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ORIGINAL ARTICLE

Pseudomonas fluorescens-like bacteria from the stomach: A microbiological and molecular study

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Abstract

AIM: To characterize oxidase- and urease-producing bacterial isolates, grown aerobically, that originated from antral biopsies of patients suffering from acid peptic diseases.

METHODS: A total of 258 antral biopsy specimens were subjected to isolation of bacteria followed by tests for oxidase and urease production, acid tolerance and aerobic growth. The selected isolates were further characterized by molecular techniques *viz.* amplifications for 16S rRNA using universal eubacterial and *HSP60* gene specific primers. The amplicons were subjected to restriction analysis and partial sequencing. A phylogenetic tree was generated using unweighted pair group method with arithmetic mean (UPGMA) from evolutionary distance computed with bootstrap test of phylogeny. Assessment of acidity tolerance of bacteria isolated from antrum was performed using hydrochloric acid from 10^{-7} mol/L to 10^{-1} mol/L.

RESULTS: Of the 258 antral biopsy specimens collected from patients, 179 (69.4%) were positive for urease production by rapid urease test and 31% (80/258) yielded typical *Helicobacter pylori* (*H. pylori*) after 5-7 d of incubation under a microaerophilic environment. A total of 240 (93%) antral biopsies yielded homogeneous semi-translucent and small colonies after overnight incubation. The partial 16S rRNA sequences revealed that the isolates had 99% similarity with *Pseudomonas* species. A phylogenetic tree on the basis of 16S rRNA sequences denoted that JQ927226 and JQ927227 were likely to be related to *Pseudomonas fluorescens* (*P. fluorescens*). On the basis of *HSP60* sequences applied to the UPGMA phylogenetic tree, it was observed that isolated strains in an aerobic environment were likely to be *P. fluorescens*, and *HSP60* sequences had more discriminatory potential rather than 16S rRNA sequences. Interestingly, this bacterium was acid tolerant for hours at low pH. Further, a total of 250 (96.9%) genomic DNA samples of 258 biopsy specimens and DNA from 240 bacterial isolates were positive for the 613 bp amplicons by targeting *P. fluorescens*-specific conserved putative outer membrane protein gene sequences.

CONCLUSION: This study indicates that bacterial isolates from antral biopsies grown aerobically were *P. fluorescens*, and thus acid-tolerant bacteria other than *H. pylori* can also colonize the stomach and may be implicated in pathogenesis/protection.

Key words: Antral biopsy; *Helicobacter pylori*; *Pseudomonas fluorescens*; HSP60; Nested polymerase chain reaction; Acid-tolerant bacteria

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium found primarily in the stomach. It has been implicated in chronic gastritis, gastric ulcers, duodenal ulcers and stomach cancers that were previously believed to be of non-microbial origin[1-3]. There was a misconception that no bacteria could live in the stomach because of its highly acidic environment. For the first time, Steer and Colin-Jones[4] published their results regarding the presence of gram-negative, oxidase- and urease-producing bacteria, but they proposed that it was *Pseudomonas* (*P.*), a contaminant and not related to peptic ulcer. In an effort to grow *H. pylori* from an antral biopsy, we could see that a peculiar type of bacterial colony was growing consistently after overnight incubation while *H. pylori* was taking 3-5 d to grow. These colonies were also oxidase and urease producers, but growing in an aerobic environment. There are reports that show the presence of a variety of bacteria in the stomach by isolation, DNA profiling and polymerase chain reaction (PCR)-based analysis, and some of them are urease producers hindering the specificity of the urea breath test[5-7]. Therefore, we aimed to characterize this type of bacterial isolate and to analyze whether they are colonizers or contaminants.

MATERIALS AND METHODS

Collection of specimens

The study subjects were patients attending inpatient services of the Department of Gastroenterology, University Hospital of Banaras Hindu University, Varanasi, Uttar Pradesh, India. This hospital provides tertiary-level health services for the eastern part of Northern India. The culture isolation, phenotypic and molecular characterizations were carried out in the Department of Microbiology, Institute of Medical Sciences.

Patients and samples

A total of 258 patients suffering from upper gastrointestinal (UGI) diseases like non-ulcer dyspepsia (NUD), peptic ulcer diseases (PUD) including gastric ulcer and duodenal ulcer, and gastric carcinoma were enrolled during a period of 3 years (2007-2010) and three antral biopsy pieces from each patient were collected. Before taking a biopsy, the endoscope was rinsed with detergent followed by water, and disinfected with 2% alkaline glutaraldehyde for 30 min then rinsed with sterile water. In a similar way, biopsy forceps were washed and sterilized and one biopsy forceps was used for one patient exclusively. The biopsy specimens were collected by endoscopic forceps from each individual with full aseptic precautions after taking well-informed consent. The work was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University. Patients with mucosal breaks greater than 5 mm in size with apparent depth were diagnosed as having ulcer and those with ulcerato-infiltrative lesions with positive histology/brush cytology were considered as having stomach carcinoma. The patients not having ulcerative lesions but suffering from dyspeptic symptoms were diagnosed as NUD. Individuals with normal endoscopic findings without gastroduodenal symptoms but having other gut problems were treated as healthy controls. In the present study, those patients were excluded who had a history of previous gastric surgery, active UGI bleeding, chronic alcoholism, intake of antibiotics and proton pump inhibitors during the last 4 wk or those taking non-steroidal anti-inflammatory drugs. Further individuals less than 18 years of age, pregnant or lactating mothers or those having illnesses like cirrhosis, chronic renal failure or ischemic heart disease were also excluded.

Microbiological processing

The three biopsy pieces were pooled and homogenized into phosphate saline buffer together in an all glass disposable homogenizer and were divided into three aliquots. The first aliquot of the tissue homogenate was transferred immediately into a rapid urease test (RUT) medium and the second was plated within 30 min of collection onto the media used for bacterial culture [Mueller Hinton agar without supplement and media containing brain heart infusion agar (Difco, Becton Dickinson, Sparks, MD, United States), supplemented with 7% sheep blood, 0.4% IsoVitaleX, and Skirrow selective supplement (vancomycin 10 μg/mL; polymixin B sulfate 2.5 IU/mL; trimethoprim lactate 5 μg/mL) (Difco, Becton Dickinson, Sparks, MD, United States)]. The non-enriched plate was incubated at 37 ℃ in an aerobic atmosphere while the other was incubated in the presence of 5% O_2, 10% CO_2, and 85% N_2 for 3-7 d. Several small colonies could be seen after overnight incubation in the first plate. The other plate was examined every alternate day after 3-7 d to see if colonies other than those observed after overnight incubation developed. Small translucent colonies developed after 5-7 d of incubation other than those originally after observed overnight incubation on non-enriched nonselective medium. These colonies were further subjected to morphological and biochemical tests *viz.* motility, oxidase, catalase, and urease. All the isolates were divided into two groups on the basis of the incubation period, *i.e.*, those isolates obtained after overnight incubation designated as group A and the other group B possessing those strains isolated after 5-7 d of incubation. The third aliquot was subjected to genomic DNA extraction.

Table 1 Primers used to study *Helicobacter pylori* and *Pseudomonas fluorescens* isolates

Target gene	Primer name	Primer sequence (5′-3′)	PCR condition (number of cycles, size of product)
16S rRNA	16S F	TTGGAGAGTTTGATCCTGGCC	94 ℃, 30 s; 59 ℃, 30 s; 72 ℃, 30 s (30, 1155 bp)
	16S R	ACGTCATCCCACCTTCTC	
HSP60			
Primary	HSP1	AAGGCATGCAATTTGATAGAGGCT	94 ℃, 30 s; 56 ℃, 30 s; 72 ℃, 30 s (35, 594 bp)
	HSP2	CTTTTTTCTCTTTCATTTCCACTT	
Nested	HSPN1	TTGATAGAGGCTACCTCTCC	94 ℃, 30 s; 56 ℃, 30 s; 72 ℃, 30 s (35, 501 bp)
	HSPN2	TGTCATAATCGCTTGTCGTGC	
Putative membrane-bound protein (PFMP)			
Primary	*PFMPF*	TCTKRYCRMGAATCRARACWRYC	94 ℃, 1 min; 50 ℃, 1 min; 72 ℃, 1 min (35, 704 bp)
	PFMPR	GKTWYTGCKCRWWKCSYTSMMC	
Nested	*PFMPNF*	TGCGYWMMWCCYWRWCCWTGA	94 ℃, 1 min; 50 ℃, 1 min; 72 ℃, 1 min (35, 613 bp)
	PFMPNR	AKCABGGTSCWGCMVRCCRBGC	
mupV			
Primary	mupVF	TGAGTTCGATGTGACCTGCCTG	94 ℃, 1 min; 55 ℃, 1 min; 72 ℃, 1 min (35 cycles, 722 bp)
	mupVR	AACTCGCCAGATTGTCGTACAC	
Nested	mupVnF	CAGCATTATCCTGCCACTGAC	94 ℃, 1 min; 55 ℃, 1 min; 72 ℃, 1 min (35, 611 bp)
	mupVnR	ATGATGTCCTGGCACACCTGATC	

PCR: Polymerase chain reaction.

RUT

For the RUT, biopsy specimens were inoculated into 1 mL of 10% urea in deionized water (pH 6.8), to which two drops of 1% phenol red solution was added, and incubated at 37 ℃ for 24 h. A positive result was recorded when the color changed from yellow to pink within 30 min. If there was mild color change within 30 min, the RUT tubes were incubated for a further 24 h.

Subculture at different temperatures

The organisms which appeared after overnight incubation were tested for growth at 4 ℃, 35 ℃ and 42 ℃ onto Muller Hinton agar; incubation was maintained for 14 d to distinguish *Pseudomonas* spp.[8,9]. Those organisms showing growth at 4 ℃ were sub cultured twice and incubated at the appropriate temperature for more than 10 d each time.

Assessment of acidity tolerance of bacteria isolated from gastric niche

Five isolates were suspended into different molar concentrations of hydrochloric acid (10^{-1} to 10^{-7} mol/L corresponding to pH 1.0 to 7.0) and a CFU was maintained as 10^6 CFU/mL. After the intervals of 0 min, 5 min, 10 min, 20 min, 30 min and 60 min, 100 μL acidic suspension (10^5 CFU) were transferred into 3 mL BHI broth. After overnight incubation optical density was recorded with the help of a spectrophotometer at 600 nm wavelength and bacterial count was expressed in CFU/mL. This experiment was repeated twice.

Preparation of genomic DNA for PCR assay

Extraction of genomic DNA from both types of bacterial isolates (A total of 320 strains including groups A + B) as well as from tissue homogenate was performed using a standard proteinase K and phenol-chloroform method[10]. To exclude the possibility of cross contamination of DNA during DNA extraction, one set of double distilled was included in each batch of DNA extraction.

Detection of H. pylori by nested PCR

Confirmation of *H. pylori* was done at a molecular level by nested PCR targeting the conserved *HSP60* gene and its restriction fragment length polymorphism. The reaction was performed in 25 μL final volume containing 10 ng of DNA, 1 U of Taq polymerase (Bangalore Genie, India), 200 mmol/L (each) deoxynucleotide triphosphate (MBI, Fermentas) and 1.5 mmol/L $MgCl_2$ in standard PCR buffer and 10 pmol of each primer as described by Singh *et al*[11]. Primer sequences and PCR conditions are displayed in Table 1. For the internal amplification, the PCR product from the primary cycle was diluted 1/50 and 1 μL was used as the template in the nested PCR. The conditions for the PCR amplification, first and nested reactions were the same. DNA from *H. pylori* reference J99 and a tube containing water in place of DNA were assayed in each PCR run as positive and negative controls, respectively.

After amplification, the PCR products (501 bp) were precipitated with 2.5 volumes of ethanol. The pellets were washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). A 10 μL precipitated amplified DNA sample was then digested with 10 U of restriction enzyme *Hind*Ⅲ in appropriate buffered solution recommended by the manufacturer (Bangalore Genie, India) and incubated for 3 h at 37 ℃. The digested DNA fragments were analyzed by electrophoresis on 2% agarose gels (Bangalore Genie, India) containing 0.5 μg of ethidium bromide per mL. The gel was run at 70 V with TBE (Tris Boric acid EDTA) buffer for 3 h and was examined by a transilluminator and photographed. The sizes of digested DNA fragments were estimated from distances of molecular weight standards and compared with in silico restriction digestion.

Amplified rDNA restriction analysis

For each group of isolates, the 16S rRNA from 8 randomly selected isolates was amplified using forward primer-16SF and reverse primer-16SR[12]. PCR amplification was performed in a thermocycler (Biometra, Germany) according to standard procedures (Table 1). After amplification by universal eubacterial primers, the PCR products (1155 bp) were precipitated with 2.5 volumes of ethanol. The pellets were washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). A 10 μL precipitated amplified DNA sample was then digested with 10 U of restriction enzyme *Eco*R1, in appropriate buffered solution recommended by the manufacturer (Bangalore Genie) and incubated for 3 h at 37 ℃. The visualization of the restriction fragment was done by the same method as described in the previous paragraph.

Sequencing

The amplified PCR products were purified from salts and primers using QIA quick PCR purification kit (Qiagen, United States). A total of 8 purified amplicons generated targeting *HSP60* and 2 amplicon 16S rRNA genes were outsourced for partial sequencing to Bangalore Genei, India. Sequences were analyzed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) to verify the identity of the sequences: whether *H. pylori* or some other microorganism.

Sequence analyses

Reference sequences of *Pseudomonas* group and other enteric pathogens used for phylogenetic analyses were retrieved from Genbank. The partial 16S rRNA sequences and HSP60 sequence for the strains were aligned with reference sequences using Clustal X version 1.81, with default parameters[13]. Phylogenetic and molecular evolutionary analyses were performed using *MEGA* version 4[14]. The phylogenetic tree was generated using the unweighted pair group method with arithmetic mean (UPGMA) from evolutionary distance computed with bootstrap test of phylogeny. The degree of statistical support for branches was determined with 500 bootstrap replicates.

Primers designed for Pseudomonas fluorescens targeting putative membrane-bound protein

The putative membrane-bound protein coding gene present in all the strains of *P. fluorescens* is available in NCBI Genbank. Due to a similarity of sequences of about 55% in *P. fluorescens,* we therefore planned to design nested degenerate primers to amplify the partial sequence of putative membrane bound protein so that it was able to amplify all strains of *P. fluorescens*. Forward and reverse oligo-nucleotide degenerate primers derived from the region located between bases 92072 and 92775 of the *Pseudomonas fluorescens* (*Pseudomonas fluorescens* Pf0-1; GenBank Accession number CP000094.2 and GI: 253992019) were synthesized. Internal primers were derived from the region between bases 92114-92726 for nested PCR. DNA extracted from *P. putida* and *P. aeruginosa* was used to monitor sensitivity against *P. putida* and *P. aeruginosa*. During the PCR assay in each batch, Mili Q water was used as a template to ensure that there was no contamination by water and PCR reagent[15].

Pseudomonas fluorescens NCIMB 10586 mupirocin biosynthetic gene

Forward and reverse oligonucleotides were derived from the conserved region located between bases 68101 and 68822 of the mupirocin biosynthetic gene cluster of *Pseudomonas fluorescens* (NCIMB 10586). An internal primer was derived from the region between bases 68796 and 68796 (GenBank Accession number AF318063.2; gene GI: 20150006). PCR amplification was similar to amplification of *HSP60* gene and conditions are described in Table 1.

Randomly amplified polymorphic DNA PCR

Fingerprinting of 71 randomly selected strains from group A was performed based on randomly amplified polymorphic DNA (RAPD) PCR methods by using primers RAPD3 5'-TACAGCTCG-3'and RAPD5 5'AGCACTGCCT-3' (this study). PCR was carried out in 25 μL volume using 10 ng of genomic DNA, 1 U of Taq polymerase (Bangalore Genie, India), and 15 pmol of each primer (Bangalore Genie), 200 mmol/L (each) deoxynucleotide triphosphate (Bangalore Genie, India) and 2 mmol/L $MgCl_2$ in standard PCR buffer. Amplification reactions were carried out in a thermal cycler (Biometra, Goettingen, Germany).

The gel images were analyzed under ultraviolet light in a gel documentation system (Alpha Innotech, United States). Cluster analysis of all the isolates was done on the basis of the fingerprint generated. Based on the presence or absence of different DNA fragments in the fingerprints of the *P. fluorescens* strains, a binary data matrix was created. Overall similarity between the pair of strains was calculated from the binary data matrix using the simple matching-dice coefficient. The resulting similarity matrix was used as the input data for cluster analysis by NTSYS pc2.0 programme of UPGMA[16].

Statistical analysis

The level of significance between the two proportions, *i.e.*, culture rates and molecular detection rates, was calculated by Fischer's Exact Probability test.

RESULTS

Bacteriological findings

Of the 258 antral biopsy specimens collected from patients, 69.4% (179/258) were found to be positive by RUT and 31% (80/258) by culture for typical *H. pylori,* after 5-7 d of incubation under a microaerophilic environment; these were gram-negative curved rods and were positive for oxidase, catalase and urease. However, 258 antral biopsies yielded 240 (93%) homogeneous semi-translucent and small colonies after overnight incubation. These isolates also grew aerobically but the colonies had

Table 2 Comparative isolation rates and prevalence of *Helicobacter pylori* and *Pseudomonas fluorescens* against rapid urease test in antral biopsies by nested polymerase chain reaction targeting *HSP60* gene and membrane bound protein *n* (%)

Diseases	Antral biopsies	RUT, positivity	*H. pylori HSP60*, positivity	*P* value[1]	*P. fluorescens* putative outer membrane protein, positivity	*P* value[2]	Isolation of different types of bacteria, positivity		*P* value[3]
							Group A	Group B	
PUD	65	51 (78.5)	59 (90.8)	< 0.001	63 (96.9)	< 0.010	61 (93.8)	23 (35.4)	< 0.001
NUD	123	92 (74.8)	109 (88.6)	< 0.001	121 (98.4)	< 0.001	119 (96.7)	39 (31.7)	< 0.001
CA	49	23 (46.9)	24 (48.9)	< 0.050	46 (93.9)	< 0.001	43 (87.5)	12 (24.5)	< 0.001
Normal	21	13 (61.9)	19 (90.4)	< 0.001	20 (95.2)	0.001	17 (80.9)	6 (28.6)	< 0.001
Total	258	179 (69.4)	211 (81.8)	< 0.001	250 (96.9)	< 0.001	240 (93.0)	80 (31.0)	< 0.001

[1]$P < 0.001$ between *Helicobacter pylori* (*H. pylori*) and nested polymerase chain reaction (PCR); [2]$P < 0.001$ between *H. pylori* and *Pseudomonas fluorescens* (*P. fluorescens*); [3]$P < 0.001$ between colonies appearing after overnight incubation group (group A) and colonies appearing after 3-7 d of incubation group (group B). Group A: Non-ulcer dyspepsia (NUD) *vs* gastric carcinoma (CA), $P = 0.02$; NUD *vs* normal, $P < 0.02$; *H. pylori*: Peptic ulcer diseases (PUD) *vs* CA, $P < 0.001$; NUD *vs* CA, $P < 0.001$; CA *vs* Normal, $P = 0.001$; Overall: Culture of *P. fluorescens* *vs* nested PCR, $P < 0.001$. Normal: Patients whose endoscopic findings were normal; RUT: Rapid urease test; RUT: Rapid urease test.

an opaque, small character contrary to the translucent one which is typical for *H. pylori* (Table 2).

Biochemical characterization

A total of 100 isolates randomly selected from group A were subjected to extensive phenotypic characterization. All of them were gram-negative, oxidase-, catalase- and urease-positive. All were non-fermenters, showed variable nitrate reduction and failed to utilize simple sugars (glucose, lactose, sucrose, mannitol and maltose). Citrate was utilized by all of them. All the tested strains were Methyl Red negative and Voges-Praskauer negative or equivocal. All the strains were oxidase- and catalase-positive. Indole test was negative but on mixing with the Kovac's reagent a typical greenish color developed. All these isolates were able to multiply at 4 ℃. These findings intimated that those isolates which appeared after overnight incubation were *P. fluorescens*.

Acid tolerance assay

The bacterial count of *P. fluorescens* was 7.3×10^8, 6.9×10^8, 7×10^8, 6.1×10^8, 7.6×10^8, 6.5×10^8 and 9.8×10^8 CFU/mL for 0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min after acid exposure (pH 1.0). Average bacterial counts were 9.3×10^8, 9.8×10^8, 1.0×10^9, 9.5×10^8 and 9.8×10^8 CFU/mL for acid tolerance of low pH 2, pH 3, pH 4, pH 5, pH 6 and pH 7, respectively, for different time intervals (0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min). Similarly, bacterial growth was approximately the same in control experiments where acidic solution was replaced by LB Broth. The exposure to acidic pH showed that *P. fluorescens* growth was not killed by exposure to lower pH 1.0 for an hour.

Amplification and RLFP of HSP60 gene

Isolates from group B, which grew under microaerophilic environment, were subjected to amplification by primers specific for *HSP60* gene of *H. pylori*. All the 80 isolates from 258 patients were positive for the 501 bp of amplicon for the corresponding gene. However, 211 (81.8%) of 258 antral biopsies were positive for *H. pylori* DNA as the 501 bp amplicon was produced by nested PCR. All nested amplicons of 501 bp were restricted into two fragments of 310 bp and 191 bp by the *Hind*Ⅲ restriction enzyme (Figures 1 and 2). Although colonies grown after overnight incubation could give amplification with first round of PCR primers, nested PCR did not generate 501 bp amplicon specific to *H. pylori*. Similarly, 240 aerobic isolates could not amplify in second round PCR.

16S rRNA amplified rDNA restriction analysis

The isolates analyzed by using *Eco*R1 restriction endonuclease enzyme on amplicons generated by 16S rRNA specific primers fell into two groups: group A isolates could not be restricted, while group B isolates which were restricted into two fragments of 635 bp and 520 bp which were similar to *in silico* restriction of *H. pylori* J99 (Figure 3).

Identification on the basis of 16S rRNA and HSP60 gene sequence

The partial nucleotide sequence of 16S rRNA of 2 isolates (GenBank accession number JQ927226 and JQ927227) from group A represented no restriction site for *Eco*R1 restriction enzyme, and comparison of the nucleotide sequences with the NCBI database showed similarity of 99% with *P. fluorescens*. Similarly, partial nucleotide sequence of unrestricted amplified *HSP60* gene (590 bp) of the isolates that grew after overnight incubation aerobically also showed 96% similarity with *P. fluorescens*.

Phylogenetic sequence analysis

The partial sequence of the 16S rRNA gene of two *P. fluorescens*-like isolated strains and sequences of *16S rRNA* gene from 34 reference strains representative of the principal *Pseudomonas* phyla and 12 other bacterial pathogens were used for comparison of a phylogenetic tree. Similarly, a partial sequence of *HSP60* gene of 8 *P. fluorescens*-like isolates and sequence of *HSP60* gene of 5 *P. fluorescens* reference strains and 6 other reference strains representative of the principal *Pseudomonas* phyla and 11 other clinical pathogens were used to prepare a phylogenetic tree. To simplify the comparisons for the resulting phylogenetic tree by the UPGMA method, we named clusters based on *16S rRNA* gene data, r-clusters, and those based on

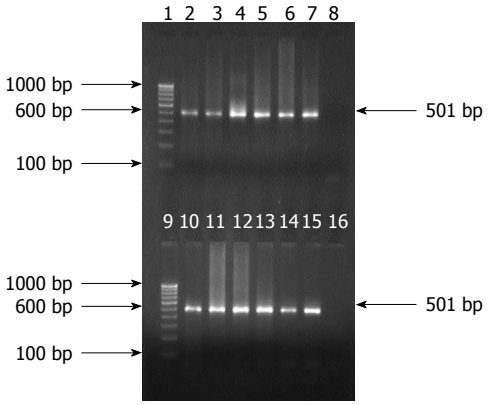

Figure 1 Amplification of partial 501 bp *HSP60* gene with specific nested primer for *Helicobacter pylori* in antral biopsies and culture isolates. Lanes 1 and 9: Molecular marker (100 bp); Lane 2: Positive control; Lanes 3 to 7: gDNA from antral biopsies; Lanes 8 and 16: Negative control; and Lanes 10 to 15: Bacterial gDNA from culture isolates.

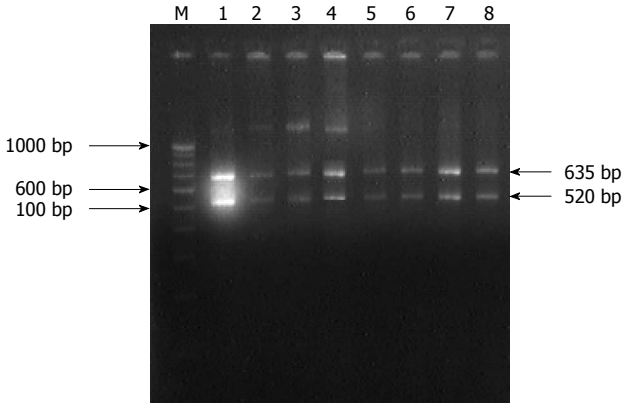

Figure 3 Gel picture of amplified rDNA restriction analysis. Lane M: 100 bp molecular marker; Lanes 1 to 8: Digested 1155 bp 16S rRNA amplicon into two fragments (635 bp and 520 bp) of culture isolates of *Helicobacter pylori*.

HSP60 data, h-clusters. The tree showed, on the basis of 16S rRNA sequences, it can be grouped clearly into three r-clusters on the basis of number of bootstraps *i.e.*, 90, 78 and 60. Cluster r-1 represents a fluorescens group along with two sequences submitted to gene bank (accession number as JQ927226 and JQ927227 for RCa25 and RCa24 respectively). Cluster r-2 represents *Pseudomonas aeruginosa, Pseudomonas syringae, Pseudomonas stuzeri, Pseudomonas chlororaphis, Pseudomonas putida, Pseudomonas pertucinogena* group and 4 *P. fluorescens* reference strains, and r-3 cluster represents other microbes and enteric pathogens rather than non-*Pseudomonas* spp. From cluster analysis it is clear that JQ927226 and JQ927227 are related to *P. fluorescens* (Figure 4).

The tree shows that, on the basis of *HSP60* sequences, three h-clusters could be observed (bootstraps number, 99, 63 and 71): the sequences of seven isolated strains from the gastric niche could be grouped in h-cluster I along with *P. fluorescens* strains while cluster II represents non-fluorescens pseudomonas species with the exception of *P. fluorescens* F113 (CP003150). The h-cluster III grouped non-*Pseudomonas* sp. including enteric pathogens along with *H. pylori*.

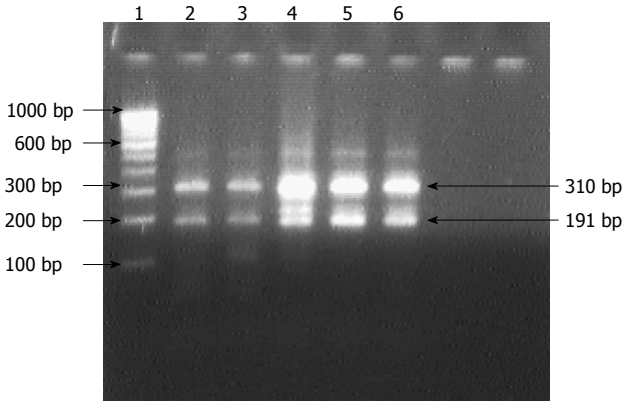

Figure 2 Electrophotograph of restriction digestion of *HSP60* gene of *Helicobacter pylori* isolates with *Hind*Ⅲ. Lane 1: 100 bp molecular marker; Lanes 2 to 6: Restricted 501 bp *HSP60* gene amplicon into two fragments (310 and 191 bp) of culture isolates of *Helicobacter pylori*.

Interestingly, one strain of *P. fluorescens* RCa 24 fell into this III cluster with closeness to *Stenotrophomonas maltophilia* and *Bordetella pertusis* (Figure 5).

Pseudomonas fluorescens specific PCR

One specific pair of primers targeting putative outer membrane protein was used to identify *P. fluorescens*. The other specific primers targeting mupirocin biosynthetic gene were used to screened out whether any *P. fluorescens*-like isolate was producing mupirocin. Genomic DNA extracted from group-A isolates and all biopsies was used as template for PCR amplification. A total of 250 (96.9%) out of 258 biopsy specimen genomic DNA samples and 240 bacterial isolates were positive for the amplification of the corresponding gene, *i.e.*, the putative outer membrane protein gene sequences (Table 2 and Figure 6). Although putative outer membrane protein gene targeting primers were degenerate, they were unable to produce the 613 bp amplicon from *Pseudomonas aeruginosa* and *Pseudomonas putida* at similar PCR conditions. The mupirocin biosynthetic gene targeting primers were unable to produce amplification of the 722 bp or 611 bp amplicon either in primary or secondary round PCR, respectively, from any of the isolates (data not shown).

RAPD

All the 71 strains tested from group-A yielded significant PCR products with RAPD primers. The strains generated approximately 3-13 well-defined bands between 150 bp to 2.5 kb sizes (9 bands on average) with each isolate yielding a unique profile of products. Cluster analysis with a RAPD-PCR based method showed that only a few isolates exhibited an identical profile. Nearly all the isolates appeared as dissimilar from each strain, but 5 strains isolated from cancer patients showed similar banding pattern at 0.0 coefficient (Figures 7 and 8).

DISCUSSION

Traditionally, the human stomach has been viewed as an

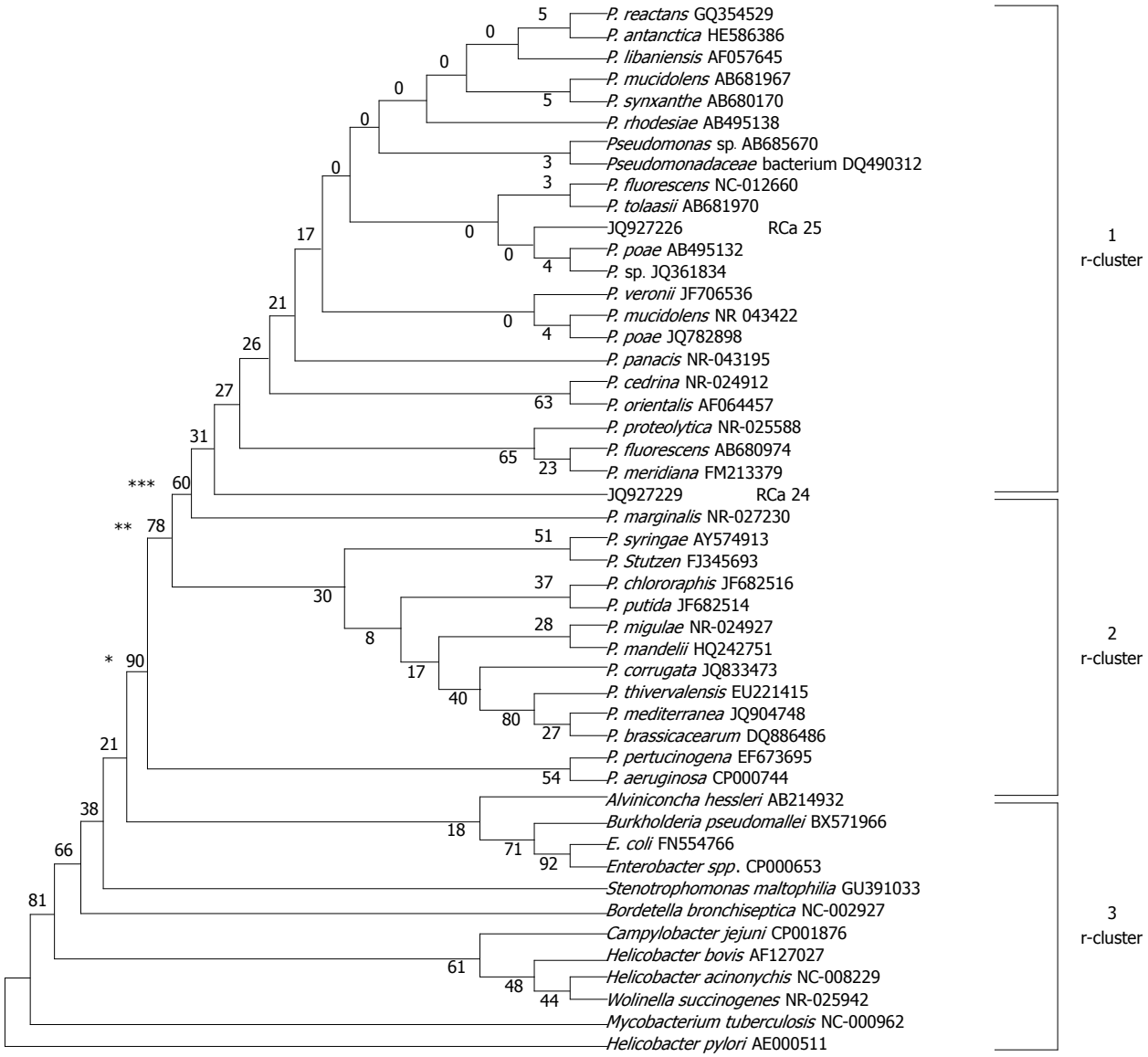

Figure 4 Phylogenetic affiliation of the *Pseudomonas fluorescens*-like isolates (*n* = 2; JQ927226 and JQ927227). The unrooted tree was generated using unweighted pair group method with arithmetic mean from evolutionary distance computed with bootstrap test of phylogeny using *MEGA* version 4 by aligning published sequences from Genbank of *16S rRNA* genes from 34 reference strains representative of the principal *Pseudomonas* (*P.*) *phyla* (accession number followed by species name in parentheses). For checking relatedness with other genera, we included *16S rRNA* gene sequences from GenBank of 12 bacterial pathogens namely *Stenotrophomonas maltophilia*, *Bordetella bronchiseptica*, *Wolinella succinogenes*, *Helicobacter pylori*, *Helicobacter acinonychis*, *Escherichia coli* (*E. coli*), *Enterobacter* spp., *Campylobacter jejuni*, *Burkholderia pseudomallei*, *Alviniconcha hessleri*, *Helicobacter bovis* and *Mycobacterium tuberculosis*. Branches found by maximum likelihood are labeled with asterisks: one asterisk if bootstrap values = 90%, two asterisks if = 78% and three asterisks if = 60%.

inhospitable environment for microorganisms because of its acidic environment along with several other antimicrobial factors. With the discovery of *H. pylori* and other gastric helicobacters, and subsequent insight into the mechanisms by which these organisms adapt to the gastric environment[17], the existence of a bacterial community adapted to this human niche seems quite plausible. This is the first report of its kind showing the presence of a *P. fluorescens*-like bacterium in the human stomach. We isolated the bacteria from NUD, gastric ulcer, duodenal ulcer and gastric carcinoma patients with the belief that only *H. pylori* is associated with acid peptic diseases. However, *P. fluorescens* grew on a simple medium like Mueller-Hinton agar without an antibiotic supplement. The small white colonies had Gram-negative slightly curved rods and produced oxidase, urease and catalase enzymes. These colonies appeared after overnight incubation in an aerobic environment. These isolates exhibited growth at 4 ℃, which is one of the key characteristics of *P. fluorescens*[9]. When these isolates were exposed to acidic pH as low as pH 1.0 for 1 h, the subsequent growth of the bacteria was not affected, indicating that they were acid tolerant. However, due to deviation from the classical *H. pylori* growth characteristics, we were prompted to consider them as non-*H. pylori* and submit them for further characterization. For the purpose, *H. pylori* specific primers for amplifying *HSP60* gene-specific sequences were applied[11]. However, an approximate 600 bp sized amplicon

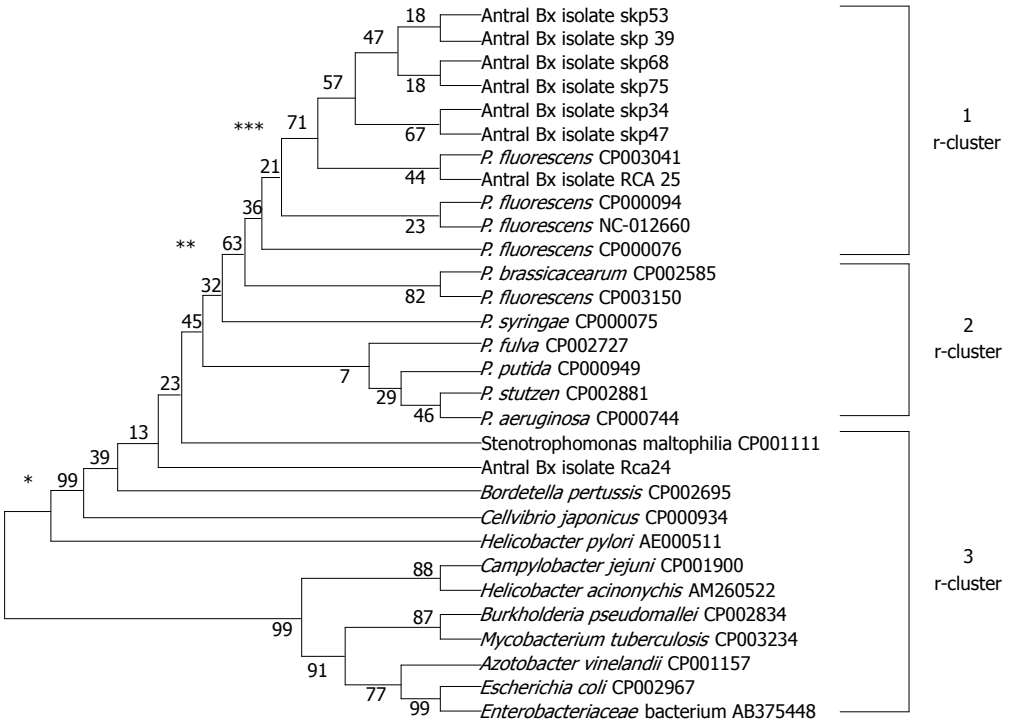

Figure 5 Phylogenetic affiliation of the *Pseudomonas fluorescens*-like isolates (*n* = 8; partial sequences of *HSP60*) on the basis of *HSP60* gene sequences. The unrooted tree was generated using unweighted pair group method with arithmetic mean from evolutionary distance computed with bootstrap test of phylogeny using *MEGA* version 4 by aligning published sequences from Genbank of heat shock protein (*HSP60*) genes from 5 reference strains of *Pseudomonas fluorescens* (*P. fluorescens*), 6 other reference strains representative of the principal *Pseudomonas phyla* (accession number followed by species name in parentheses). For checking relatedness with other genera, we included *HSP60* gene sequences from GenBank of 11 bacterial pathogens, namely *Helicobacter pylori*, *Helicobacter acinonychis*, *Campylobacter jejuni*, *Enterobacter* spp., *Escherichia coli*, *Bordetella pertussis*, *Burkholderia pseudomallei*, *Stenotrophomonas maltophilia*, *Cellvibrio japonicus*, *Azotobacter vinelandii* and *Mycobacterium tuberculosis*. Branches found by maximum likelihood are labeled with asterisks: one asterisk if bootstrap values = 99%, two asterisks if = 63% and three asterisks if = 71%.

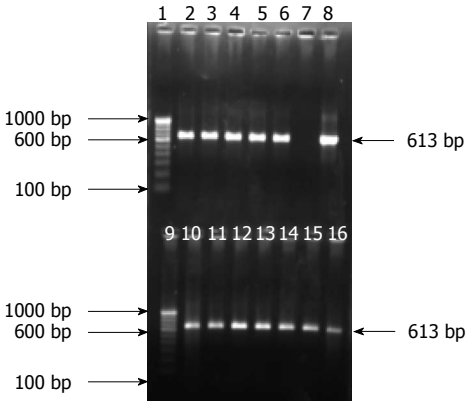

Figure 6 Amplification of putative outer membrane protein gene with specific nested primer generating 613 bp amplicon for *Pseudomonas fluorescens* of antral biopsies and culture isolates. Lanes 1 and 9: Molecular marker (100 bp); Lanes 2 to 6: gDNA from antral biopsy specimens; Lane 7: Negative control; Lane 8: Positive control; and Lanes 10 to 16: Bacterial gDNA from culture isolates.

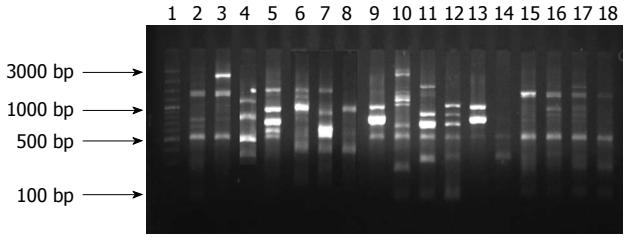

Figure 7 Representative gel showing amplification of *Pseudomonas fluorescens* genomic sequences by randomly amplified polymorphic DNA primers. Randomly amplified polymorphic DNA (RAPD) 3 5'-TACAGCTCG-3' and RAPD5 5'-AGCACTGCCT-3'. Lane 1: 100 bp molecular marker; Lanes 2 to 18: *Pseudomonas fluorescens* isolates isolated from stomach.

was produced by the first round of PCR, but a restriction site specific for *H. pylori* for *Hind*Ⅲ enzyme was absent in these amplicons. Further, none of these isolates could yield a 501 bp amplicon for *H. pylori* by nested PCR primers. Thus, the possibility of these isolates being *H. pylori* was excluded. On the other hand, the typical *H. pylori* isolates which grew after 5-7 d of incubation yielded 501 bp amplicon by nested PCR protocol targeting the *HSP60* gene.

Identification based on partial nucleotide sequencing of 16S rRNA of the representative isolates showed 99% sequence similarity with γ-*protobacteria*. On the basis of enzyme restriction analysis as well as partial nucleotide sequencing of 16S rRNA, the strains isolated from the acidic environment of the stomach either could be grouped in the genus *Pseudomonas* or with a closely related new genus. Further, on the basis of blasting of partial nucleotide sequences of *HSP60* gene sequences on the NCBI gene data bank, isolated strains could be grouped with *P. fluorescens*. On the basis of h cluster based on the

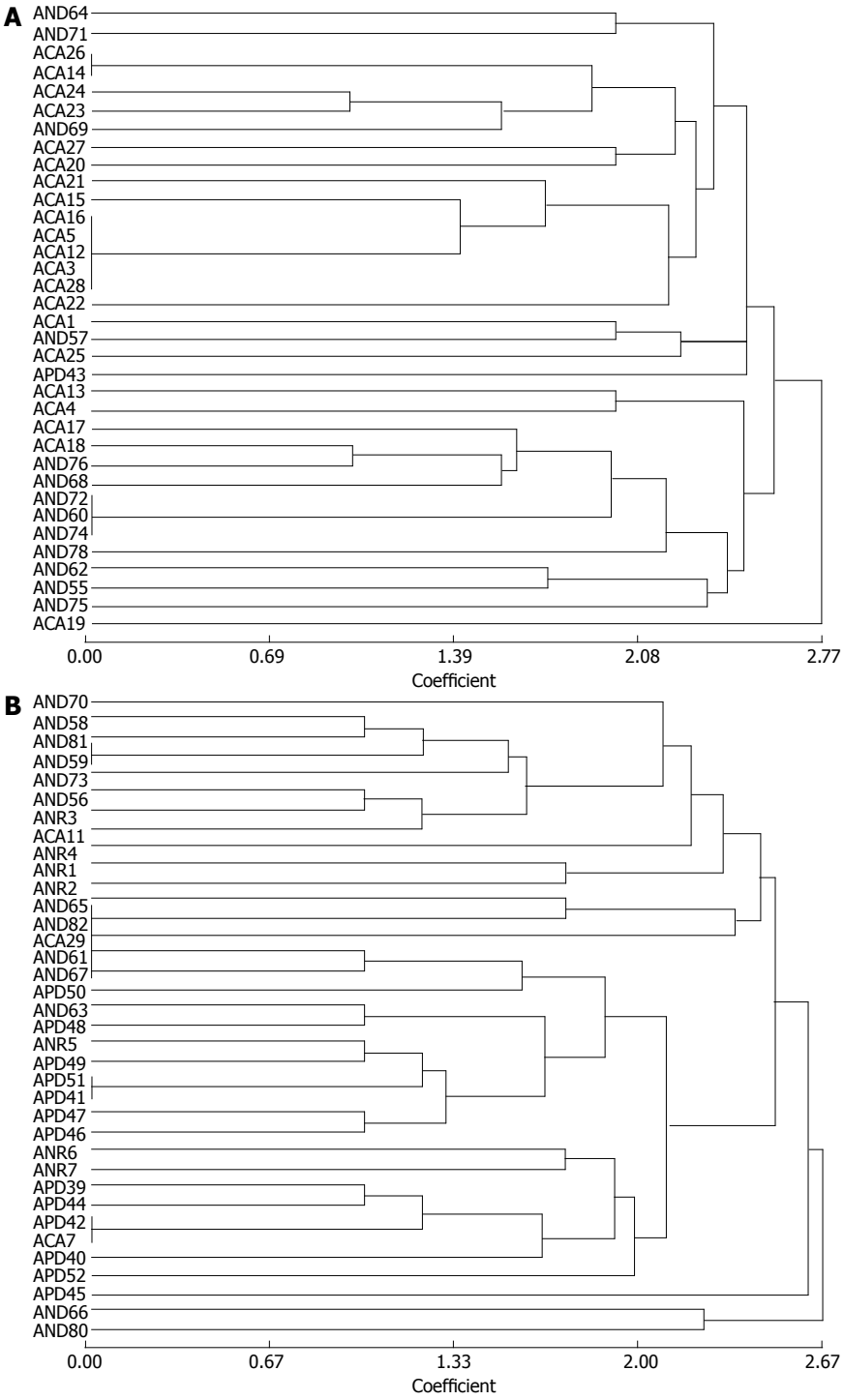

Figure 8 Dendrogram generated by unweighted pair-group method, arithmetic mean on basis of banding pattern of randomly amplified polymorphic DNA for *Pseudomonas fluorescens*. A: 1-35 out of 71 randomly selected isolates of *Pseudomonas fluorescens* from group A (n = 35); B: 36-71 out of 71 randomly selected isolates of *Pseudomonas fluorescens* from group A (n = 36).

UPGMA phylogenetic tree, it may be concluded that the *HSP60* gene sequence has better discriminatory power than 16S rRNA. Further, as one of the isolates could be grouped with *Stenotrophomonas maltophilia* and *Bordetella pertusis,* the possibility of the presence of bacteria other than *P. fluorescens* may not be denied.

The relatively conserved *16S rRNA* gene of *P. fluorescens* has been targeted for PCR-based amplification in culture isolates, but with variable specificity[18]. Therefore, we decided to target a conserved putative membrane-bound protein gene of *P. fluorescens*. The putative membrane-bound protein specific primer screened in the present study was found to be specific for *P. fluorescens* and it yielded an amplicon of 613 bp, confirming the isolates as being *P. fluorescens*. Mupirocin is known for its antibacterial activity and has been reported in only one strain of *P. fluorescens* (NCIMB 10586). A mupirocin-producing gene cluster could not be traced in any of the *P. fluorescens*

isolates despite confirmation by 3 genes, *i.e.*, 16S rRNA, *HSP60* and putative membrane bound protein genes. None of our isolates showed the presence of *mup*V (mupirocin). It is quite possible that this gene might be either absent in all the strains or have too much polymorphism at the loci of primer annealing[19]. We could isolate the *P. fluorescens* strain from 93% of antral biopsies of the patients suffering from gastric diseases. These isolates were further confirmed by PCR-based amplification targeting 3 conserved genes. In DNA of antral biopsy tissue also, 98.4% of NUD and 93.9% of gastric cancer patients were found to be positive for the *P. fluorescens* DNA by nested PCR, while 95.2% of normal stomach had *P. fluorescens* and 96.9% of patients with PUD were found to have the bacterium. Thus our observation indicates a high prevalence and density of these non-*H. pylori* bacteria in the gastric mucosa of patients. Further, to ascertain that the isolated *P. fluorescens* were not contaminants, we carried out whole genome fingerprinting of the 71 randomly selected isolates by using RAPD[20,21] with two RAPD primers. Cluster analysis indicated that almost all the strains had different banding patterns with only a few exceptions, confirming that the isolates were of different clones and thus ruling out the possibility of cross contamination. Moreover, from time to time, we did specific PCR amplification for the *P. fluorescens* on DNA extracted from samples of tap water and washouts of the endoscope, which never yielded the required amplicon even by nested protocol for putative outer membrane protein.

It is interesting to mention that before the implication of *H. pylori* in acid peptic diseases and stomach cancer, Steer and Collin-Jones[4] reported that 80% of antral biopsies have *Pseudomonas* spp. There are reports showing the presence of non-*Helicobacter* bacteria in gastric biopsies of patients suffering from gastric atrophy[22,23]. In cases of reduced gastric acidity due to antacids, it also has been reported that there is presence of several other bacteria[24-26]. Two more studies based on profiling of bacterial flora by temperature gradient gel electrophoresis, 16S rRNA sequence analysis[7] and molecular analysis of the bacterial microbiota[27] have shown presence of several bacterial species including *Pseudomonas* in the stomach. This bacterium seems to be able to colonize the stomach due to its ability to produce urease enzyme. Similarly, it was reported that a non-*H. pylori* bacterium, *Ochrobacrum anthropi,* could be implicated in causation of gastritis in the Squirrel monkey[6].

Such a high prevalence of *P. fluorescens* in human stomach raises many questions: Is it prevalent in the stomach of patients of other subcontinents and continents? Does it have any pathogenic role? Does it have some protective role? Is it simply a part of commensal flora of human stomach? There is a study from Venezuela[5] reporting that *Pseudomonas* strains may interfere with the identification of *H. pylori*. They suggested that one should not rely on rapid urease, catalase and oxidase tests for identification of *H. pylori*. However, studies are needed to observe and identify the presence of *P. fluorescens* in other parts of the world also. With regard to its pathogenic potential, *P. fluorescens* is known as an unusual pathogen of humans. It has been reported as causing septicemia in humans, especially associated with transfusion and cancer[28-30]. Moreover, there is a report indicating that *P. fluorescens* encodes the Crohn's disease-associated I^2 sequence and T cell super antigen, thus implicating it in the pathogenesis of Crohn's disease[31].

However, its commensal state in the stomach may be speculated strongly due to its prevalence in stomach at such a high level and density (it could be isolated in the majority of the antral biopsy). Assumptions may be made that *P. fluorescens* might be producing some antibacterial substances, such as is produced by *P. aeruginosa*, *P. aeruginosa* is known for producing 4-hydroxy-2-alkylquinoline which is inhibitory *in vitro* to *H. pylori*[32]. Moreover, one of the *P. fluorescens* strains is already known for production of mupirocin which is very effective against methicillin-resistant *Staphylococcus aureus*. This bacterium has been stated to have a probiotic role in the gills of fish[33]. Furthermore, low isolation of *H. pylori* and fewer incidences of acid peptic diseases including gastric cancer in North Indians may also be speculated on the basis of the probiotic activity of *P. fluorescens* in the stomach. In addition, there are reports of the unique property of *P. fluorescens* to inhibit the growth of other bacteria, fungi and nematodes causing plant pathology[34-40].

In view of the suggestions made by previous studies[41-43], there is a strong need to explore the exact role of *H. pylori* in stomach diseases because the commensal role of *H. pylori* cannot be rejected outright. In a similar way, the observations made in the present study strongly indicate that further exploration of the different aspects of associations of *P. fluorescens* with human disease and health should be carried out. The pathogenic potential may be explored in animal models like gerbils.

This study concludes that *P. fluorescens* is as common as *H. pylori* in the stomach of humans. Colonies that appeared on enriched BHI agar after 72 h of strictly microaerophilic incubation were *H. pylori* while those growing faster in an aerobic atmosphere were different. These different growths could be identified as *P. fluorescens*. The activity of *P. fluorescens* in the stomach may be speculated to be either pathogenic or probiotic.

COMMENTS

Background

Although *Helicobacter pylori* (*H. pylori*) has been implicated in acid peptic diseases along with stomach cancer, there are reports indicating the presence of several other bacterial species in the stomach. Antral biopsies from North Indian subjects frequently yielded a bacterial growth on selective, non-enriched simple medium in an aerobic environment at 37 ℃ of small, low convex, and pinhead-size translucent colonies. Presence of these types of growth provoked questions about its characterization and its status: whether it was a contaminant from the environment during antral biopsy collection.

Research frontiers

The big question to be answered is further characterization of these isolates and to ensure that they are actual colonizers of the stomach. Do these acid tolerant isolates have pathogenic potential if they are real colonizers?

Innovations and breakthroughs

Pseudomonas fluorescens (*P. fluorescens*)-like bacteria colonize the stomach quite frequently of North Indian patients at high density, as polymerase chain reaction (PCR)-based detection and isolation rates were both comparable. In contrast, the density of *H. pylori* seems to be quite low as nested PCR-based detection is significantly high as compared to the isolation rate of the bacterium. *P. fluorescens* isolates are urease producers and acid tolerant. Although the mupirocin gene could not be detected in any of the *P. fluorescens* isolates, the probiotic (inhibitory to *H. pylori*) role of the bacterium in the stomach may be speculated.

Applications

The potential of *P. fluorescens* as a probiotic may be explored because despite very high prevalence of *H.pylori* in India the incidences of acid peptic diseases and stomach cancer are quite low.

Terminology

Nested polymerase chain reaction is a modified technique of PCR intended to increase sensitivity and specificity of primer and to reduce the contamination in amplicons due to the amplification of undesired primer annealing sites. Phylogenetics is the study of evolutionary relations among groups of organisms such as strains or species, which are based on molecular sequencing data matrices. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, *e.g.*, lactic acid bacteria.

Peer review

The study is well carried out from the methodological perspective. This manuscript characterized the bacterial isolates growing aerobically from antral biopsies were *P. fluorescens*, which was the acid tolerant bacteria other than *H. pylori*. On the basis of 16S rRNA and HSP60 sequence and from phylogenetic sequence analysis these organisms were closely related to *P. fluorescens*. The authors also reconfirmed this unknown bacterium as *P. fluorescens* by PCR positivity of *P. fluorescens* specific conserved putative outer membrane protein gene. Finally, the authors concluded that *P. fluorescens* is as common as *H. pylori* in the human stomach.

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