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A STUDY ON CHARACTERIZATION OF *H. PYLORI* STRAINS ISOLATED FROM GASTROESOPHAGEAL REFLUX DISEASE (GERD) AND NON GASTROESOPHAGEAL REFLUX DISEASE (GERD) PATIENTS

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ABSTRACT
25% to 40% of the world's population suffers from gastroesophageal reflux disease (GERD), which is caused by the reflux of gastric acid into the esophagus. Reflux esophagitis (RE) increases the risk of Barrett's esophagus and esophageal adenocarcinoma in patients. In patients with GERD, the risk factors include age, body mass index, hiatal hernia, NSAID use, alcohol consumption, and cigarette smoke. Helicobacter pylori (H. pylori) infection, which affects 70 to 90 percent of the developing world's population, is the primary cause of peptic ulceration and gastric cancer globally. The virulence genes cytotoxin-associated antigen (cagA), vacuolating cytotoxin (vacA), and induced upon contact with epithelium (iceA) are involved in the pathogenesis of H. pylori. The polymorphism of these virulent genes has been linked to distinct pathological characteristics in H. pylori-infected adults with gastrointestinal diseases. CagA and vacA polymorphisms, alone or in combination, are associated with H. pylori-related pathogenesis. The association between H. pylori and peptic ulcer disease, MALT lymphoma, and gastric adenocarcinoma is well-established, whereas its significance in gastroesophageal reflux disease (GERD) remains debatable. There are frequent reports of an inverse relationship between H. pylori and GERD; however, population-based studies with high-quality data to support this supposition are scarce and contradictory. In addition, there are few reports on the epidemiology of GERD in India. The prevalence of gastroesophageal reflux disease (GERD) in India is probably between 8% and 19%, which is comparable to GERD prevalence rates reported in western nations pylori infections can be treated by eradicating the bacteria with a combination of a proton pump inhibitor and multiple antibiotics. Metronidazole, furazolidone, clarithromycin, amoxicillin, levofloxacin, and tetracycline are the most frequently employed antibiotics against H. pylori. Resistance to antibiotics is a significant issue with H. pylori, and it is rapidly increasing and reaching alarming levels. The prevalence of dual and multidrug resistance has also increased significantly in a number of countries and has become a significant barrier to the eradication of the H. pylori infection. Clarithromycin is a macrolide that has been recommended by the majority of consensus meetings and is commonly used in first-line therapy. Clarithromycin should not be used to treat H. pylori infection due to the high prevalence of clarithromycin resistance (greater than 15-20%). Clarithromycin is a bacteriostatic antibiotic that inhibits bacterial protein synthesis by binding to domain V of the 23S rRNA gene in the 50S ribosomal subunit of microorganisms. According to a mutagenesis analysis, the most prevalent 23S rRNA mutations that confer resistance to this macrolide are adenine-to-guanine transitions at positions 2142 and 2143. The imperative need to develop new non-antibiotic antibacterial agents for H. pylori infection is a result of the escalating complications of conventional combination therapies. In this investigation, the novel bioactive preparations of natural origin are lichen Parmelia perlata, also known as stone flower, which is commonly used to enhance the flavor and taste of food. In ancient folklore, it was used as a cosmetic and was said to have medicinal properties. It was used to treat dysentery, diarrhea, wound healing, and dyspepsia.

KEYWORDS: *H. Pylori* Strains, Gastroesophageal Reflux Disease (Gerd).

1. INTRODUCTION
Humans have been infected with H. pylori, a spiral, flagellated, microaerophilic, gram-negative, gastric pathogen, for over 60,000 years. The first association between the presence of H. pylori in the gastric mucosa and antral gastritis in adults was reported by Marshall and Warren in 1984. The World Health Organization's

International Agency for Research on Cancer classifies H. pylori as a group I human carcinogen.

This study investigates the potential association between H. pylori infections and its virulent markers (cagA, vacA, and iceA gene) and gastroesophageal reflux disease (GERD). In the published literature over the past decade, the relationship between H. pylori and GERD has been contentious. Due to the reflux of gastric contents into the esophagus, 25-40% of the population suffers from gastroesophageal reflux disease. H. pylori infection has been associated with a significantly reduced risk of developing GERD, Barret's esophagus, and esophageal adenocarcinoma, the protective function of H. pylori infection in the development of GERD for the first time. Subsequently, McColl and colleagues hypothesized that H. pylori infection shields atrophic gastritis patients from GERD. Several other studies, however, contradicted Labenz's findings, demonstrating that H. pylori eradication may not cause GERD symptoms and that the infection may not be linked to the condition.

According to previous research, risk factors for gastroesophageal reflux disease (GERD) include age, obesity, hiatal hernia, and an unhealthy lifestyle, which includes alcohol consumption and nicotine use. In this study, we also analyzed the relationship between GERD symptoms and a number of background variables, concentrating primarily on lifestyle factors and the gender of participants in a health screening program. the most prevalent manifestations of H. pylori infection are antral gastritis, duodenal ulcer, and peptic ulcer diseases. Infection with H. pylori is a risk factor for gastric malignancies including adenocarcinoma and MALT lymphoma. The virulence markers of bacteria are cag PAI (cytotoxin-associated gene pathogenicity island), vacA (vacuolating cytotoxin gene), and iceA (induced upon contact with epithelium gene). In H. pylori-infected individuals with gastrointestinal disease, polymorphisms in cagA, vacA, and iceA have been linked to a variety of pathological outcomes. The presence of the cagA gene, which codes for the 145 kDa cagA protein, indicates the presence of cag PAI. CagA protein is injected into epithelial cells via type IV secretion system encoded by some genes in cagPAI; after internalization, it is phosphorylated at tyrosine residue within the repeated penta amino acid sequence EPIYA motif present at the variable 3' end of the cagA gene, which has been linked to gastroduodenal disease, including gastric cancer. Active VacA is a toxin that induces substantial vacuolization of epithelial cells in vitro. Due to sequence heterogeneity within the vacA gene at the 5' end signal (s) region and the middle (m) region, each allele s1/s2 and m1/m2 has a different biological activity. Another restriction endonuclease homolog, iceA, was discovered, and DNA sequences revealed two families, iceA1 and iceA2, which are associated with overt disease and benign infection, respectively. Given the high genetic and geographic variability that characterizes H. pylori (Salamaet al., 2000) and the fact that the role of virulent markers (cagA, vacA, and iceA) of the bacteria with GERD has not been evaluated from North India, the purpose of this study is to evaluate the potential association between H. pylori infection and its virulent genes and GERD in India using an endoscopic examination of biopsy specimens.

H.pylori infection is acquired during childhood, frequently before the age of 5 years, via fecal-oral, oral-oral, or gastro-oral transmission. Without treatment, the duration of the infection could be permanent. The bacterium must be eradicated in patients with symptomatic H.pylori infection associated with duodenal and gastric ulcers, lymphoma, or atrophic gastritis with intestinal metaplasia. Since their proposal at the first Maastricht conference, triple drug regimens containing a proton pump inhibitor (PPI), amoxicillin, and either clarithromycin or metronidazole have been widely used, with the aim of achieving eradication rates of 80-90%. Several H. pylori eradication trials undertaken in India had lower eradication rates than comparable trials conducted in the West. Resistance to commonly used antibiotics, such as metronidazole (MTZ), clarithromycin (CLR), amoxicillin (AMX), tetracycline (TET), levofloxacin (LEV), and furazolidone (FZ), is the leading cause of anti-H. pylori therapy failure. To fill this knowledge gap regarding H. pylori isolates in India, the present study was conducted to ascertain the antibiotic resistance pattern throughout the country. There is an urgent need to develop new treatment strategies for H. pylori infection in light of the inadequate cure achieved with conventional treatment due to the increasing resistant strains, undesirable side effects, the cost of antibiotic regimens, noncompliance among patients, and a few other factors for antibiotic inefficacy.

Since antiquity, spices and condiments have been regarded as indispensable to the culinary arts in India, as they are used to flavor foods. In addition, the physiological and therapeutic properties of these substances were acknowledged. The broad-spectrum efficacy of Paederia foetida and Parmelia perlata may provide a suitable foundation for novel anti-H. pylori therapies. This new information would facilitate the development of enhanced diagnostic, therapeutic, or preventative measures to help eliminate the negative effects of infection and improve the health of individuals in nations that continue to carry and transmit the infection.

2. RESEARCH METHODOLOGY

Sample size

The purpose of sample size calculation for a study is to determine an adequate number of samples to accurately estimate the population prevalence. The sample size was determined using the following formula:

$$n = \frac{Z^2 P (1-P)}{d^2}$$

n =Sample size,

Z = Z statistic for Confidence level at 95% (standard value of 1.96),
P = Estimated prevalence of measure (in proportion of 1) *H. pylori* prevalence 80% or 0.8; GERD prevalence 20% or 0.2
d = Precision or margin of error (in proportion of 1; 5% = 0.05),
n = ~250 patients
Zstatistic (Z): For the conventional level of confidence of 95%, the Z value is 1.96. In the current investigations, results were accompanied by confidence intervals (CI) of 95%.

This is the proportion (prevalence) that was estimated by prior research or literature. Scale of Pi ranges from 0 to 1, and sample size varies based on the value of P. In prior studies, the prevalence of H. pylori in India was estimated to be 80% (Thirumurthi and Graham, 2012), while the prevalence of GERD in India was approximately 20% (Kumar and Shivalli, 2014).

Precision (d): The formula suggests that the sample size is inversely proportional to the square of the precision (d2). At the conclusion of an investigation, we must present the prevalence along with its 95% confidence interval. In the present investigation, the prevalence of H. pylori and GERD in India are, respectively, 80% and 20%. It indicates that the study estimated the proportion of H. pylori-infected individuals to be between 70% and 90%, and the prevalence of GERD to be between 10% and 30%. Here, the precision (d) for this estimate is 10%, indicating that the width of the confidence interval (CI) is twice the precision (CI width = 2d). If the width of the CI is large, the estimate may be deemed inaccurate. To attain a more precise CI, we must design a study with a smaller d (good precision or smaller estimate error). Consequently, for a CI width of 10% (0.1), d should be set to 0.05.

3. STUDY POPULATION

a total of 483 patients (mean age = 44.5) suffering from various gastro-duodenal diseases (male:female ratio = 261:222 or 1:0.9) were recruited from two regions in North India. One is from the Yashoda superspeciality hospital in Ghaziabad, India (Delhi-NCR region), and the other is from the Maulana Azad medical college in Delhi. Patients who were taking antibiotics or any other medication, who had a bleeding ulcer or acute hemorrhage from other sites in the upper gastrointestinal tract, or who had undergone stomach surgery were excluded from the study.

4. RESULTS AND DISCUSSION

4.1 Characteristics of study population

The total research population was divided into age groups 18-40yrs, 41-60yrs, and 61-80yrs, with 215, 186, and 82 patients, respectively, in each age group (Table 1).

Table-1: Characteristics of the total study population.

Characteristics		No. of patients n (%)
Study Population		483
Age (in yrs.)	18-40	215/483 (44.5)
	41-60	186/483 (38.5)
	61-80	82/483(17.0)
Gender	Female	222/483 (46.0)
	Male	261/483 (54.0)
Disease Diagnosis	RE	158/483 (32.7)
	NERD	155/483 (32.1)
	NUD	50/483 (10.4)
	AG	28/483 (5.8)
	DU	14/483 (2.9)
	Control	64/483 (13.3)
	Fungal/viral esophagitis	5/483 (1.0)
	Miscellaneous/Malignancy	9/483 (1.9)
Reflux Esophagitis Grade	LA grade A	99/158 (62.7)
	LA grade B	38/158 (24.1)
	LA grade C	18/158 (11.4)
	LA grade D	3/158 (1.9)

4.2 Classification of the study population based on disease outcomes

On the basis of FSSG questionnaire and endoscopy-based clinical symptoms, Reflux esophagitis (RE), Non-erosive reflux disease (NERD), and Non-ulcer dyspepsia (NUD) were described as follows: RE was considered in patients with endoscopic evidence of esophagitis and an FSSG score greater than 7. NERD was considered in patients with a normal endoscopy whose FSSG score was greater than 7.NUD was evaluated when a patient's endoscopy was normal and their FSSG score was less than 7. Patients without GERD symptoms who underwent endoscopy for other reasons, such as diarrhea or chronic liver disease (CLD), are considered controls. Endoscopic examination classified other patients as

having duodenal ulcer, antral gastritis, fungal/viral esophagitis, and other diseases.

The total sample population was distributed as follows by disease category: RE – 32.7% (158/483), NERD – 32.1% (155/483), NUD – 10.4% (50/483), antral gastritis (AG) – 5.8% (28/483), duodenal ulcer (DU) – 2.9% (14/483), control –13.3% (64/483), Fungal/viral esophagitis (FE) – -1.0% (5/483) and miscellaneous –1.

4.3 Characterization of *H. pylori* strains isolated from GERD and Non-GERD patients

In India, numerous communities and religions coexist with a remarkable amalgamation of ethnicities and cultures; consequently, the genetic characteristics of its population vary. In the present study, we endeavor to identify unique genetic characteristics and to characterize virulent marker genes carried by H. pylori strains isolated from GERD and non-GERD patients in North India. Using PCR and DNA extracted from H. pylori cultures/biopsies, the presence of virulence genes including cagA, vacA subtypes, and iceA were determined.

Genotyping of cagA

The presence or absence of the cag PAI was determined using PCR with specific primers and extracted DNA from cultured strains. Using cagA-specific primers designed from the 5' end conserved region of this gene, products indicative of the cag PAI were obtained. The cultures, which lacked any cag PAI-specific PCR product, produced an empty-site 550-bp product with primers from the flanking regions (Lunil 1 and R5280) of the cag PAI, indicating the absence of the cag PAI (Akopyants et al., 1998). The prevalence of the cagA gene in North Indian isolates was 86/151 (57.0%), while the remaining 65/151 (43%), yielded a 550 bp amplicon of a vacant site, indicating the absence of cag PAI.

Out of a total of 151 H. pylori isolates, cagAgene was detected in 86 H. pylori isolates, with the following distribution in different diseases: 49 (GERD), 13 (NUD), 4 (AG), 4 (DU), 1 (FE), 4 (Other) and 11 (control) patients were diagnosed. Compared to GERD patients (49.5%), cagA-positive H. pylori isolates are significantly associated with DU (100%) and NUD (76.5%; p 0.05;) In contrast, cagA-negative H. pylori isolates were strongly linked to GERD. As opposed to GERD patients, no significant association was found between other gastro-duodenal diseases and control patients (p-value > 0.05).

Table-2: Correlation of *cagA*positive/negative*H.pylori* isolates from GERD patients with *cagA* positive/negative isolates from Non-GERD patients (different disease outcome.

Disease outcome	*cagA* positive n (%)	*cagA* negative n (%)	P-value
GERD	49 (49.5)	50 (51.5)	0.03*
NUD	13 (76.5)	4 (23.5)	
GERD	49 (49.5)	50 (51.5)	0.69
AG	4 (57.1)	3 (42.9)	
GERD	49 (49.5)	50 (51.5)	0.016*
DU	6 (100.0)	0 (0.0)	
GERD	49 (49.5)	50 (51.5)	0.085
control	11 (73.3)	4 (26.7)	
GERD	49 (49.5)	50 (51.5)	0.183
miscellaneous	4 (80.0)	1 (20.0)	
GERD	49 (49.5)	50 (51.5)	0.989
Fungal esophagitis	1 (50)	1 (50)	

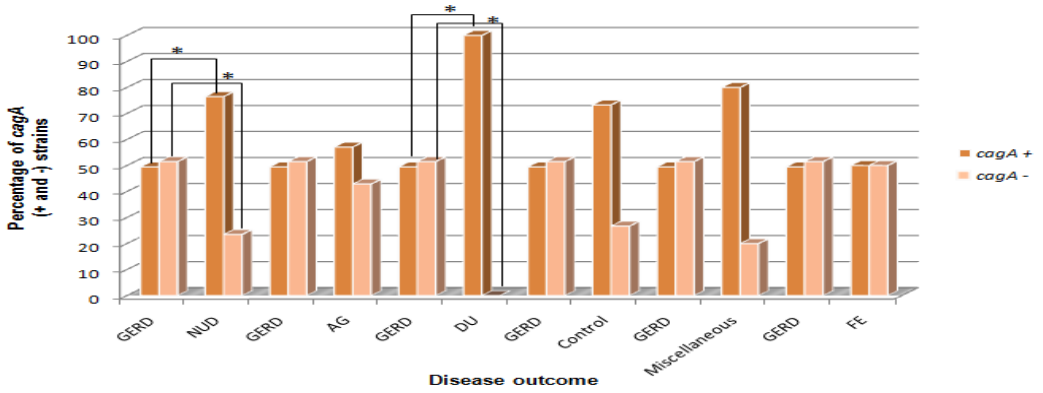

Figure-1: Correlation of *cagA*positive/negative*H.pylori* isolates from GERD patients with *cagA* positive/negative isolates from Non-GERD patients (different disease outcome.

***vacA* genotyping**

In this study, the signal sequence alleles s1 (generally virulent) and s2 (generally non-virulent) of the vacA gene were identified using primers designed for the 5' end of the gene (Atherton et al., 1995).Among the 151 samples of H. pylori DNA analyzed in this study from North India, 107 (70.9%) yielded the typical 259 bp amplicon for the s1 allele of the vacA gene, whereas the remaining 44 (29.1%) strains yielded the 286 bp amplicon for the s2 allele (Figure-**2).**

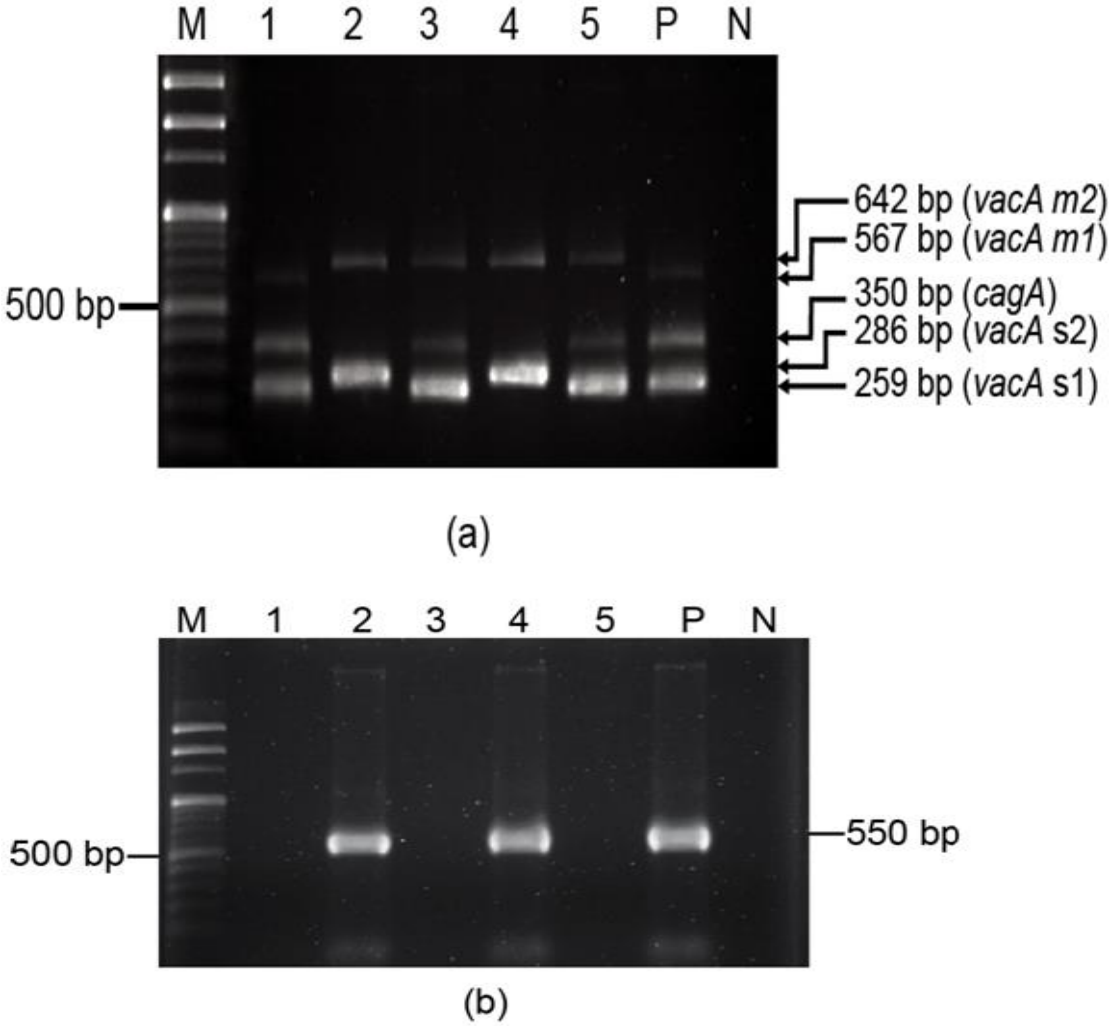

Figure-2: (a) Multiplex PCR for the detection of *vacA*s1, *vacA* s2, *vacA* m1, *vacA* m2 subtypes and the *cagA* gene of *H. pylori* isolated from North India. Lane M, 100 bp marker (Bangalore Genei); Lane 1, 3,5 shows *vacA* s1m1 *cagA*positivegenotype; Lane 2, 4 shows *vacA* s2m2 *cagA*negative genotype. Lane P shows Positive control; Lane N shows Negative control. (b) Empty site PCR: All the strain which failed to give amplicon for *cagA* gene gave 550bpproduct for cag empty site.

There are various alleles in the middle region of vacA, which varies geographically. Using primers VAG-F and VAG-R, the prevalence of the alleles of the middle (m) region of vacA was also determined by PCR. 49 (32.5%) of the 151 H. pylori DNA samples from North India analyzed in this study yielded a typical amplicon of 560 bp for the m1 allele of the vacA gene, while the remaining 102 (67.5%) samples yielded a typical amplicon of 632 bp for the m2 allele (Figure-2).

The distribution of the vacA s1m1 genotype in disease is as follows: 49/151 (32.5%) H. pylori isolates carried the vacA s1m1 genotype. 26 (GERD), 6 (NUD), 2 (AG), 5 (DU), 7 (control), 3 (other), and 0 (Fungal esophagitis). vacA s1m2 was present in 58/151 (38.4%) participants, with the following distribution: 38 (GERD), 1 (DU), 7 (NUD), 4 (AG), 6 (control), 1 (other) and 1 (fungal esophagitis) were diagnosed. vacA s2m2 was detected in 44/151 (29.1%) H. pylori isolates, with the following distribution: 35 (GERD), 0 (DU), 4 (NUD), 1 (AG), 2 (Control), 1 (Other), and 1 (Fungal esophagitis) were the diagnoses.

Compared to GERD patients (26.3%), s1m1 vacA genotype was significantly linked to DU patients (83.3%; p 0.05). In contrast, the s2m2 vacA genotype was substantially associated with GERD (35.5%), whereas DU was not (Table-3, Figure-2).In contrast, no

significant association was found between vacAgene of H. pylori in patients with other diseases and GERD (p > 0.05).

Table-3: Correlation of vacA genotype of *H. pylori* isolates from GERD patients with *vacA* genotype of *H. pylori* isolates from Non- GERD patients (different disease outcome)

Disease outcome	s1m1 n (%)	s1m2 n (%)	s2m2 n (%)	p-value
GERD	26 (26.3)	38 (38.4)	35 (35.4)	0.588
NUD	6 (35.3)	7 (41.2)	4 (23.5)	
GERD	26 (26.3)	38 (38.4)	35 (35.4)	0.485
AG	2 (28.6)	4 (57.1)	1 (14.3)	
GERD	26 (26.3)	38 (38.4)	35 (35.4)	0.01*
DU	5 (83.3)	1 (16.7)	0 (0)	
GERD	26 (26.3)	38 (38.4)	35 (35.4)	0.147
control	7 (46.7)	6 (40.0)	2 (13.3)	
GERD	26 (26.3)	38 (38.4)	35 (35.4)	0.260
Miscellaneous	3 (60.0)	1 (20.0)	1 (20.0)	
GERD	26 (26.3)	38 (38.4)	35 (35.4)	0.701
Fungal Esophagitis	0 (0.0)	1 (50.0)	1 (50.0)	

Note: Asterisk mark (*) showed the significant relationship

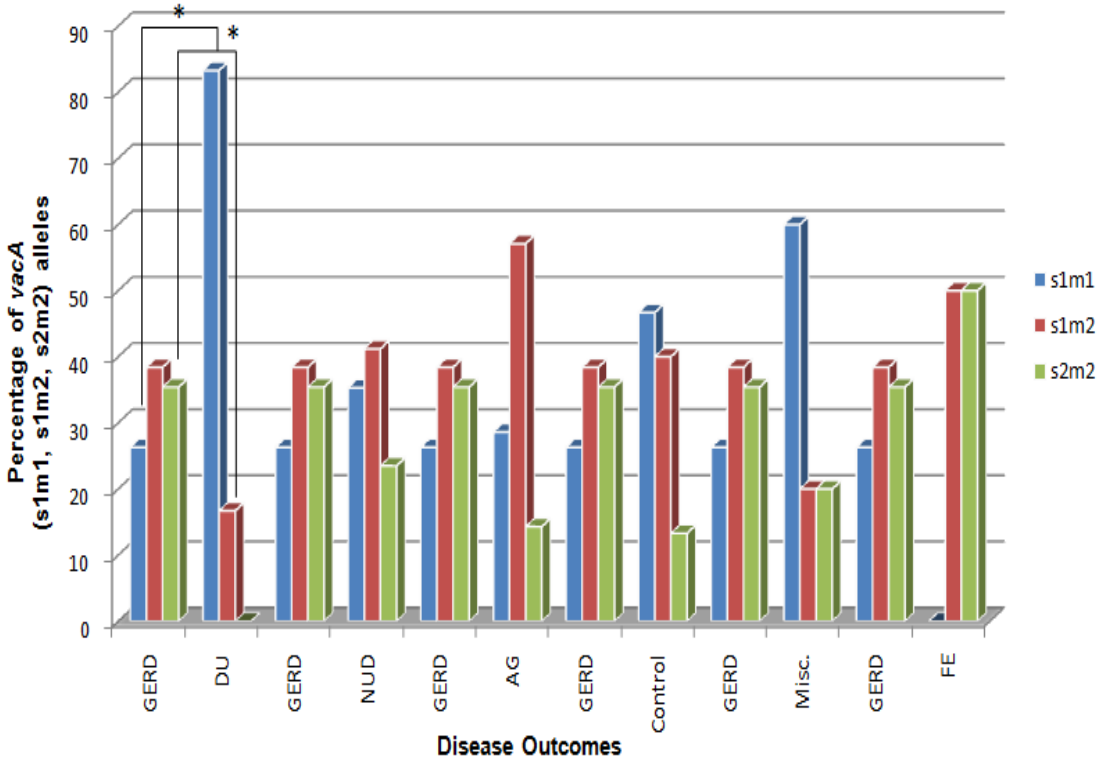

Figure-3: Correlation of *vacA* genotype of *H. pylori* isolates from GERD patients with *vacA* genotype of *H. pylori* isolates from Non- GERD patients (different disease outcome). Asterisk mark (*) above the bars showed the significant relationship.

5. CONCLUSION

In Western countries, gastroesophageal reflux disease (GERD) has traditionally been regarded as a significant clinical outcome with a wide range of clinical manifestations. Presence of GERD symptoms is the major risk factor for Barrett's esophagus and esophageal adenocarcinoma, the incidence of which is increasing at an alarming rate relative to other cancers. The proton pump inhibitor (PPI) is an effective treatment for GERD and its complications, which reduce the quality of life of patients. GERD is a costly medical condition, and its complications decrease the quality of life of patients.

It is difficult to quantify the prevalence of GERD because those who seek medical attention represent only the top of the iceberg. Consequently, the current understanding of the epidemiology of GERD is primarily founded on cross-sectional population studies conducted

in western nations. the prevalence of GERD in western populations ranges from 10 to 44%. Several studies indicate that 14-24% of adults experience heartburn and acid regurgitation at least once a week, and the frequency has recently increased to about one-third of the adult population.

Consequently, the purpose of the present research was to generate objective data on the prevalence of GERD and its risk factors in a defined cohort. Endoscopy (invasive procedures) and symptom frequency (FSSG Questionnaire) were used to diagnose GERD in the current study. According to the Montreal workshop report (Vakil N et al., 2006), GERD is comprised of both reflux esophagitis (RE) and endoscopy-negative reflux disease, also known as non- erosive reflux disease (NERD). In this study, 64.8% (313/483) of all patients were diagnosed with GERD, including both RE and NERD, which is a higher prevalence than studies conducted in other Asian and Western countries. RE was found in 32.7% (158/483) of the study population, and NERD was found in 32.1% (155/483). 13.3% (21/158) of patients had more severe erosive esophagitis with LA grades C and D, whereas 86.7% (137/158) of patients had less severe erosive esophagitis with LA grades A and B.

The prevalence of gastroesophageal reflux disease (GERD) in India is higher than previously estimated and appears comparable to that of western countries. In this swiftly developing society, the prevalence of gastroesophageal reflux disease (GERD) can be affected by a number of factors, including dietary and lifestyle changes, rising obesity rates, and smoking.

This increase in GERD prevalence was anticipated given the distinct genetic characteristics of the Indian population compared to those of the Western and other Asian populations, as well as the rapid changes in the socioeconomic environment. With an improved understanding of the risk factors, it may be possible to target an intervention at the root cause of India's GERD epidemic. In addition, future research should conduct physiological studies and endoscopic evaluations of GERD in order to obtain a deeper understanding of its causes and complications.

Currently, eradication with a proton pump inhibitor-based triple therapy is used to treat H. pylori infection.Even though it has an 80 to 90 percent success rate, problems such as treatment failure, inconsistencies for some patients, and the rapid emergence of drug resistance in H. pylori strains during treatment with various antibiotics pose a significant barrier to the development of effective eradication therapies. For eradicating H. pylori strains resistant to antibiotics, the search for safe and effective nonantibiotic compounds that inhibit H. pylori growth is intensifying. In the Indian system of traditional medicine, a number of plants and plant products are recognized as possessing potent medicinal properties, indicating their therapeutic value. Recent research has demonstrated that the leaf and stem extract of Paederia has anti-H. pylori potential.This prompted us to investigate its antimicrobial potential against geographically distinct Indian H. pylori strains that differ from East Asian and Western strains. In addition, the majority of the Indian population is infected with H. pylori, and many of them suffer from H. pylori-related gastrointestinal diseases. In the present study, we primarily demonstrated that mother extract and its reacted products (R1, R2, and R3) inhibited the in vitro growth of all H. pylori strains isolated from patients with gastrointestinal disorders. It is notable that one of the H. pylori isolates was resistant to metronidazole (MIC>8g/ml). Therefore, our results suggest that the methanolic extract of Paederia leaf and stem inhibits the growth of H. pylori by distinct mechanisms distinct from those of these antibiotics. This study provides novel insights into the therapeutic potential of Paederia extract against H. pylori infections, but additional research is necessary to extrapolate its effect to humans.

Dual drug-resistant H. pylori strain was included in this study. Both methanolic and ethanolic P. perlata extracts are investigated. In the present study, we discovered that methanolic and ethanolic extracts of P. perlata inhibited the in vitro growth of H. pylori isolates obtained from an infected patient with NERD and GERD. Notable is the fact that one of the H. pylori strains (HP1) was resistant to both clarithromycin and metronidazole, whereas another strain (HP2) was only resistant to metronidazole, which is an essential component of the H. pylori treatment's first-line therapy. Therefore, our findings suggest that the methanolic and ethanolic extracts of P. perlata inhibit the proliferation of H. pylori by distinct mechanisms distinct from those of these antibiotics. To determine the compounds accountable for antibacterial activity against H. pylori, additional research is required. The findings also suggest that scientific studies conducted on medicinal plants with traditional efficacy goals can yield fruitful outcomes.

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