

# Exploring the Prevalence of Virulence Genes of *Helicobacter pylori* and their Association with Gastrointestinal Diseases in South Indian Tamils

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## Abstract

*Helicobacter pylori*, a Gram-negative gastric pathogen and a class I carcinogen harbors key virulence genes such as *vacA*, *cagA*, *ureC*, *homb* and *oipA* that contributes to gastrointestinal diseases. The study assessed the prevalence of *H. pylori* infection and their correlation with virulence genotypes with clinical outcomes in South Indian Tamils. Gastric antral biopsies from 500 patients were assessed for *H. pylori* prevalence by RUT followed by genomic DNA extraction and PCR. Molecular confirmation employed 16S rRNA, a preliminary marker, with the identification of virulent genes *cagA*, *vacAs1*, *vacA m1/m2*, *oipA*, *ureC*, *homb* are validated by sequencing. *H. pylori* were confirmed in 491 patients where *vacA s1* (71.89%) was correlated with gastritis (OR-1.68;95% CI: 1.11-2.53; p = 0.013) and pangastritis (OR-4.79;95% CI: 2.02-11.34; p = 0.0004) while *vacA s1/m1* and *vacA s1/m2* alleles were found to be 51.53% and 14.26%, respectively. Gastritis was associated with *s1/m1* (OR-2.34; 95%CI: 1.29-4.23; p = 0.004), *s1/m2* (OR-2.03; 95%CI: 1.04-3.95; p = 0.03) and pangastritis (OR-2.61;95%CI: 0.97-7.02; p = 0.05) was associated with *s1/m1*. The *cagA* gene (31.36%) was correlated to gastric cancer (OR-3.45; 95%CI: 1.20-9.89; p = 0.02) and *oipA* gene (64.97%) was associated with PUD (OR = 7.87; 95% CI: 1.02–60.40; p = 0.04), *homb* (24.03%) with gastritis (OR-1.62; 95%CI: 1.01-2.59; p = 0.04) and *ureC* (59.67%) with no disease association. Therefore, our study provides genotypic prevalence of *H. pylori* infection in the South Indian Tamil population, with *vacA s1* being the predominant genotype reported and significantly associated with gastritis and pangastritis. Hence, *vacA s1* can serve as a potent virulent marker for gastrointestinal disease manifestations.

**Keywords:** Gastric Cancer, Gastritis, *Helicobacter pylori*, PUD, South Indian Population, *vacA*

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## INTRODUCTION

*Helicobacter pylori* is a Gram-negative bacterium that typically colonizes in the antral region of the human stomach. The pathogen was considered to be a class I carcinogen, as classified by the WHO, and was associated with various gastric complications such as gastritis, peptic ulcer disease (PUD), dyspepsia, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer (GC).<sup>1</sup> The frequency of *H. pylori* infection in Asia was reported high, reaching almost 80% of the population with the prevalence rate surpassing 50% by the age of 10, thereby rising to 90% in adulthood, while in developed countries like Japan, Western Europe and Oceania, it was estimated to be between 30% and 50%. Moreover, a recent meta-analysis report among 15 different states of Indian population have shown a marginal regional variation in the prevalence of infection among the states, where Rajasthan reports highest prevalence of 70% and the lowest prevalence was reported in Gujarat (9%). Also, the report has analyzed the pooled prevalence of *H. pylori* among people with GI disorders was 54% in comparison with 61% of no clinically diagnosed GI disorders.<sup>2-4</sup> Studies have reported that the recommended initial treatment for *H. pylori* infection includes a seven-day vonoprazan-based triple therapy and a non-bismuth quadruple therapy for about ten to fourteen days. These regimens have achieved eradication rates of about 90%, even in regions with high prevalence of antimicrobial-resistant strains. Nonetheless, these treatments still have certain limitations, such as the potential to increase antimicrobial resistance and causing imbalances in gut microbiota.<sup>5</sup> The pathogenesis and adverse effects of the disease are influenced by complex interactions between bacterial virulence, host responses, and environmental factors. Over time, these virulence factors contribute to the persistence and colonization of *H. pylori*. The recognized virulence factors of *H. pylori* are vacuolating cytotoxin A (*vacA*), induced by contact with the epithelium (*iceA*), outer membrane proteins such as helicobacter outer membrane proteins (*hom*), outer inflammatory protein A (*oipA*), *urease* (*ure*),

*cagPAI* (*cag* pathogenicity island) and blood group antigen-binding adhesion (*babA*) which have the potential to interfere with gastric epithelial cell signaling pathways thereby inducing gastric diseases.<sup>6-8</sup>

*cag* PAI of *H. pylori*, comprising 40 kb of DNA, housing for 27-31 genes, a significant virulence factor which codes for *cagA* has been identified in adverse cases of gastrointestinal ailments, such as acute gastritis, PUD and GC. The *cagA* gene represents part of the type IV secretion system (T4SS), which acts as translocation complex to deliver the *cagA* protein into the cytoplasm of the infected gastric cells, triggering cytoskeletal changes, increased cell proliferation, and release of interleukin-8 (IL-8).<sup>9</sup> *vacA*, a 140 kDa pore-generating toxin, encoded by a 3.9 kb gene and induces the formation cytoplasmic vacuoles, mitochondrial fragmentation, permeabilization of cell membranes and kinase enzyme activation. The *vacA* gene has two allelic forms namely, signal peptide (*s1*, *s2*), and middle region (*m1*, *m2*). Moreover, the combination of the allelic forms also known to cause higher levels of toxins as produced by *s1/m1* type strains in comparison to *s2/m2* are associated with gastric cancer and premalignant conditions. It has been reported that the *vacA s1m1* genotype was reported to be highly dangerous because of the high level of toxin production, secretion of inflammatory chemicals, and an increased risk of GC.<sup>10</sup> The *oipA* gene plays a vital role in triggering IL-8 secretion and enhancing bacterial invasion in the stomach.<sup>11</sup> The regulation of *oipA* gene was mediated *via* slipped-strand mispairing, which is contingent on the number of CT repeats. The expression of this toxin leads to apoptosis, cellular stress, and alteration in cytoskeletal of gastric epithelial cells, causing significant gastrointestinal disorder.<sup>12</sup> The *ureC* gene encodes an open reading frame that originated from the urease structural gene (*ureAB*) of *H. pylori*, a phosphoglucosamine mutase derivative.<sup>13</sup> An investigation indicates that the gene has been strongly associated with gastric cancer.<sup>14</sup> The *hom* family represents a small group of paralogous proteins characterized by signal sequences in the C-terminal and interchanging hydrophobic motifs. The *homA* and *homB* genes

are 90% identical with a variation in the central domain. Recent studies reported that *homb* has a close association with IL-8 secretion and a significant association with gastritis, PUD and gastric cancer.<sup>15</sup>

In 2022, India was ranked third globally for stomach cancer incidence and second for gastric cancer mortality.<sup>16</sup> The recent metanalysis study on *H. pylori* infection highlighted a significant regional disparities, among various parts of India with prevalence ranging from 9%-70%.<sup>4</sup> Based on the higher prevalence of *H. pylori* infection in India and its well-established role in gastrointestinal diseases, our study aims to investigate the genotypic profile of *H. pylori* virulent strains isolated from gastric biopsy sample of South Indian Tamil population. By determining the prevalence of key virulence genes and analyzing their association with specific gastrointestinal conditions, our study will provide the insights into strain-specific risks. Also, the findings of our study may enhance the understanding of disease progression to aid in the identification of molecular markers for detection and targeted therapeutic interventions.

## MATERIALS AND METHODS

### Study subjects and sample collection

The Institutional Ethical Committee has granted approval for the proposed study (EC Reg No. 2394/IEC/2021) and informed consent was acquired from the *H. pylori* infected patients prior to their recruitment. Also, the committee has granted approval for study objective which demands patients tested positive for *H. pylori* during the endoscopic procedure and restricted the inclusion of a true *H. pylori* negative control group. Blood and gastric biopsy tissues were procured from 500 patients who underwent upper gastrointestinal endoscopy at SRM Medical College and Research Centre, Tamil Nadu, India. *H. pylori* infection was confirmed through the rapid urease test (RUT) and ELISA to determine IgG levels against the bacteria (HPIgG) (Data not shown). A cutoff value of  $\geq 30$  U/mL was considered positive for active *H. pylori* infection.<sup>17</sup> Two biopsy samples were taken from the patient during the endoscopic procedure: one for histopathological

study and the other for further molecular studies. The biopsy tissue samples were transported to the laboratory under ice-cold conditions in a transport medium containing 0.6 ml of Brucella broth and 15% glycerol and stored at -80 °C until further analysis.<sup>18</sup>

### Investigation of biopsy samples

#### Rapid Urease Test

The tissue samples collected from each subject were placed on an RUT strip purchased from Gastrohub in Kolkata, India. Two drops of sterile water were added, and was observed for the colour change from yellow to pink or red within 2 h was considered positive. The test has detected the presence of *H. pylori* infection through its urease activity.<sup>19</sup>

#### *H. pylori* confirmation by histopathology

Histopathologic confirmation was performed by fixing the biopsy in 10% formalin, embedded in paraffin, sectioned and stained using giemsa staining solution to detect spiral or rod-shaped bacteria on the gastric epithelium.<sup>20</sup>

#### DNA extraction and genotyping of *H. pylori* virulence genes

Genomic DNA was extracted using the QIAamp® DNA Mini kit, from the stomach tissues according to the manufacturer's protocol and stored in -20 °C until use. The genotyping analysis was performed with 16S rRNA and gene-specific primers with the PCR cycling conditions mentioned in Table 1.<sup>21-26</sup> PCR was performed in a 20 µl reaction mixture containing 10 µl Taq DNA Polymerase 2x Master Mix RED, 2 µl (10-50 ng) of genomic DNA, 1 µl of 10 pm forward and reverse primers for *vacA s1*, *m1/m2*, *oipA*, *ureC*, *homb* and 2 µl of 2.5 pm primers of *cagA* and the rest nuclease-free water. PCR products were visualized on 2% agarose gel stained with ethidium bromide. The clinical isolates were subjected to nucleotide sequencing analysis to confirm the genotyping assay.

#### Statistical Analysis

All the statistical analysis were performed using IBM SPSS Software (version 23). The Chi-test

( $\chi^2$ ) was used to analyze significant differences in the demographic characteristics (sex, age, smoking/alcohol and tobacco consumption) in our study population. Odds Ratios (ORs) with 95% confidence intervals (CIs) were calculated to evaluate the link between gastrointestinal diseases and the presence of virulence genes with statistical significance at p-values  $\leq 0.05$ .

## RESULTS

### Confirmation of *H. pylori* from biopsy samples

The study examined 500 patients who underwent *H. pylori* detection via RUT, correlated with various gastrointestinal disorders

(Table 2). Giemsa staining was performed on the gastric biopsies, confirming the presence of rod-shaped bacilli within the gastric epithelial cells, as illustrated in Figures 1a-d, revealing *H. pylori* infection. Further confirmation involved PCR analysis for the identification of the 16S rRNA gene specific to *H. pylori* which showed amplification at 522 bp in 491 samples (Figure 2a). The sequence of the 16S rRNA gene was subsequently analyzed using Geneious Software showing 97.33% similarity to the reference strain (CP079530 NCBI), with minor variation as shown in Figure 2b and Figure 2c. The demographic analysis revealed that 279 participants were male with a mean age of  $47.16 \pm 15.74$  year, while 221 were

**Table 1.** Primer sequences and PCR conditions of *H. pylori* 16S rRNA, virulent genes and their PCR product size

Genes	Primer sequence (5'→3')	PCR Condition	Size of PCR Product (bp)	Ref.
16S rRNA	5'-GCGCAATCAGCGTCAGGTAATG-3' 5'-GCTAAGAGAGCAGCCTATGTCC-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 53 °C and extension for 45 Secs @ 72 °C (40 cycles)	522 bp	22
<i>VacA s1</i>	5'-ATGGAATACAACAAACACAC-3' 5'-CTGCTGAATGCGCAAAC-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 55 °C and extension for 40 Secs @ 72 °C (35 cycles)	259 bp	23
<i>CagA</i>	5'-GTTGATAACGCTGTCGCTTCA-3' 5'-GGGTTGATGATATTTCCATAA-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 55 °C and extension for 40 Secs @ 72 °C (35 cycles)	351 bp	24
<i>vacA m1/m2</i>	5'-CAATCTGTCCAATCAAGCGAG-3' 5'-GCGTCAAATAATTCCAAGG-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 56 °C and extension for 30 Secs @ 72 °C (35 cycles)	587 bp/ 642 bp	23
<i>ureC</i>	5'-CCCTCACGCCATCAGTCCCAAAA-3' 5'-AAGAAGTCAAAAACGCCCAAAA-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 63 °C and extension for 40 Secs @ 72 °C (35 cycles)	413 bp	25
<i>homB</i>	5'-AGAGGGTGTGTTGAAACGCTCAATA-3' 5'-GGTGAATCTTCTGCGGTTTG-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 63 °C and extension for 30 Secs @ 72 °C (35 cycles)	161 bp	21
<i>oipA</i>	5'-CAAGCGCTTAACAGATAGGC-3' 5'-AAGGCGTTTTCTGCTGAAGC-3'	Denaturation for 30 Secs @ 94 °C; Annealing for 30 Secs @ 63 °C and extension for 40 Secs @ 72 °C (35 cycles)	428 bp	26

female with a mean age of  $47.87 \pm 14.35$  years. Also, the social behaviour of both the groups was analyzed and we identified a significant association between smoking/alcohol, tobacco consumption and *H. pylori*, as shown in Table 3.

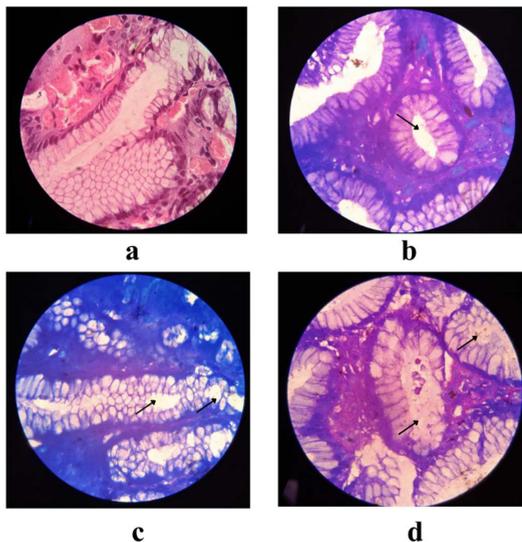
### Genotypic status of virulence genes of *H. pylori* in different clinical manifestations

Genotypic study of 491 *H. pylori* isolates revealed distinct prevalence patterns for key virulence genes as described in Table 2. The *vacA s1* gene exhibited the highest prevalence at 71.89% (353 of 491;  $p = 0.0003$ ) of samples (Figure 3a), showing 99.2% similarity and isolates varying from the reference strain CP079377 (Figure 3b and Figure 3c). The *cagA* gene was found in 31.36% (154/491;  $p = 0.199$ ) of the patients, as shown in Figure 4a, and exhibited 96.33% identity (Figure 4b), though variations existed among isolates when compared to the CP032037 standard (Figure 4c). Amplification of the *vacA m1/m2* gene was shown in Figure 5a., accounted for approximately 51.52% (253/491;  $p = 0.0003$ ) of *vacA m1* and 21.18% (104/491;  $p = 0.044$ ) of *vacA m2* gene strains and

showed a 91.2% similarity and variability against their reference (CP078468) (Figure 5b and Figure 5c). While Figure 6a shows the amplification of the *ureC* gene, with 59.6% (293/491;  $p = 0.727$ ) of the samples being positive, which showed 97.5% similarity and variation in comparison to strain CP079049 (Figures 6b-c) The *oipA* gene of *H. pylori* was detected in 64.97% (319/491;  $p = 0.0003$ ) of the patient cohort (Figure 7a), showing 90.8% similarity variations compared to its reference (CP122953) as depicted in Figure 7b and Figure 7c. The *hombB* was identified in 24.03% (118/491;  $p = 0.03$ ) as illustrated in Figure 8a, with 96.7% similarity to its reference CP078863 (Figures 8b-c).

### Clinical Impressions and their association with *H. pylori* genotypes

A higher frequency of *H. pylori* infection was detected for the virulence genes *vacA s1* (71.89%), *oipA* (64.77%), *vacA m1* (51.53%) and *ureC* (59.67%). The combination of *vacA s1/m1* strain was 36.05% and *vacA s1/m2* was 14.26%, as presented in Table 4. The *vacA s1* was most common in adults between 18 to 35 years (24.9%;  $p = 0.048$ ), while the *cagA* gene (39.61%;  $p = 0.02$ ) and *vacA m2* (15.38%;  $p = 0.036$ ) was common among adults of 46 to 60 years (data not shown). It was inferred from our findings that the *vacA s1* genotype has shown significant threat of acquiring gastritis (OR-1.68;95%CI: 1.11-2.53;  $p = 0.013$ ) and pangastritis (OR-4.79;95%CI: 2.02-11.34;  $p = 0.0004$ ) whereas our study population has reported that the *vacA s1* genotype has reduced the risk for conditions such as gastric cancer (OR-0.13; 95%CI: 0.04-0.2;  $p = 0.0006$ ), gastric ulcer (OR-0.10; 95%CI: 0.02-0.51;  $p = 0.005$ ) and PUD (OR-0.27; 95% CI: 2.202-11.34;  $p = 0.01$ ). In addition, the *vacA s1/m1* combination strain has shown a higher incidence of gastritis (OR-2.34; 95%CI: 1.29-4.23;  $p = 0.004$ ) and pangastritis (OR-2.61; 95%CI: 0.97-7.02;  $p = 0.05$ ) and the *vacA s1/m2* combination significantly correlated with gastritis (OR-2.03; 95%CI: 1.04-3.95;  $p = 0.03$ ). The *cagA* strain showed a increased likelihood of GC (OR-3.45; 95%CI: 1.20-9.89;  $p = 0.02$ ) and the *oipA* strain had a greater incidence of PUD (OR-7.87; 95%CI: 1.02-60.40;  $p = 0.04$ ). The *vacA m1*, *vacA m2*, and *oipA* as well as *vacA s1/m1* and *vacA s1/m2* combinations showed no association with

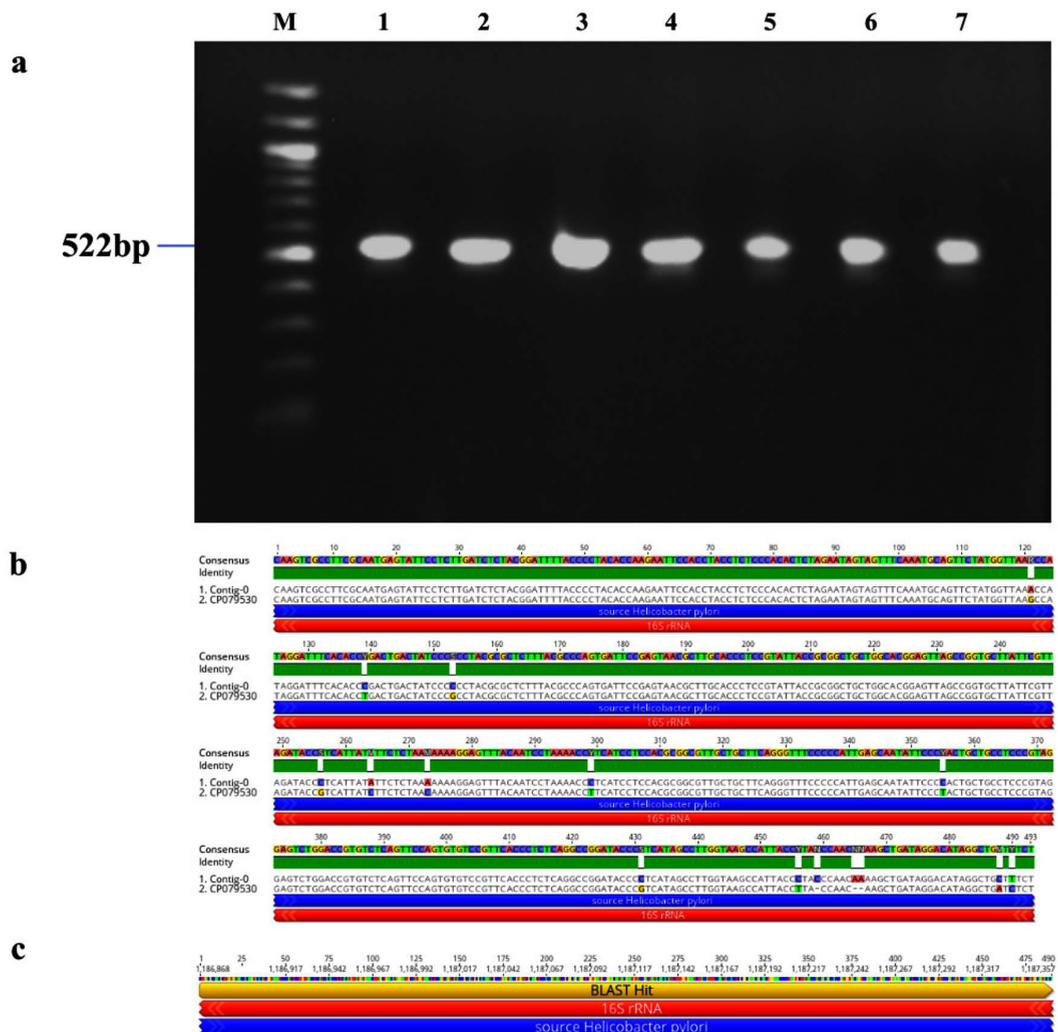


**Figure 1.** Histopathological images from clinical isolates representing the level of infection by *H. pylori*, with black colour arrow representing the colonization of *H. pylori* (a) Normal Gastric mucosa (b) Mild infection by *H. pylori* (c) Moderate infection by *H. pylori* (d) Severe infection by *H. pylori*

gastric cancer as shown in Table 4. Also, the *vacA s1/m1* have shown lower risk for gastric ulcer (OR-0.06; 95%CI: 0.007-0.56;  $p = 0.01$ ) whereas, *hombB* gene had an increased incidence of gastritis (OR-1.62; 95%CI: 1.01-2.59;  $p = 0.04$ ) and a markedly reduced risk for the development of duodenal ulcer (OR-0.17 95% CI: 0.04-0.72;  $p = 0.01$ ).

## DISCUSSION

*H. pylori*, a challenging bacterium to cultivate and identify due to its meticulous nature, which requires significant time and effort. Therefore, molecular techniques that offer high sensitivity and specificity for detecting these



**Figure 2.** Identification and sequence analysis of 16S rRNA gene from *H. pylori* isolated biopsies. (a) Agarose gel electrophoresis of 1% agarose to identify 16S rRNA gene amplified at 522 bp from *H. pylori* isolated biopsies where M represents 100 bp ladder and lanes represent the positive samples. (b) Pairwise identity and nucleotide sequence for 16S rRNA compared with the standard strain: CP079530 (NCBI) with 97.33% identity (c) Blast Hit of 16S rRNA amplified region from 1,186,868 →1,187,357, relative to the original gene of the reference strain

**Table 2.** Particulars of endoscopic findings of *H. pylori*-infected cases based on admission diagnosis and their percentage prevalence of virulence genes associated

Endoscopic Findings (n = 500)	PCR Positive for 16S rRNA	<i>cagA</i> Positive (%)	<i>vacA s1</i> Positive (%)	<i>ureC</i> Positive (%)	<i>oipA</i> Positive (%)	<i>vacA m1</i> Positive (%)	<i>vacA m2</i> Positive (%)	<i>homb</i> Positive (%)	<i>vacA s1/m1</i> Positive (%)	<i>vacA s1/m2</i> Positive (%)
Gastritis (339)	333	96 (19.5)	251 (51.1)	199 (67.9)	217 (68.2)	175 (69.2)	73 (70.2)	89 (75.5)	124 (70.1)	52 (74.3)
Duodenal Ulcer (36)	36	12 (2.4)	21 (4.2)	24 (8.2)	30 (9.4)	21 (8.3)	9 (8.7)	2 (1.7)	11 (6.2)	4 (5.7)
Gastric cancer (9)	15	9 (1.8)	4 (0.8)	11 (3.8)	4 (1.3)	3 (1.2)	2 (1.9)	3 (2.5)	3 (1.7)	2 (2.9)
Gastric ulcer (15)	9	5 (1.01)	2 (0.4)	6 (2.05)	9 (2.83)	3 (1.19)	4 (3.85)	0	1 (0.56)	0
Pangastritis (82)	79	23 (4.6)	66 (13.4)	42 (14.3)	42 (13.2)	41 (16.2)	14 (13.5)	23 (19.5)	33 (18.6)	12 (17.1)
PUD (4)	15	7 (1.4)	6 (1.2)	9 (3.07)	14 (4.4)	9 (3.5)	2 (1.9)	1 (0.8)	4 (2.2)	0
Nodular Gastritis (15)	4	2 (0.4)	3 (0.6)	2 (0.6)	3 (0.9)	1 (0.4)	0	0	1 (0.6)	0
<b>Total (%)</b>	<b>491</b>	<b>154 (31.36)</b>	<b>353 (71.89)</b>	<b>293 (59.6)</b>	<b>319 (64.97)</b>	<b>253 (51.52)</b>	<b>104 (21.18)</b>	<b>118 (24.03)</b>	<b>177 (36.05)</b>	<b>70 (14.26)</b>

Endoscopic findings (n = 500), PCR confirmation by 16S rRNA specific for *H. pylori* (n = 491) and virulence genes prevalence was calculated from PCR-positive isolates

bacteria are becoming increasingly popular for studying their prevalence in various experimental samples.<sup>27</sup> The diverse genetic makeup of virulence factors among *H. pylori* strains worldwide contributes to a broad spectrum of gastrointestinal diseases. Increasing evidence suggests that *H. pylori* genetic diversity has significant clinical implications.<sup>28</sup> The present study provides a comprehensive molecular and histopathological evaluation of *H. pylori* infection in gastric biopsy tissues by employing RUT, Giemsa staining and PCR amplification by 16S rRNA gene. The identification of *H. pylori* in 98.2% of the samples by PCR underscores a greater sensitivity and specificity of molecular strategies compared to conventional diagnostic methods. Our findings were consistent with previous studies conducted by Gantuya et al. who, reported 94.3% detection rate of *H. pylori* using 16S rRNA specific PCR and Han et al. observed 92.2% positivity rate.<sup>29,30</sup> Similarly, Elnosh et al. reported a 78.6% detection rate, emphasizing that PCR targeting the 16S rRNA gene was one of the most reliable results for molecular detection of *H. pylori*.<sup>31</sup>

*H. pylori*, the most genetically diverse bacterial pathogen, exhibiting variable virulence genotypes across populations from different geographic origins. Molecular typing methods have significantly enhanced our insights into the epidemiology of *H. pylori* infection.<sup>32</sup> In our study, various virulence genes of *H. pylori* were analyzed, for the presence of virulence-associated genes, particularly *vacA* (*s1*, *m1/m2*), *cagA*, *oipA*, *ureC* and *homb*.

The *vacA* gene, an important virulence factor, displays a mosaic structure characterized by two groups of allelic variations: the signal sequence (*s1*, *s2*) and the mid-region (*m1*, *m2*).<sup>33</sup> Extensive investigations have been conducted on allelic variation as a virulence determinant in *H. pylori* strains. All *H. pylori* strains harbour the *vacA* gene; however, the difference in the signal and mid-regions alter cytotoxicity. The variation in *vacA* genotypes (allelic variations) was notably distinct across different countries, with prior studies highlighting significant geographic disparities in these virulence factors.<sup>23</sup> In our investigation, the *vacA s1* genotype was predominant in 71.89% of cases, which was consistent with previous

**Table 3.** Demographic characteristics of patients upon confirmation by 16S rRNA specific for *H. pylori*

Variable	Subjects n (%)	<i>H. pylori</i> Positive n (%)	<i>H. pylori</i> Negative n (%)	p-value
All	500 (100)	491 (98.2)	9 (1.8)	
<b>Sex</b>				0.988
Male	279 (55.8)	274 (55.8)	5 (55.6)	
Female	221 (44.2)	217 (44.2)	4 (44.4)	
<b>Age</b>				0.797
<35	133 (26.6)	131 (26.7)	2 (22.2)	
35-45	111 (24.4)	108 (22)	3 (33.3)	
46-60	152 (30.4)	149 (30.3)	3 (33.3)	
>60	104 (20.8)	103 (21)	1 (11.1)	
<b>Smoking/Alcohol</b>				<b>0.053*</b>
Yes	96 (19.2)	94 (18.8)	2 (0.4)	
No	404 (80.8)	397 (79.4)	7 (1.4)	
<b>Tobacco</b>				<b>0.018*</b>
Yes	49 (9.8)	48 (9.6)	1 (0.2)	
No	451 (90.2)	443 (88.6)	8 (1.6)	

n (%), represents the number (percentage) of patients with the demographic conditions

\*Chi-square test was performed to compare demographic variables and patients with a positive result for *H. pylori*. Highlighted values denote a statistically significant result at the  $p < 0.05$

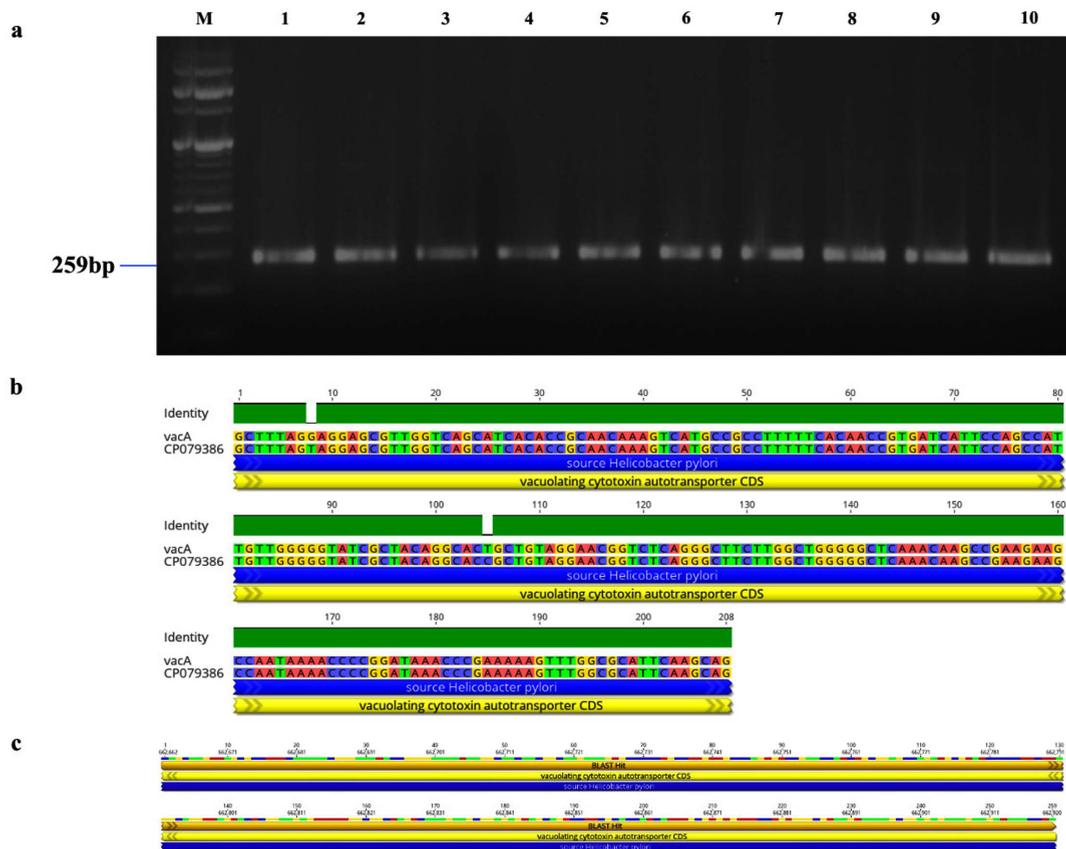
reports highlighting its strong association with gastroduodenal diseases. Notably, *vacA m1* strains, which have been implicated in increased cytotoxic activity and enhanced gastric epithelial cell vacuolation, were reported in 51.53% of cases, while the *vacA s1/m1* was reported in 36.05% and the *vacA s1/m2* was reported in 14.26% of our cases. Hence, these findings have revealed the higher incidence of *vacA* gene among *H. pylori*-infected patients which aligns with studies conducted in various regions of India such as Hyderabad, Varanasi, Coimbatore and Chennai.<sup>34-36</sup> Similarly, our study correlated with studies reported for *H. pylori* virulence toxins *vacA s1* exhibiting high prevalence rates in Asia, South Africa, Northern Europe, South America, and Southern Europe<sup>37,38</sup> and studies conducted in Brazil and the Middle East indicate that *vacA s1* or *m1* genotypes are linked to the advancement of stomach cancer.<sup>39</sup> Also, Mukhopadhyay et al. observed notable prevalence of the *vacA s1m1* allele in Kolkata.<sup>40</sup> In addition, the findings of our study aligns with the research conducted on patients in the

Khuzestan province of Iran, which reported higher *vacA s1/m1* genotypes.<sup>33</sup> Nonetheless, a study by Udayakumar et al. has indicated that the *vacA m2* allele was more commonly reported in Chennai.<sup>34</sup> Moreover, our findings have discovered notable correlations between particular *H. pylori* genotypes and a range of gastrointestinal disorders where our analysis revealed that the association of *vacA s1* with specific gastric conditions was predominantly noted in patients diagnosed with gastritis (OR = 1.68; 95% CI 1.11-2.53;  $p = 0.013$ ) and pangastritis (OR = 4.79; 95% CI 2.02-11.34;  $p = 0.0004$ ), indicating increased odds of these inflammatory conditions in individuals harboring *s1*-positive strains. Similarly, *vacA s1/m1* genotypes demonstrated increased odds of gastritis (OR - 2.34; 95%CI: 1.29-4.23;  $p = 0.004$ ) and pangastritis (OR - 2.61; 95%CI: 0.97-.02;  $p = 0.05$ ), indicating its contribution to mucosal inflammation. These results align with previous studies involving Iranian and South Korean populations.<sup>41,42</sup> Also, the *vacA s1/m2* genotype was significantly associated with increased odds of gastritis (OR - 2.03; 95%

CI: 1.04-3.95;  $p = 0.03$ ) and inversely linked with other conditions and they align with previous studies conducted.<sup>23,34,40</sup> Interestingly, although *vacA* genotypes are globally associated with more severe disease outcomes such as duodenal ulcer, gastric cancer and gastric ulcer our study exhibited a lower risk of these conditions among *vacA*-positive individuals. This inverse association was likely influenced by very small sample sizes among these gastric conditions, leading to imprecise odds ratio estimates and wide confidence intervals. Therefore, *vacA* being an important virulence marker for the gastrointestinal conditions in our South Indian population, these findings should be interpreted with caution. Furthermore, the

results highlight how the pathogenic potential of globally significant virulence factors can manifest differently across specific regional populations.

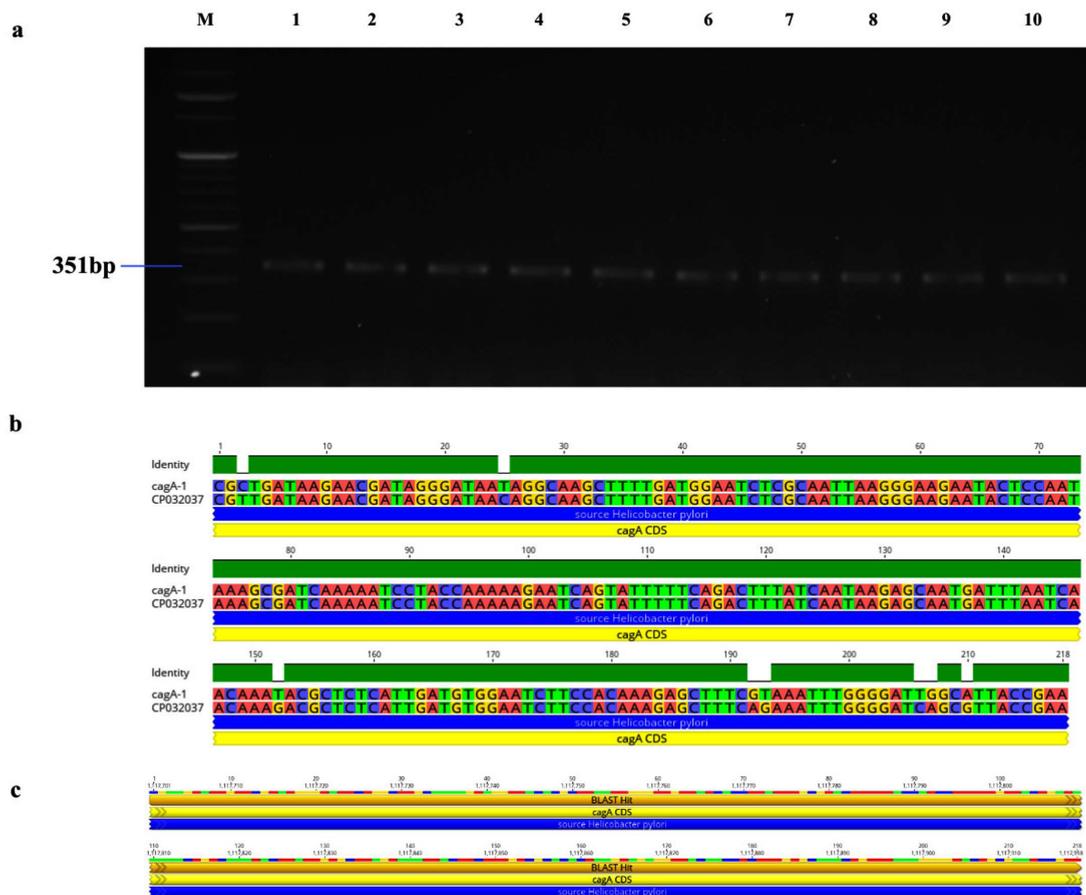
The *cagA* gene was identified as a notable virulence factor, potentially elevating the risk of drug resistance and carcinoma in affected individuals. The correlation between *cag* PAI and infection status in East Asia was approximately 60%, whereas in India, it is around 90%. However, our study found that the *cagA* genotype, an indicator for *cag* PAI presence, was 31.36% indicating a moderate prevalence within the studied population and was significantly associated with an elevated risk of GC (OR-3.45; 95%CI: 1.20-9.89,  $p \leq 0.02$ ), reinforcing its role



**Figure 3.** Identification and sequence analysis of *vacA* gene from *H. pylori* isolated biopsies. (a) Agarose gel electrophoresis of 2% agarose for the identification of *vacA* gene amplified at 259 bp from *H. pylori* isolated biopsies (b) *vacA* gene pairwise identity was 99.23% compared with the standard strain (CP079386) (c) The Blast Hit of *vacA* (259 bp) amplified area 662,662 → 662,920, relative to the original gene of the reference strain

as a critical virulence determinant in *H. pylori*-induced carcinogenesis. These findings were similar to a study on the comparative analysis of *H. pylori* isolates from North and South India, which demonstrated significant variations in the integrity of the *cagA* gene and *cagPAI* between the two regions. Approximately 34% of the isolates obtained from Varanasi and 46% of the isolates from Hyderabad exhibited the complete *cagPAI*. In comparison, 65.2% of the isolates from Varanasi exhibited a partially deleted *cagPAI*, while 53.5% of the isolates from Hyderabad showed the same characteristic features. The frequency of this

gene exhibits considerable variation worldwide, with reported values between 60% and 80%.<sup>43,44</sup> However, numerous studies have reported that the *cagA* gene has been linked to gastritis, PUD and duodenal ulcer; however, our findings have discovered that there was a lower correlation with any of these gastric conditions.<sup>45,46</sup> The findings reinforce the critical role of *cagA* as a virulence determinant in *H. pylori*-induced carcinogenesis, even when its overall prevalence is lower in our population. Although, *cagA* has been widely associated with gastritis, duodenal ulcer in many populations, our study did not



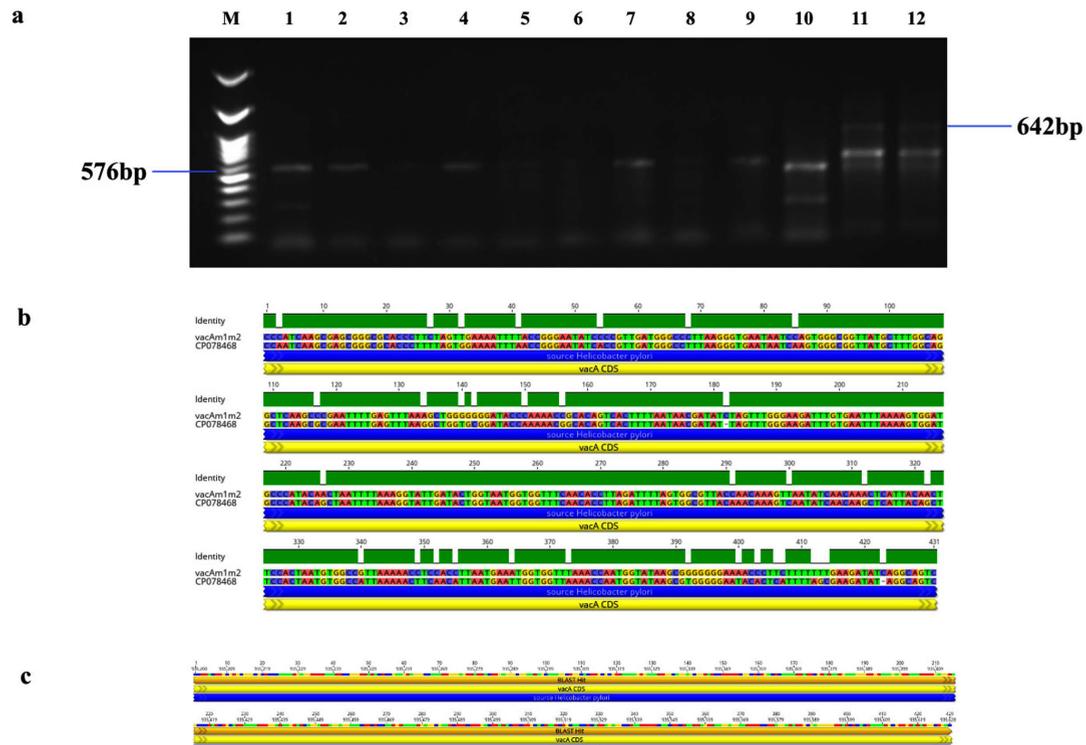
**Figure 4.** Identification and sequence evaluation of the *cagA* gene derived from *H. pylori* isolated biopsies. (a) Agarose gel electrophoresis utilizing a 2% agarose medium to identify the *cagA* gene, which was amplified at 351 bp from *H. pylori* isolated biopsies. (b) The pairwise identity of the *cagA* gene was determined to be 96.3% in comparison to the reference strain (CP032037). (c) The Blast Hit of the *cagA* (351 bp) amplified region spans from 1,117,564 → 1,121,211, relative to the original gene of the reference strain

observe such correlations. This discrepancy likely reflects the regional variations in *cagA* gene that includes EPIYA motifs or partial deletion within the pathogenicity island which could diminish its functional activity. The characterization of the predominant circulating strains and their disease associations was indispensable for developing region-specific clinical guidelines and contributes to the global understanding of the *H. pylori* virulence markers. Hence, the establishment of this genotypic baseline provides the essential scientific framework for future mechanistic studies emphasizing South Indian strains.<sup>47-49</sup>

The identification of *ureC*, a prerequisite for bacterial survival in acidic environments, was observed in 59.6% of cases, underscoring the adaptability of *H. pylori* within the gastric niche. Our study has reported a higher prevalence of the *ureC* gene in comparison with a study carried out

in Gujarat, India, where they reported 18.18% of *H. pylori*-positive samples with *ureC* gene and there was no correlation with the gastric conditions.<sup>50</sup> However, a study conducted in Taiwan showed that the Mbol-defined genotype 3 *ureC* gene exhibits a closer association with gastric cancer and gastric ulcer. Thus, the genetic variations in *ureC* may impact the pathogenesis of gastric diseases.<sup>14</sup>

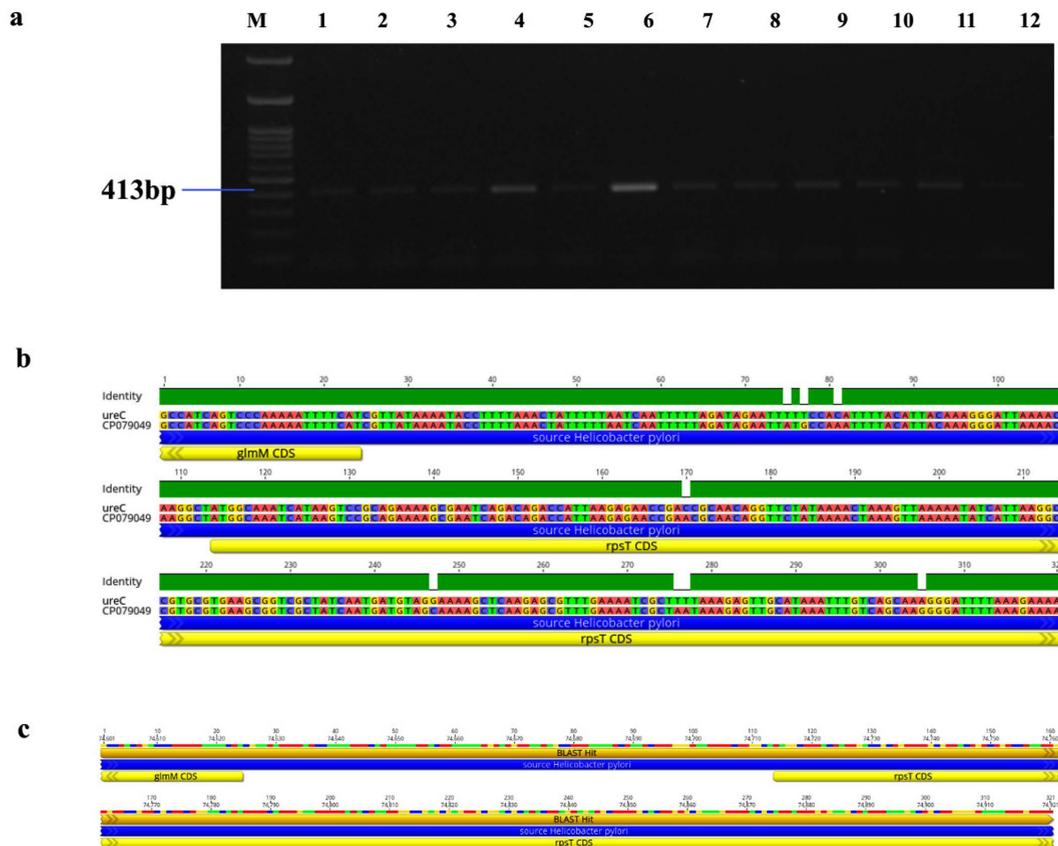
*oipA* and *hombB* are well-known outer inflammatory proteins (OMPs) of *H. pylori* that play a crucial role for immune response stimulation, cell attachment, and initiation of infection. *oipA*, an important adhesion associated with bacterial colonization and inflammation, was identified in 64.97% of cases and showed a correlation with PUD (OR - 7.87; 95%CI: 1.02-60.40; p = 0.04) in our study population however, this extremely wide confidence interval indicates low precision due to decreased sample size of PUD cases, as



**Figure 5.** Identification and sequence analysis of *vacA* m1/m2 gene from *H. pylori* isolated biopsies. (a) Agarose gel electrophoresis of 1% agarose to identify *vacA* m1/m2 gene amplified at 576/642 bp from *H. pylori* isolated biopsies where M represents 100 bp ladder and lanes represent the positive samples (b) *vacA* m1/m2 gene pairwise identity was 92.11% compared with the standard strain (CP078468) (c) The Blast Hit of *vacA* m1/m2 (576/642 bp) amplified area 935,200 → 935,628, relative to the original gene of the reference strain

that of the study reported by Rezaei et al., with the prevalence of 56% *oipA* gene. In addition, the study investigated by El-Sayed et al. in the Egyptian population, where the *oipA* virulence gene showed a higher association with gastroduodenitis.<sup>9,51</sup> However, the *oipA* gene reported by Singh et al. and Esteghamati et al. have found no significant association with distinct gastrointestinal diseases such as DU, GERD, NERD and gastritis & PUD respectively. While *oipA* was identified at a high frequency and demonstrated a significant association with PUD in our population, this finding must be approached with caution due to the limited number of PUD cases, which led to a very wide confidence interval and restricted statistical

precision. The variability in the functional ‘on/off’ status of *oipA*, along with regional strain diversity and host-specific inflammatory responses, may contribute to the inconsistencies observed across studies. Despite these limitations, *oipA* was recognized as a significant mediator of gastric inflammation, and our findings suggest that it may serve as a virulence biomarker in patients with PUD. Therefore, comprehensive studies with broader spectrum of ulcer cases are necessary to elucidate its accurate clinical significance among the South Indian population and to facilitate the formulation of targeted antimicrobial strategies.<sup>52,53</sup>



**Figure 6.** Identification and sequence analysis of ureC gene from *H. pylori* isolated biopsies. (a) Agarose gel electrophoresis of 1% agarose to identify ureC gene amplified at 413 bp from *H. pylori* isolated biopsies where M represents 100 bp ladder and lanes represent the positive samples. (b) ureC gene pairwise identity was 97.51% compared with the standard strain (CP079049) (c) The Blast Hit of ureC (413 bp) amplified area 74,601 → 74,921, relative to the original gene of the reference strain

**Table 4.** Association of clinical impressions with *H. pylori* virulent genes

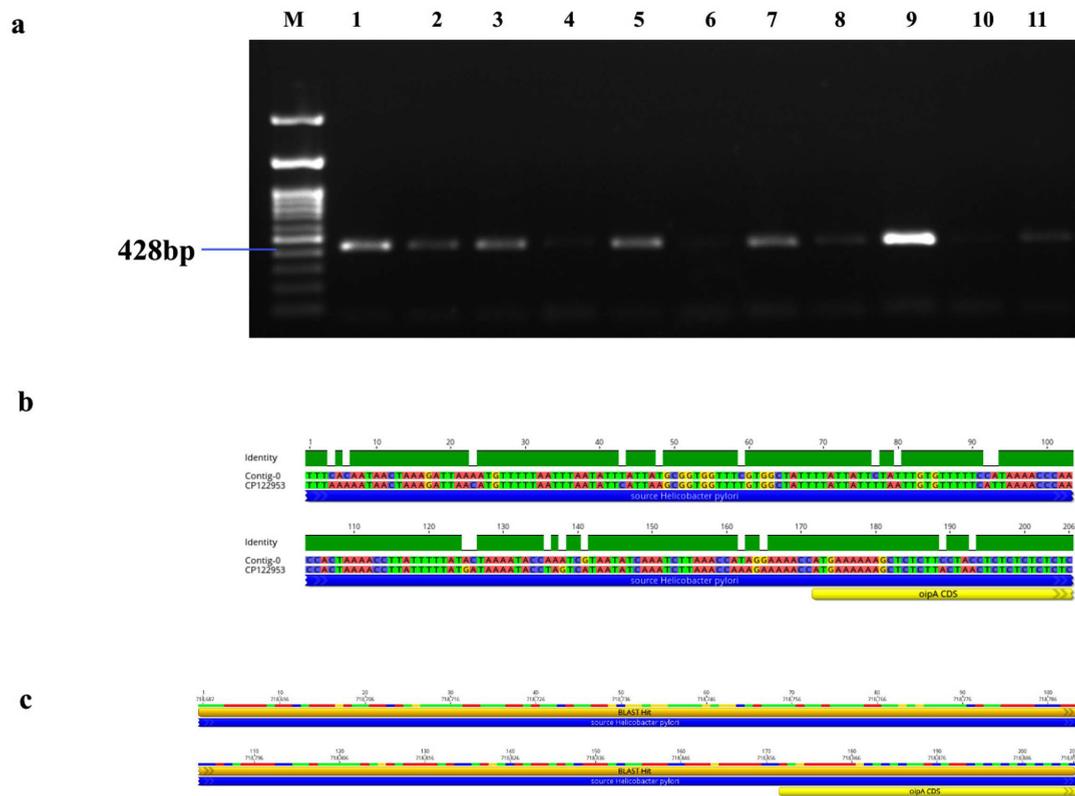
Genes	Prevalence (%)	Clinical Impressions						
		Gastritis OR (95% CI)	Duodenal Ulcer	Gastric cancer	Gastric Ulcer	Pangastritis	PUD	Nodular Gastritis
<i>vacA s1</i>	71.89	<b>1.68</b> <b>(1.11-2.53)</b>	0.52 (0.25-1.03)	<b>0.13</b> <b>(0.04-0.42)</b>	<b>0.10</b> <b>(0.02-0.51)</b>	<b>4.79</b> <b>(2.02-11.34)</b>	<b>0.27</b> <b>(0.05-1.42)</b>	1.11 (0.12-11.38)
<i>cagA</i>	31.16	<b>0.013</b> 0.71 (0.48-1.07)	0.063 1.11 (0.54-2.28)	<b>0.0006</b> <b>3.45</b> <b>(1.20-9.89)</b>	<b>0.005</b> 2.82 (0.074-10.65)	<b>0.0004</b> 0.89 (0.52-1.51)	<b>0.01</b> 1.97 (0.70-5.55)	0.9 2.22 (0.31-15.94)
<i>vacA m1</i>	51.53	1.13 (0.77-1.65)	1.34 (0.67-2.67)	<b>0.22</b> <b>(0.06-0.81)</b>	0.46 (0.11-1.87)	1.01 (0.62-1.64)	1.42 (0.49-4.06)	0.31 (0.03-3.00)
<i>vacA m2</i>	21.18	1.15 (0.71-1.84)	1.26 (0.57-1.26)	<b>0.028</b> 0.91 (0.25-3.31)	0.281 3.05 (0.80-11.59)	0.942 0.79 (0.42-1.48)	0.105 0.56 (0.12-2.54)	0.313 - -
<i>ureC</i>	59.67	0.56 1.01 (0.68-1.48)	0.56 1.38 (0.67-2.83)	0.89 1.89 (0.59-6.02)	0.1 1.35 (0.33-5.49)	0.46 0.72 (0.44-1.18)	0.1 1.01 (0.35-2.89)	0.67 (0.09-4.82)
<i>oipA</i>	64.77	1.05 (0.71-1.56)	1.16 (0.56-2.37)	<b>0.18</b> <b>(0.05-0.59)</b>	-	<b>0.55</b> <b>(0.34-0.91)</b>	<b>7.87</b> <b>(1.02-60.40)</b>	-
<i>homb</i>	24.03	0.78 <b>1.62</b> <b>(1.01-2.59)</b>	0.68 <b>0.17</b> <b>(0.04-0.72)</b>	<b>0.004</b> 0.78 (0.21-2.82)	-	<b>0.01</b> 1.37 (0.80-2.34)	<b>0.04</b> 0.21 (0.02-1.68)	-
<i>vacA s1/m1</i>	36.05	<b>0.04</b> <b>2.34</b> <b>(1.29-4.23)</b>	<b>0.01</b> 0.75 (0.25-2.26)	0.711 <b>0.08</b> <b>(0.02-0.29)</b>	<b>0.06</b> <b>(0.007-0.56)</b>	0.24 <b>2.61</b> <b>(0.97-7.02)</b>	0.14 <b>0.02</b> <b>(0.003-0.18)</b>	0.34 (0.02-5.62)
<i>vacA s2/m2</i>	14.26	<b>0.004</b> <b>2.03</b> <b>(1.04-3.95)</b>	0.61 0.57 (0.17-1.89)	<b>0.0002</b> 0.24 (0.05-1.15)	<b>0.01</b> -	<b>0.05</b> 1.74 (0.72-4.22)	<b>0.0004</b> -	0.45 -
		<b>0.03</b>	0.51	0.07		0.21		

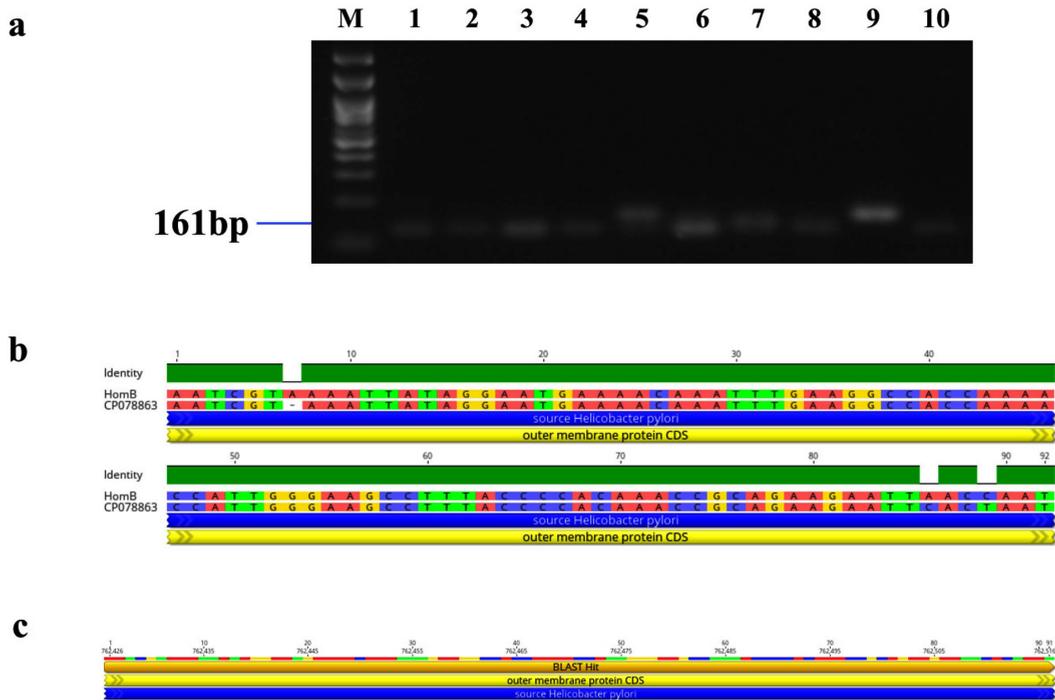
OR odds ratio was carried out to establish the association between clinical conditions with *H. pylori* virulent genes; % denotes the percentage of each virulent gene present in the patients. 95% CI: 95% Confidence interval; Values highlighted represent the statistical significance at the  $p < 0.05$  level

Our findings are concordant with previous studies by Oleastro et al. in the French population and Bajwa et al. in the Pakistani population, which showed a significant association with PUD and gastritis for the prevalence of *homb*, where we reported 24.03% and others reported 58% and 38.8%, respectively. Consistent with earlier studies, we observed that *homb*-positive strains were more common in patients with gastritis (OR - 1.62; 95%CI: 1.01-2.59; p = 0.04).<sup>21,54</sup> However, in our study, the *homb* did not show any significant correlation with GC, unlike the study reported by Talebi et al., in Iranian population. This discrepancy could indicate regional variations in circulating *H. pylori* lineages, differences in the distribution of *homa/homb* paralogues, or host immune responses that influence the expression the gene.

The limited number of gastric cancer cases in our cohort likely restricted the statistical power to identify associations with less prevalent virulence markers. Although *homb* appears to contribute for inflammatory diseases in our population, its involvement in malignant outcomes necessitates additional research through larger, multi-centre studies.<sup>55</sup>

Therefore, in our study we found that the presence of specific virulence genes significantly influences the development of various gastrointestinal diseases and PCR-based detection in our study revealed diverse distributions of *vaca*, *cagA*, *oipA*, *homb* and *ureC* genotypes across various gastric conditions, highlighting the importance of genotype-specific risk assessment to guide clinical management and treatment





**Figure 8.** Identification and sequence analysis of homB gene from *H. pylori* isolated biopsies. (a) Agarose gel electrophoresis of 1% agarose to identify homB gene amplified at 161 bp from *H. pylori* isolated biopsies where M represents 100 bp ladder and lanes represent the positive samples. (b) Pairwise identity and nucleotide sequence for homB compared with the standard strain: CP078863 (NCBI) with 96.7% identity (c) Blast Hit of homB amplified region from 762,426 → 762,516, relative to the original gene of the reference strain

strategies. Consequently, the genotypic diversity identified among the *H. pylori* isolates suggests that various strains with differing pathogenic potential may exist within a population, leading to disease heterogeneity.

**Limitations**

Our study was conducted in a single medical facility; therefore, the potential for selection bias cannot be rule out. As this study was confined to the South Indian Tamil population, the ability to extrapolate its findings to a wider range of global cohorts was limited. Although several significant associations were observed between the virulence genotypes and clinical outcomes, the unequal distribution of cases across clinical outcomes may have contributed to highly variable odds ratio estimates and wide confidence intervals, warranting cautious interpretation of genotype-disease associations. Furthermore, the cross-sectional design restricts the ability to infer

causality between the presence of virulence genes and disease progression. The highly sensitive PCR-based molecular method detected only the presence of virulence genes and did not evaluate their expression levels, functional activity, host-pathogen interaction or genotype-phenotype patterns, which could provide a more precise indication of pathogenic potential. The inability to characterize mixed infections or allelic diversity-common in *H. pylori* populations also represents an additional limitation. These constraints provide essential context for interpretation and highlight the need for larger, multicentric, and mechanistic studies to enable the inclusion of appropriate control groups in near future.

**CONCLUSION**

*H. pylori* strains demonstrated significant genotypic diversity, with *vacA s1* identified as the most common virulence genotype in our

population. Significant associations were identified between *vacA s1* and *vacA s1/m1* genotypes with gastritis and pangastritis, while *cagA* showed a strong correlation with gastric cancer. The prevalence of virulence factor genes was reported in the increasing order of *vacA s1* > *oipA* > *ureC* > *vacA m1* > *vacA s1/m1* > *cagA* > *vacA m2* > *vacA s1/m2*, from isolates of patients infected with *H. pylori* infection. These findings suggest that *vacAs1* can may serve as one of the potent virulent markers for gastrointestinal disease manifestation. Also, our findings highlight the genetic diversity among *H. pylori* strains and their effects on gastric pathology. However, given the cross-sectional design and limited subgroups sizes, the association should be interpreted cautiously. Further multicentre and longitudinal studies, including gene expression analyses are needed to emphasizes the pathogenic significance of genotype and to enlighten clinical management and treatment strategies for *H. pylori*-associated gastrointestinal diseases.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

MJ conceptualized and designed the study. NAR recruited the cases for the study and performed endoscopies. SKSR performed sample collection. SKSR performed the experiments. SKSR and MJ performed data analysis. MJ and SKSR wrote the manuscript. MJ reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

#### FUNDING

None.

#### DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

#### ETHICS STATEMENT

This study was approved by the Institutional Ethical Committee, SRM Medical College and Research Center, Kattankulathur, Chennai, India, dated 25/03/2021 with EC Reg. No. 2394/IEC/2021.

#### INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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