbiology. For example, are they present in other geographic regions? Do they have a positive (protective) or negative (virulent) role or are they part of the commensal microbiota? A study from Patel et al. [21] reported that urease-producing bacteria (like *Pseudomonas*) might hinder initial diagnosis using the urease test. Similar findings have also been reported in squirrel

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monkeys with *O. anthropic*; hence, this study sets the groundwork for several interesting avenues in characterizing human gastric microbial diversity.

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Competing interests

None declared.

Ethical approval

Not required.

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