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IDENTIFICATION OF CagA HARBORING *HELICOBACTER PYLORI* FROM BUCCAL SWAB COLLECTED FROM TOBACCO ADDICTED WOMEN SUBJECTS

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ABSTRACT

Helicobacter pylori is recognized as the major etiological agent of chronic gastritis, gastric adenocarcinoma and lymphoma. The present study was carried out to investigate the presence of *Helicobacter pylori* in buccal salivary sample collected from tobacco addicted women from India and to study certain virulence properties including detection of the presence of cancer producing gene *cagA*. Here, sixteen bacterial isolates were identified as *Helicobacter pylori* and they were different in their colony morphology i.e., rugose (37.5%), opaque (62.5%). All of them were motile, protease positive, highly biofilm forming. All of them were found resistant to chloramphenicol, tetracycline but sensitive to ampicillin and kanamycin. But, resistance was found to amoxicillin, trimethoprim and streptomycin for 50%, 25% and 12.5% isolates, respectively. PCR analysis revealed five isolates to possess *cagA* gene. Results of the present study predicted that these *Helicobacter pylori* isolates may be a potential threat for developing gastric infection in future.

KEY WORDS: Saliva, *Helicobacter pylori,* antibiotic susceptibility, biofilm*, cagA*.

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INTRODUCTION

Helicobacter pylori has been reported as the etiological agent of chronic gastric inflammation which is one of the most common infections of humans that can progress further to a variety of diseases such as peptic ulcer and mucosa-associated lymphoid tissue (MALT) lymphoma or adenocarcinoma [1,2]. Report shows that over 80% of population in some developing countries with poor sanitation get infected [3]. Additionally it is also proposed that *Helicobacter pylori*-infected persons have high risk for developing gastric cancer as compared with uninfected persons and World Health Organization characterized *Helicobacter pylori* as a class 1 carcinogen [4,5]. Prevalence of *Helicobacter pylori* in gastric infection is high all over the world as antibiotic resistance property is increased among *Helicobacter pylori* [6]. It is suggested that *Helicobacter pylori* is mainly transmitted within families, especially from mother to child and also through the contaminated eating devices [7,8]. *Helicobacter pylori* is a helical, microaerophilic bacterium that inhabits the stomach of more than 50% of the world's population and humans are appeared to be the main reservoir of *Helicobacter pylori* in nature [9,10]. To survive in the acidic microenvironment of the stomach, *Helicobacter pylori* has urease activity, considered as the major virulence factor [11]. *Helicobacter pylori* also produces toxic ammonia, protease certain phospholipase, oxidase, catalase to ensure its survival within the stomach as well as progress of the disease process [12,13]. On the other hand, motility is appeared to be a determinant of *Helicobacter pylori* pathology as it enhances colonization to stomach [14,15]. *Helicobacter pylori* are also found in water distribution systems in several countries and it may exist as a biofilm (most likely mixed species) on surfaces exposed to water [16,17]. Biofilm is described as matrix enclosed bacterial population adherent to each other or attached to solid surface and partly composed of highly hydrated exopolysaccharides. *Helicobacter pylori* have been reported to produce biofilm which facilitates their survival both in vivo and during transmission. Moreover, biofilm confers protection against antibiotics, fluctuation in pH [18,19,20,21,22]. In *Helicobacter pylori* two putative bacterial markers of virulence i.e., the cytotoxin-associated gene pathogenicity island (*cag* PAI, it is of 40-kb DNA segment) and the vacuolating cytotoxin gene (*vacA*) play a crucial role in determining the clinical outcome of *Helicobacter* infections [23,24,9]. The gene *cagA* (marker for *cag* PAI) which is associated with severe clinical outcomes is not present in every *Helicobacter pylori* strain. Moreover, the gene *cagA* encodes CagA protein, recognized as an important virulence marker for the development of gastritis and gastric carcinoma [25,26,24,27]. Depending on this background we conducted present study to investigate the presence of *Helicobacter pylori* in buccal salivary samples collected from the women subjects of age group around 50 years from Midnapore, West Bengal who swallowed tobacco and kept it in their mouth for a long time during working period. Our aim was to further characterize the *Helicobacter pylori* isolates in respect of colony morphology, motility, protease activity, antibiotic susceptibility, biofilm forming ability and also to detect the presence of *cagA* which is recognized as a potential threat for developing gastric carcinoma.

MATERIALS AND METHODS

1. Collection of samples

Buccal salivary samples were collected during the period of February to May, 2011 from women subjects of age around 50 years residing in Midnapore town, southern part of state of West Bengal, India who used to take and keep tobacco in their buccal cavity for 16-18 hours continuously during their occupational and also domestic work period. These women belonged to low socioeconomic background and they were usually involved occupationally as women labors in different unorganized sectors. The collected samples were further processed by the method mentioned by Zhu et al.[28] and

Dhaenens et al.[29]. The women subjects from same socioeconomic status having the age around 50 years and neither addicted in buccal tobacco usage nor had any tobacco practice at all, were used as control group.

2. Bacterial strains and culture methods [29]

Bacterial colonies were identified as *Helicobacter pylori* by both biochemical tests including rapid urease reaction, peroxidase test, oxidase test and Gram's staining. Following primary isolation the organisms were grown on Columbia agar (Himedia Laboratories Pvt. Ltd., India) plates supplemented with 10% horse blood in a microaerobic atmosphere at 37° C. Freshly grown cells were inoculated in brain heart infusion (BHI) broth (Himedia Laboratories Pvt. Ltd., India) supplemented with 7% fetal calf serum and 25 mM desferrioxamine (Sigma).

3. Detection of motility [30]

Motility of the *Helicobacter pylori* isolates were tested using swarm plate containing 0.3% Columbia agar supplemented with 10% horse blood through inoculation of the isolates at the center. Spreading of bacterial culture from central point towards periphery was considered as the bacteria to be motile while confinement of the culture at a central point was considered as the bacteria to be non-motile.

4. Detection of protease activity [31]

Protease activity of the isolated *Helicobacter pylori* strains were detected by using a single-diffusion technique on Columbia agar plate containing skim milk (Himedia Laboratories Pvt. Ltd., India) as a substrate supplemented with 10% horse blood. Sample solution (15 µl) was added to wells of 3 mm in diameter, and the plates were incubated at 37° C for over night. There was a linear correlation between the concentration of the sample and the diameter of the zone of clearing.

5. Antibiotic susceptibility test [32]

Antibiotic susceptibility test was done against the *Helicobacter pylori* isolates by disk diffusion method. Antibiotics (Himedia Laboratories Pvt. Ltd., India) used for this study were trimethoprim (0.045 mg/ml), amoxicillin (40 µg/ml), ampicillin (50 µg/ml), streptomycin (100 µg/ml), chloramphenicol (20 µg/ml), tetracycline (20 µg/ml) and kanamycin (40 µg/ml). Characterization of the resistance or susceptibility profile of the isolates was determined by measuring inhibitory zone.

6. Biofilm formation assay [33]

Helicobacter pylori strains were initially allowed to grow from frozen stocks on Columbia agar plates containing 5% sheep blood in an atmosphere of 10% CO_2, 5% O_2 and 85% N_2 for 2 days and then bacterial culture was transferred into brain heart infusion (BHI) broth. Cultures were adjusted to 5×10^7 cells/ml and 1 ml was inoculated per well into twelve-well microtiter trays containing glass frit (approximately 2- to 5-mm diameter). Each well also contained a sterile 25-mm borosilicate coverslip that had been placed at an angle in the chamber in order to allow biofilm formation at the air-liquid interface and the cultures were kept in gentle shaking condition at 37° C for 2 to 6 days in the same atmosphere as described above. The glass frit containing adherent *Helicobacter pylori* cells was washed twice with phosphate-buffered saline (PBS). A 1.5-ml aliquot of sterile 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) (Sigma) in BHI broth was added to the glass frit and the biofilms were incubated with gentle shaking in the same atmosphere as described above for 24 h. The solution was aspirated from the frit containing the adherent, purple-stained bacteria. Further, 1.5 ml of isopropanol (Himedia Laboratories Pvt. Ltd., India) was added to the frit to extract the formazan (reduced MTT) and the isopropanol suspension from biofilm was pelleted. Finally supernatant was removed and the absorbance of the supernatant was measured at 550 nm using spectrophotometer (Shimadzu, Japan). This experiment was performed with at least two repetitions.

7. DNA isolation [34]

Bacterial culture of *Helicobacter pylori* was harvested in PBS. Then cells were pelleted in 300 µl SET (25% sucrose, 1 mM EDTA, 10 Mm Tris HCL) (Himedia Laboratories Pvt. Ltd., India), lysozyme (10 mg/ml, dissolved in SET) (Bangalore Genei, India), EDTA (0.5M) (Himedia Laboratories Pvt. Ltd., India), proteinase K (10 mg/ml) (Bangalore Genei, India), 10% Sodium dodecylsulphate (Himedia Laboratories Pvt. Ltd., India) and mixture was incubated at 65° C for 1-2 hours. Then DNA was extracted using phenol-chloroform (Merck), Na- acetate (Merck), ethanol (Merck).

8. Detection of virulence marker [35,36]

A total of sixteen DNA samples were amplified through PCR with upstream primer: 5' ATGACTAACGAAGCCATT 3' and downstream primer: 5'TTAAGATTTTTGGAAACC 3' to detect presence of *cagA* as a virulence marker. PCR reaction mixture was carried out in 25 µl volume using 10 ng of genomic DNA, 1U of Taq polymerase (Bangalore Genei), and 10 pmol of each primer (Sigma), 0.25 mM (each) deoxynucleotide triphosphate (Bangalore Genei), and 2-3 mM $MgCl_2$ in PCR buffer. PCR amplification reactions were performed in a thermal cycler (Eppendorf) with a programme consisting of initial denaturation at 94° C for 1min and 35 cycles of denaturation at 94° C for 1 min, annealing at 59° C for 1 min, extension step at 72° C for 1.3 min and final extension step at 72° C for 20 mins. Amplified PCR products were analyzed by 1% agarose gel (Himedia Laboratories Pvt. Ltd., India) containing Ethidium Bromide (EtBr) (Himedia, India) in Tris Acetate buffer.

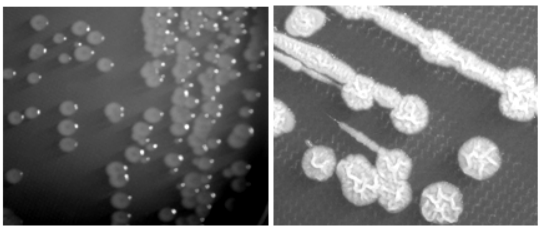
RESULTS AND DISCUSSIONS

Helicobacter pylori is a micro-aerophilic bacterium residing principally in the stomach and is associated with peptic ulcer disease, gastric MALT (mucosa- associated lymphoid tissues) lymphoma, and distal gastric cancer [37,38].

1. Identification and characterization

We isolated thirty five bacteria from buccal swabs of the subjects chosen and performed urease reaction, peroxidase test and Gram's staining to identify the presence of *Helicobacter pylori* organism. Thus, we could confirm sixteen bacterial isolates as *Helicobacter pylori* who were Gram-negative as well as positive in urease reaction and peroxidase test. But these tests could not confirm the presence of *Helicobacter pylori* in the saliva of control subjects. Further, we characterized these *Helicobacter pylori* isolates in respect of colony morphology, motility and protease activity, and found 37.5% isolates to have rugose colony morphology and rest to form opaque colonies (Table 1 & Fig 1). Phenotypic appearance has a great importance for pathogenicity in bacterial life cycle as it was reported earlier that opaque colony is more virulent than the translucent one [39,40]. Rugosity may be due to excess expression of exopolysaccharides (EPS) which is significant for their adaptation as it protects the organism from host defense systems [41]. We also observed all *Helicobacter pylori* isolates to be motile (Table 1), which indicates involvement of the *Helicobacter pylori* isolates in chronic infection of human gastric mucosa as motility has been reported to enhance colonization potential to stomach [42]. Moreover, we found 75% *Helicobacter pylori* isolates to be able to hydrolyze casein (Table 1). This shows that these isolates may have virulence potential to produce gastro-intestinal infection as protease produced by *Helicobacter pylori* was reported to cause degradation of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF beta) and platelet derived growth factor (PDGF) for their survival within the environment of stomach as well as for the induction of gastric infection [43].

Colony morphology of Helicobacter pylori



A **B**

Figure 1

Bacterial cells were grown on Columbia agar plate supplemented with 10% horse blood in a microaerobic atmosphere at 37° C. (A) Opaque colony & (B) Natural rugose colony of Helicobacter pylori.

2. Antibiotic susceptibility

Although the current treatments against *Helicobacter pylori* infection have good efficacies in general, but re-infections are common feature. High proportions of these organisms show antimicrobial resistance leading to the treatment failures of all infected persons [44,45,46,6]. We found all the *Helicobacter pylori* isolates to be resistant to chloramphenicol, tetracycline but sensitive to the ampicillin and kanamycin (Table 1). Furthermore, we also observed 75% of these isolates to be sensitive to trimethoprim, 87.5% to be sensitive to streptomycin and 50% to be resistant to amoxicillin (Table 1). Previous reports suggest that tetraclycine is a potent antibiotic against *Helicobacter pylori* [47] and Singh et al.[36] reported prevalence of amoxicillin resistant and tetracycline sensitive *Helicobacter pylori* strains in North India. In contrast, we find tetracycline resistant but ampicillin and kanamycin sensitive strains from these subjects. Here, we predict that the shift in antibiotic responsiveness may be significant in terms of treatment of the gastric infections and may be a result of horizontal gene transfer among the *Helicobacter pylori* populations as suggested by McClelland et al [48].

Table 1
Characterization of Helicobacter pylori isolates

Sample	Colony morphology	Motility	Protease activity	Antibiotic susceptibility
SBH1	Rugose	+	+	amp^s, kan^s, $amoxi^s$, $strep^r$, $trimeth^r$, chl^r, tet^r
SBH2	Rugose	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH3	Opaque	+	+	amp^s, kan^s, $amoxi^s$, $strep^r$, $trimeth^s$, chl^r, tet^r
SBH4	Rugose	+	+	amp^s, kan^s, $amoxi^s$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH5	Rugose	+	-	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH6	Opaque	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^r$, chl^r, tet^r
SBH7	Opaque	+	+	amp^s, kan^s, $amoxi^s$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH8	Rugose	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH9	Opaque	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^r$, chl^r, tet^r
SBH10	Opaque	+	-	amp^s, kan^s, $amoxi^s$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH11	Rugose	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH12	Opaque	+	-	amp^s, kan^s, $amoxi^s$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH13	Opaque	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^r$, chl^r, tet^r
SBH14	Opaque	+	+	amp^s, kan^s, $amoxi^s$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH15	Opaque	+	-	amp^s, kan^s, $amoxi^s$, $strep^s$, $trimeth^s$, chl^r, tet^r
SBH16	Opaque	+	+	amp^s, kan^s, $amoxi^r$, $strep^s$, $trimeth^s$, chl^r, tet^r

N.B. s: sensitive; r: resistant; amp: ampicillin; kan: kanamycin; amoxi: amoxicillin; strep: streptomycin; trimeth: trimethoprim; Cl: chloramphenicol; tet: tetracycline

3. Biofilm formation by the Helicobacter pylori isolates

Biofilm, a three dimentional multicellular-conformation is the adaptive surface growth that confers the bacterial cells in its fitness for survival by overcoming the adversities. In *Helicobacter pylori* biofilm, which is dependent upon the expression of a matrix material termed exopolysaccharide (EPS) by individual cell facilitate their survival both in vivo and during transmission [19,20,21,22]. In the present study we made an attempt to investigate the long persistence ability among the *Helicobacter pylori* isolates and observed that most of the *Helicobacter pylori* isolates were able to form large amount of biofilm (Fig 2) which indicates that they had the capability to persist in the environmental reservoir as well as within the host environment. Besides, according to Falk et al.[49] this biofilm forming ability can be correlated with their pathogenicity to the host body.

Biofilm formation by the different Helicobacter pylori isolates

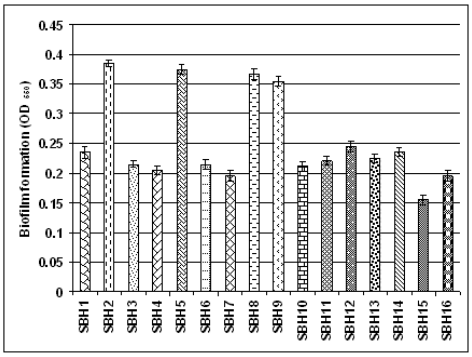


Figure 2

Biofilm formation ability by the different Helicobacter pylori isolates are measured by biofilm formation assay at 37°C for 24-hr. Figure shows biofilm formation by (A) SBH1, SBH2, SBH3, SBH4, SBH5, SBH6, SBH7, SBH8, SBH9, SBH10, SBH11, SBH12, SBH13, SBH14, SBH15 and SBH16 Helicobacter pylori isolates. OD_{550} values indicate quantity of biofilm produced. Error bars are standard deviations.

4. Presence of virulence marker

Pathogenicity of *Helicobacter pylori* depends on the presence of two virulence markers i.e., *vacA* and *cagA PAI*. The gene *cagA* (marker for the presence of the *cag* PAI) encodes CagA protein which contributes to the development of cancer to the epithelial cells of stomach [2]. Accordingly, we developed an interest to detect the presence of *cagA* among these *Helicobacter pylori* isolates and we found that only five of them harbored *cagA* but rest were negative for *cagA* through PCR analysis (Table 2). Two of them contained *cagA* of 1.2 kb in size and other two isolates possessed *cagA* of 0.3 kb. Rest of them was found to have *cagA* of 0.7 kb. Different studies on *Helicobacter pylori* collected from different sources, demonstrate the presence of *cagA* of different molecular sizes as Tiwari et al.[50] reported existence of *Helicobacter pylori* (collected from bile) containing *cagA* gene of 349 bp in length and Bindyana et al.[35] isolated *Helicobacter pylori* from faeces harboring *cagA* gene of 400 bp in size. Besides, reports suggest that *cagA* positive strains can significantly increase the risk for developing severe gastritis and gastric carcinoma compared with *cagA* negative *Helicobacter pylori* strains [51,52,53]. Moreover, gastrointestinal infection associated with *Helicobacter pylori* is found in high frequency among smokers than the non smokers as smoking is reported to be involved in inducing atrophic gastritis and intestinal

metaplasia in patients infected with *Helicobacter pylori* [54,55,56]. Okuda et al.[57] reported that *Helicobacter pylori* has only a transient presence in the oral cavity as growth of the organism is inhibited by the antagonist effects of oral microflora [57]. On the other hand, it is also evident that both chewing and smoking of tobacco reduces salivation and alter the normal oral microflora [58]. Moreover, some other reports revealed that tobacco was significantly associated with presence of *Helicobacter pylori*, as tobacco modulates the periodontal defenses and thus favors the colonization of this organism in buccal cavity [59]. On the basis of these prior reports we predict that similar underlying reasons may be responsible for significant occurrence of *Helicobacter pylori* isolates in buccal swabs of tobacco-using women in the present study in comparison to tobacco non-using control women who did not demonstrate presence of *Helicobacter pylori* in their buccal swabs. Additionally, it was also proposed that the oral cavity may act as a reservoir of *Helicobacter pylori* for developing infection in the stomach [60,61,62]. Hence we predict that these *cagA* positive *Helicobacter pylori* isolates those had colonized in the buccal mucosa of tobacco-addicted women may be significant for increasing chance for developing gastric infection as well as carcinoma in future.

Table 2
Identification of virulence marker among different Helicobacter pylori isolates

Samples	Presence of *cagA* gene	Length of the band
SBH1	+	1.2kb
SBH2	-	-
SBH3	-	-
SBH4	-	-
SBH5	-	-
SBH6	+	1.2kb
SBH7	+	0.7kb
SBH8	-	-
SBH9	+	0.3kb
SBH10	+	0.3kb
SBH11	-	-
SBH12	-	-
SBH13	-	-
SBH14	-	-
SBH15	-	-
SBH16	-	-

CONCLUSIONS

It can be concluded that *Helicobacter pylori* isolated from buccal swab of the tobacco addicted women were motile, protease positive, resistant to tetracycline, but sensitive to ampicillin and kanamycin. Besides, they were also able to persist within the host environment for long period and five of them were *cagA* positive. Finally, it can also be proposed that tobacco usage may be related to the occurrence of *Helicobacter pylori* in the oral cavity of tobacco addicted women subjects and the *cagA* positive *Helicobacter pylori* may be potential threat for promoting the development of gastric infection in future.

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