



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>
Journal DOI: [10.21474/IJAR01](https://doi.org/10.21474/IJAR01)

INTERNATIONAL JOURNAL
OF ADVANCED RESEARCH

RESEARCH ARTICLE

EXPRESSION OF CagA GENE IN HELICOBACTER PYLORI ISOLATED FROM DENTAL PLAQUE OF MALE SNUFF USERS.

*Debnarayan Jana¹ and Anup Shamsher Budhathoki².

1. Lecturer, Department of Physiology, National Medical College and Teaching Hospital, Birjunj, Nepal.
2. Lecturer, Department of Biochemistry, National Medical College and Teaching Hospital, Birjunj, Nepal.

Manuscript Info

Manuscript History:

Received: 14 March 2016
Final Accepted: 29 April 2016
Published Online: May 2016

Key words:

Helicobacter pylori, Snuff, CagA, Oral cancer.

*Corresponding Author

Debnarayan Jana.

Abstract

Helicobacter pylori is recognized as the major etiological agent of chronic gastritis, gastric adenocarcinoma and lymphoma. Stomach lining along with Saliva have some carcinogenic *Helicobacter pylori*. The present study was carried out to investigate the presence of *Helicobacter pylori* in dental plaque sample collected from snuff addicted male person (who have been using snuff for >20 years) from Raxaul, Bihar and to study the probable relation between snuff and expression of CagA gene. Here, sixty three bacterial isolates from study group were identified as *Helicobacter pylori*. Out of them forty two were CagA positive. Only twenty one bacterial isolates from control group were identified as *Helicobacter pylori* and two of them possess CagA gene. Result of this study conclude that snuff may stimulate the expression of CagA gene and may one of the potential threat for the development of oral cancer.

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Introduction:-

Helicobacter pylori is one of the important gastric pathogenic bacteria, responsible for gastritis, peptic ulcer disease¹ and Stomach Cancer². *Helicobacter pylori* is a spiral shaped, gram negative, lophotrichous bacterium having 3 µm in length and 0.5 µm in diameter^{3,4}. The mode of transmission and other epidemiology of *Helicobacter pylori* infection is unclear till today. Though some researchers think that the oral cavity harbors *Helicobacter pylori* and may be the source of infection and transmission. *Helicobacter pylori* shows large diversity of strains. Most of the strain have an approximately 40 KB long Cytotoxic Associated Gene Pathogenicity Island (Cag PAI). This Island has about 30 genes out of these 29 genes are associated with the synthesis of Complex -Type IV secretion system and remains one gene code for a highly virulent protein CagA (1186 amino acid), has capability to cause cancer⁵ by deregulating gastric epithelial SHP2 oncoprotein. *Helicobacter pylori* also contain UreA, UreB and UreC genes in their genome that is responsible for the synthesis of an enzyme Urease^{6,7} that neutralize the acidic environment for their survival within stomach lining. Previous research proposed that long term use of snuff are linked with cancer of cheek, gums and inner surface of lips. Neluka Frenando et. al. found that, oral cavity of betel chewers provide more suitable environment for colonization of *Helicobacter pylori* compared to the oral cavity of non-betel chewers⁸. The purposes of this study was to identify the presence of *Helicobacter pylori* strain in dental plaque sample from snuff users (have been using snuff for > 20 years) and to identify the expression of CagA gene by isolated *Helicobacter pylori*.

Materials & Methods:-

Subject selection and collection of sample:-

Total 77 male subject were selected randomly in this study from slum area 5km away from Raxaul, Bihar with mean age group 47.8 years who were using snuff throughout the working hour. Snuff users for less than 20 years were excluded from our study. From the same area, 52 male subject were selected who never took any kind of snuff during their entire life and no report of gastritis or peptic ulcer disease were considered as a control group. The study

was carried out from August 2015 to November 2015. In the early morning before washing mouth, the dental plaque were collected by using sterile forceps and sterile cotton and then dipped into Brain heart Infusion (BHI) Broth (Himedia Laboratories Pvt. Ltd. India) supplemented with 7% fetal calf serum and 25mM desferrioxamine (sigma) and stored in ice bag for further study in laboratories.

All the test tubes with culture broth and sample were placed within a shaking incubator at 37°C for 24 hours then by Quadrant streak Plate Method (KRYSTAL- Biomedical Engineering, Florida Institute of technology) we Separated different type of bacterial colony on Columbia Agar plate supplemented with 10% horse blood in a micro-aerobic atmosphere at 37° C.

Isolation of *Helicobacter pylori* by Biochemical tests:-

Bacterial colonies were identified as *Helicobacter pylori* by biochemical tests including Gram staining, Urease Reaction Test and Catalase Test.

Gram staining:-

Gram staining was carried out by using crystal violet solution, Lugol's iodine, saffranin, iodine acetone solution and PBS solution. At first a loop of bacterial growth were mixed with a drop of PBS solution on a glass slide (for all individual colony used individual slide separately) followed by fixing by low flame burner, crystal violet for 1 min, excess stain removal and covered by lugol's iodine for 1 min, hold in running laminar flow of tap water, covered with saffranin for 30sec and again wash in running tap water. Finally observed under compound light microscope.

Urease test:-

For each set four tube were taken and marked as S, UC, BC, B (S=sample, UC=Urea control, BC=bacteria control, B=blank). The S and UC marked tubes were 1/3 filled with media (Brain heart Infusion (BHI) Broth supplemented with 7% fetal calf serum and 25mM desferrioxamine) contain urea and BC & B marked tubes were 1/3 filled with only media without urea and allow them for solidification. Only BC and S marked tubes inoculated with bacterial sample. Urea hydrolysing bacteria shows red or pink coloration on S marked test tube and other than urea hydrolysing bacteria shows no changes of colouration on S marked test tube.

Catalase test:-

Catalase test was carried out by using a specific protocol evolved by "Jackie Reynolds, Richland College, BIOL 2421"

Determination of virulence properties of isolated *H. pylori*:-

To detect the virulence property of *Helicobacter pylori* we performed Motility test Protease test by using a specific protocol evolved by "Jackie Reynolds, Richland College, BIOL 2421"

Bio-film Assay:-

Biofilm assay⁹ were performed by method used by C. Ghosh and S. Biswas et al¹⁰

DNA isolation & Detection of *CagA* gene:-

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), lysozyme (10 mg/ml, dissolved in SET), EDTA (0.5M), proteinase K (10 mg/ml), 10% Sodium dodecylsulphate and mixture was incubated at 65° C for 1-2 hours. Then DNA was extracted using phenol-chloroform, Na- acetate, ethanol. A total of 63 DNA samples from study group and 21 DNA samples from control group were amplified through PCR with upstream primer: 5' ATGACTAACGAAGCCATT 3' and downstream primer: 5'TTAAGATTTTGGAAACC 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, and 10 pmol of each primer, 0.25 mM (each) deoxynucleotide triphosphate, and 2-3 mM MgCl₂ in PCR buffer. PCR amplification reactions were performed in a thermal cycler 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 containing Ethidium Bromide (EtBr) in Tris Acetate buffer.

Results & Discussion:-

We identified the isolates from study group and control group as *H. pylori* by using Columbia Agar media with specific microaerobic environment and by selective biochemical tests (Gram staining, Urease Reaction Test, Catalase test). In study group out of 77 isolates 63 were found as gram-ve bacteria. Morphologically they were Round opaque

(74.02%) and Round translucent(9.09%). Only 3.9% were found as gram +ve Rugose colony. In control group out of 52 isolates 21 were found as gram –ve, and morphologically 17.3% were round opaque , 11.5% were round translucent and 71.15% were found as rugose colony. On the basis of protease and motility test we did not find any significant difference of virulence property in between round opaque and round translucent colony. 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 one¹¹ and several recent studies reports that *H. pylori* form Biofilm either in vitro¹² or in vivo¹³. In our study round opaque colonies were more adherent to the hydrophobic glass surface compared to round translucent colonies among the study group. In control group the adherent property of both type of bacterial colonies were less compared to the study group (**Table1.1**). In study group and control group opaque colonies were found to be more adherent compared to round translucent colonies. Adherent property of bacteria is one of the determining factor for virulence. We found Round Opaque colony possesses more virulent property(**Table1.2**).

Table:1.1. Identification of *Helicobacter pylori* and Detection of virulence property by following Gram staining and Biochemical tests

Group	No. of colonies	Morphology	No. of colonies	Gram stain		Urease test for gram –ve colonies	Catalase test for gram–ve colonies	Motility test for gram–ve colonies	Protease test for gram–ve colonies
				+ve	-ve				
Study group	77	Round opaque small	38	02	36	36	36	36	36
		Round opaque large	26	05	21	21	21	21	21
		Round translucent small	10	03	07	06	06	06	06
		Rugose	03	03	00	00	00	00	00
Control group	52	Round opaque small	04	00	04	04	04	04	04
		Round opaque large	05	00	05	05	05	05	05
		Round translucent small	06	00	06	06	06	06	06
		Rugose	37	37	00	00	00	00	00

Table:1.2. Optical density of morphologically different type colonies of *H.pylori* after performing Biofilm.

Group	Type of colony	Optical density(Mean±SD)
study group	Round opaque	3.7±0.254
	Round translucent	2.9±0.158
control group	Round opaque	0.76±0.147
	Round translucent	0.45±0.08

Pathogenicity of *Helicobacter pylori* depends on the presence of *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. The international agency for research into cancer(IARC) has classified *H.pylori* as class-I carcinogen. The aim of our study was to identify the *CagA* gene in *Helicobacter pylori* isolates collected from dental plaque of male snuff users. From the study group out of 63 *Helicobacter pylori* isolates, 21 were found to be negative for *CagA* gene and 42 carried *CagA* gene, out of them 29 carried *CagA* gene of 1.2kb, 08 carried *CagA* gene of 0.7 kb and 05 carried *CagA* gene of 0.3kb(**Table.1.3**). Nineteen isolates from the control group were negative for *CagA* gene and 02 possesses *CagA* gene of 0.3kb long. Different studies on *Helicobacter pylori* collected from different sources, demonstrate the presence of *CagA* gene of different molecular sizes^{14,15}. Previous studies suggest that *CagA* positive strains can significantly increase the risk for developing severe gastritis and gastric carcinoma compared with *CagA* negative *Helicobacter pylori* strains^{16,17,18}. *Helicobacter pylori* has only a transient presence in the oral cavity as growth of the organism is inhibited by the antagonist effects of oral microflora¹⁹. It is also evident that both chewing tobacco reduces salivation and alter the normal oral microflora²⁰. Some other reports

revealed that tobacco was significantly associated with presence of *Helicobacter pylori*, as tobacco modulates the periodontal defences and thus favours the colonization of this organism in buccal cavity²¹. On the basis of these prior reports we predict that similar underlying reasons may be responsible for significant occurrence of *Helicobacter pylori* isolates in dental plaque of male snuff users study group incompared to snuff non-using control male who did not demonstrate significant presence of *Helicobacter pylori* in their dental plaque sample.

Table:1.3. Identification of CagA gene among Helicobacter pylori isolates

Group	No.of Helicobacter pylori colonies	CagA gene			
		Absent	Present with length		
			1.2kb	0.7kb	0.3kb
study group	63	21	29	8	5
control group	21	19	00	00	02

Conclusion:-

From this study we conclude that *CagA* gene is significantly express in male snuff users but we did not conduct any studies to demonstrate the direct role of snuff in *CagA* gene expression and we predict that these *CagA* positive *Helicobacter pylori* isolates from dental plaque of male snuff users may be significant for increasing chance to develop oral infection as well as carcinoma in future.

Acknowledgement:-

We are grateful to all the volunteers for their dental plaque sample and our thanks to National Medical College and Teaching Hospital, Birgunj, Nepal for supporting our research work.

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