DETECTION OF HELICOBACTER PYLORI VIRULENCE GENES BY POLYMERASE CHAIN REACTION IN GASTRIC BIOPSIES AMONG THE DYSPEPSIA PATIENTS

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ABSTRACT

Helicobacter pylori infection is very common in Pakistan as compared to other developing countries. The aim of this study was to detect the virulence genes of *H. pylori cagA, vacA, ureA* and *ureC* genes in gastric biopsy isolated from dyspepsia patients belonging to Peshawar district. A total 80 samples of gastric biopsy were collected from dyspepsia patients with different age groups during endoscopy from June to October 2021 in Peshawar district. Genomic DNA was extracted after isolation of *Helicobacter pylori* from the biopsy culture. All the virulence genes of *H. pylori* were amplified by process of polymerase chain reaction as well as compared with a reference database. The prevalence rate of *Helicobacter pylori* among the dyspepsia patients was reported 75% (60/80). The detection of virulence genes: *cagA*, *vacA*, *ureA* and *ureC* were 52 (65.0%), 39 (48.7%), 35 (43.7%), and 18 (22.5%) in gastric biopsies, respectively. It was found that the *CagA* gene is more specific for the detection of *H. pylori* than *vacA*, *ureA* and *ureC* gene. The statistical difference (P>0.05) was not found between the investigated genes and gastric disorders, probability of the alternative (P>0.05) as per hypothesis was rejected and null hypothesis was accepted, which was detected among the dyspepsia patients.

KEY WORDS: *Helicobacter pylori*, Gastric biopsy, PCR, Virulence genes, *CagA, VacA, UreA* and *ureC* gene, prevalence

INTRODUCTION

A gram-negative bacterium called *Helicobacter pylori* exists in the stomach mucosa with around 50% of people throughout the world [1]. *H. pylori* infection causes chronic gastritis and raises the risk of gastric cancer, peptic ulcer, and mucosa-associated lymphoid tissue (MALT) lymphoma [2]. The spiral organisms would have seen in the gastric mucus layer for a long time ago of *H. pylori* discovery, increased interest in the pathogenesis of gastrododenal diseases, and relatively regular availability of clinical samples through endoscopic biopsy, all contributed to significant advancement in medical care [3]. The gram negative bacteria *H. pylori* has a length of 2 to 4 pm and width 0.5 to 1pm. The body has 2 to 6 flagella and because of their motility the flagella move may quickly through viscous media like mucus layer of the gastric epithelial cell [4]. *H. pylori* lack fimbriae adhesins, unlike many other gastrointestinal tract bacteria. The growth takes place between 34 and 40°C with a maximum 37°C all these requirements is fulfill in the gastrointestinal tract of mammals. Therefore, bacteria pH4 exposure, however growth was only accelerated lower pH range of 5.5 to 8.0, with neutral pH being the best for growth [5, 6].

According to the most recent global cancer data from 2018 [7], gastric cancer has the second-highest mortality rate of any type of cancer globally and the sixth-highest incidence rate [8].

The risk factors for *H. pylori* infection have been shown to include cigarette smoking, alcohol use, nutrition, occupational exposure, and personal genetic makeup [9]. Both invasive and non-invasive diagnostic procedures were employed to find *H. pylori* in patients. Analyzing material from stomach biopsies provides the foundation for invasive diagnostics like the rapid urease test, culture and PCR assays that are non-invasive, such as urea breath tests, serological testing, blood or urine tests, and tests for the presence of antigen in *H. pylori* stool samples [10].

2. METHODS AND MATERIALS

Gastric biopsy samples were taken from dyspepsia patients at the Shaukat Khanum Hospital in Peshawar with the help of gastroenterologists. The patients consisted of 57 males and 23 females with different age groups. The gastric biopsy samples obtained between June 2021 and September

2021 were used as a basis for the PCR analysis. The patients suffered from severe diseases such as gastritis, gastric ulcers, duodenal ulcers, and stomach cancer. The most commonly observed symptoms were abdominal pain, heartburn, vomiting, nausea, bloating, belching, regurgitation and fever. A patient of *H. pylori* infection was considered to be confirmed by the detection of cagA, vacA, ureA, and ureC in stomach biopsy samples. The result was analyzed by using of SPSS (statistical package) computer program which came out in ratio and percentages. Furthermore, the groups were compare by chi-square test and statistically significant level regarded at P-value <0.05.

Table 1 Primers sequences and base pair of the product size

Genes	Primers	Primer sequence (5'-3')	Product size
CagA	Forward Reverse	AGTAAGGAGAAACAATGA AATAAGCCTTAGAGTCTTTTTGGAAATC	1,320
VacA	Forward Reverse	GCTTCTCTTACCACCAATGC TGTCAGGGTTGTTCACCATG	1,162
UreA	Forward Reverse	GAGAATGAGATGAAACTCACCC TTGTCTGCTTGTCTATCAACC	627bp
UreC	Forward Reverse	TGGGACTGATGGCGTGAGGG AAGGGCGTTTTTAGATTTTT	820bp

3. RESULTS

A total of 80 samples collected from the infected individuals. All of the affected individuals had abdominal pain, heart burn, bloating, belching, nausea, vomiting, regurgitation and fever. The patients were proved to have gastric cancer, gastric ulcer, duodenal ulcer and gastritis by endoscopic examination. The presence of many other symptoms of gastrododenal diseases was identified by medical aspects.

The maximum incidence of *H. pylori* infection was recorded 88.8% in age group I (10-20 years) and then 79.3% recorded in age group III (31-40 years) while the minimum incidence was recorded 45.4% in age group V (51-60 years). Maximum number of patients were in age III (31-40 years) next were in age group IV (41-50 years) then age group II (21-30 years) and then age group V (51-60 years) and then age group I(10-20 years). Data about prevalence showed that its prevalence was high among the age group 1 (10-20 years) which was 88.8% and then second most susceptible group was age group 3(31-40 years) which was 79.3% and significance difference was not observed (p=0.1) as shown in table 3. Out of 80 patients, 60 samples showed proof and confirmation of *H. pylori* infection so prevalence of *H. pylori* recorded was 72 %. Regarding *H. pylori* positivity 42 out of 57 (73.6%) males were *H. pylori* +ve and 15 out of 57 (26.3%) were *H. pylori* -ve. In case of females 18 out of 23 (78.2%) were *H. pylori* +ve and 5 out of 23 (21.7%) were *H. pylori* (table-5) without statistical difference (p=0.6) as shown in table 4

The fluorescent bands of DNA observed under U-V light by using method of Gel Doc system as shown in fig 2. Primer for amplication bulging result was observed on 1.5 % agarose gel electrophoresis and the resultant amplicon was 1,340 base pair by comparison of 100bp DNA marker. Out of 80 patients CagA gene has been identified in 52 individuals who are affected as shown in fig 3. Primer for amplication bulging result was observed on 1.5% agarose gel electrophoresis and the resultant amplicon was 1,162 base pair by comparison of 100bp DNA marker. Out of 80 patients vacA gene has been identified in 39 individuals who are affected. The positive predictive values of VacA gene found to be 48.7% as shown in figure 4. Primer for amplication bulging result was observed on 1.5 % agarose gel electrophoresis and the resultant amplicon was by comparison of 100bp DNA marker. The resultant amplicon was 627 base pair as compare to DNA marker. Out of 80 patients ureA gene has been identified in 35 individuals who are affected. The positive predictive values of UreA gene are found to be 43.7% as shown in fig 5. Primer for amplication bulging result was observed on 1.5 % agarose gel electrophoresis and the resultant amplicon was 820 base pair by comparison of 100bp DNA marker. Out of 80 patients ureC gene has been identified in 18 individuals who are affected. The positive predictive values of UreC gene were found to be 22% as shown in fig 6.

Table 2 Clinical and laboratory comparison of dyspepsia patients (n=80) with H. pylori and without H. pylori

Symptoms	No of patient (%)	*H. pylori* positive (%)	*H. pylori* negative (%)	P value
Abdominal pain	73 (91.2%)	57 (78%)	16 (21.9%)	0.31
Heart burn	66 (82.5%)	42 (63.6%)	24 (36.3%)	0.99
Bloating	57 (71.2%)	34 (59.6%)	23 (40.3%)	0.99
Belching	26 (32.5%)	8 (30.7%)	18 (69.2%)	0.001
Nausea	71 (88.7%)	57 (80.2%)	14 (19. 7%)	0.1
Vomiting	69 (86.2%)	53 (76.8 %)	16 (23.1%)	0.71
Regurgitation	34 (42.5%)	16 (47%)	18 (52.9%)	0.2
Fever	12 (15%)	4 (33.3%)	8 (66.6%)	0.22

Table 3 Incidence of H. pylori infection with respect to age

Group	Arrange group	No of patient	*H. pylori* positive (%)	*H. pylori* negative (%)	P value
I	10-20 years	9	8 (88.8%)	1 (11.1%)	
II	21-30 years	13	10 (76.9%)	3 (23%)	
III	31-40 years	29	23 (79.3%)	6 (20.6%)	
IV	41-50 years	18	14 (77.7%)	4 (22.2%)	
V	51-60 years	11	5 (45.4%)	6 (54.5%)	0.1

Table 4 Prevalence of H. pylori in different sex

Sex	No of patient %	*H. pylori* +ve	*H. pylori* –ve	P
Male	57	42 (73.6%)	15 (26.3%)	0.6
Female	23	18 (26.5%)	5 (21.7%)	

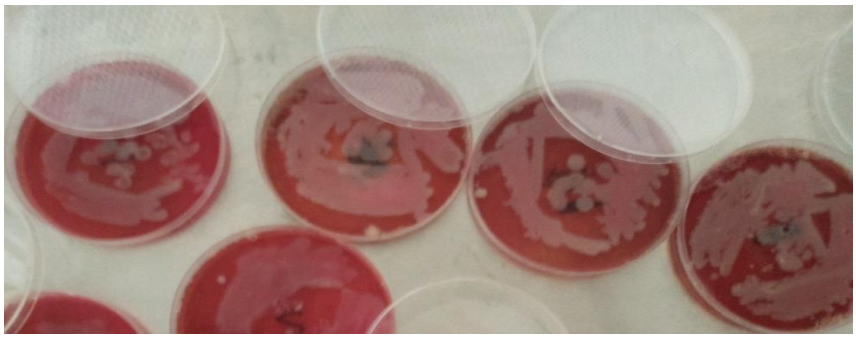

Figure 1 Colonies of anaerobic H. pylori on the blood agar media

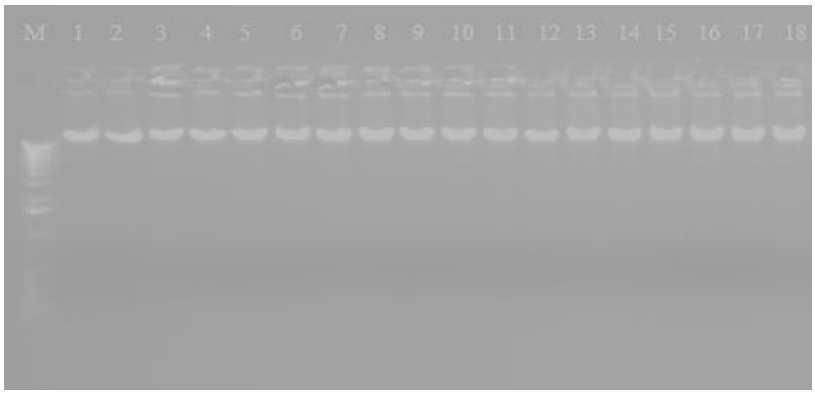

Figure 2 Gel photo indicate the Purified DNA of biopsy samples

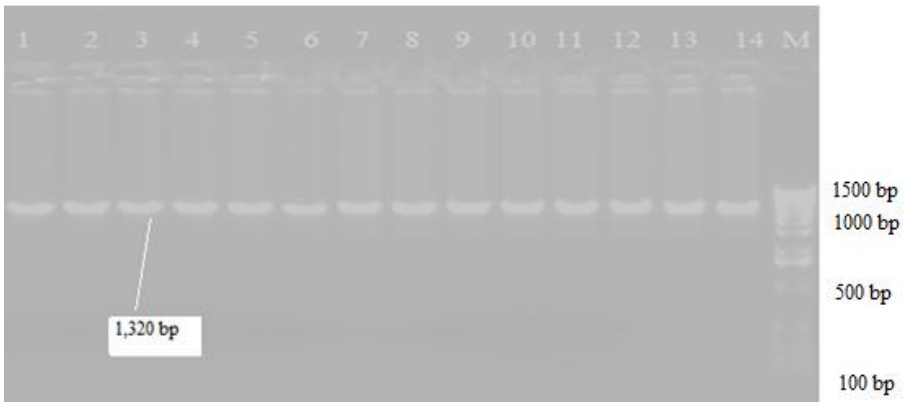

Figure 3 Gel photo showing specific detection of H. pylori CagA gene by PCR method which indicate amplify product of 1,320bp

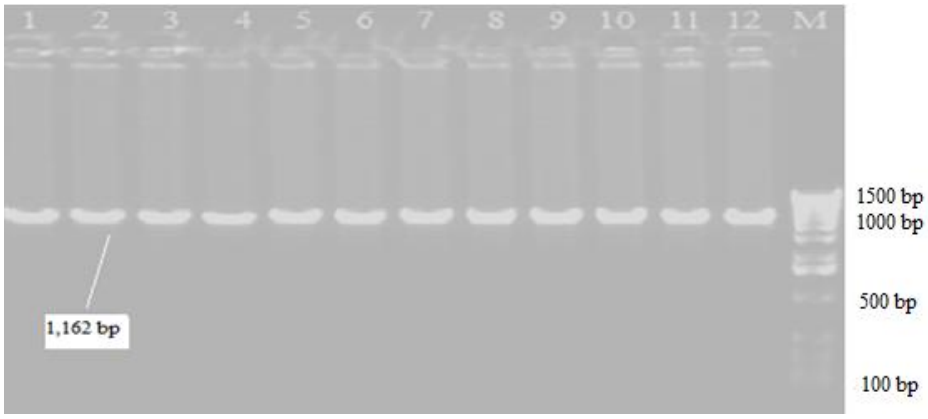

Figure 4 Gel photo showing specific detection of H. pylori VacA gene by PCR method which indicate amplify product of 1,162bp

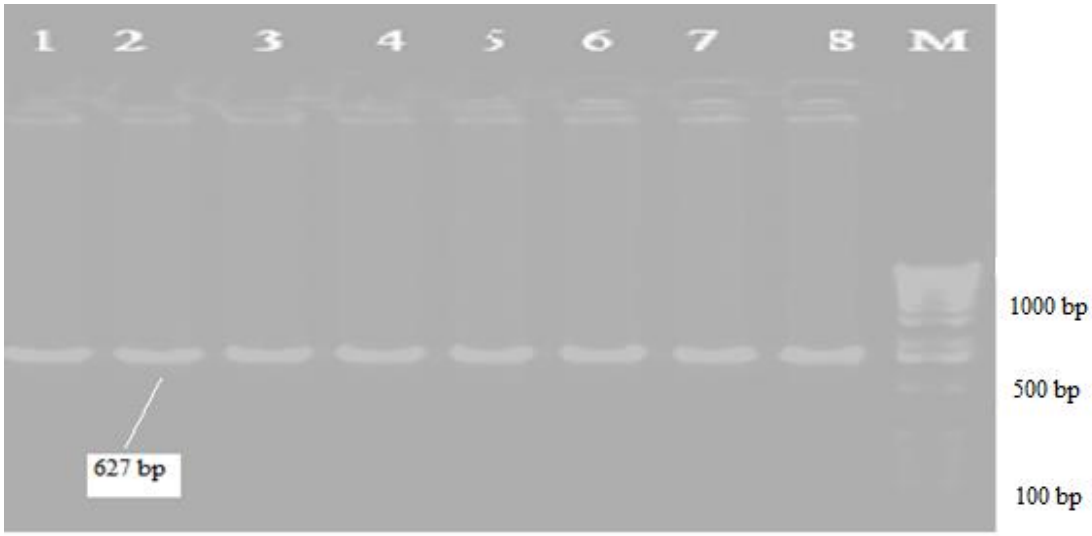

Figure 5 Gel photo showing specific detection of H. pylori UreA gene by PCR method which indicate amplify product of 627bp

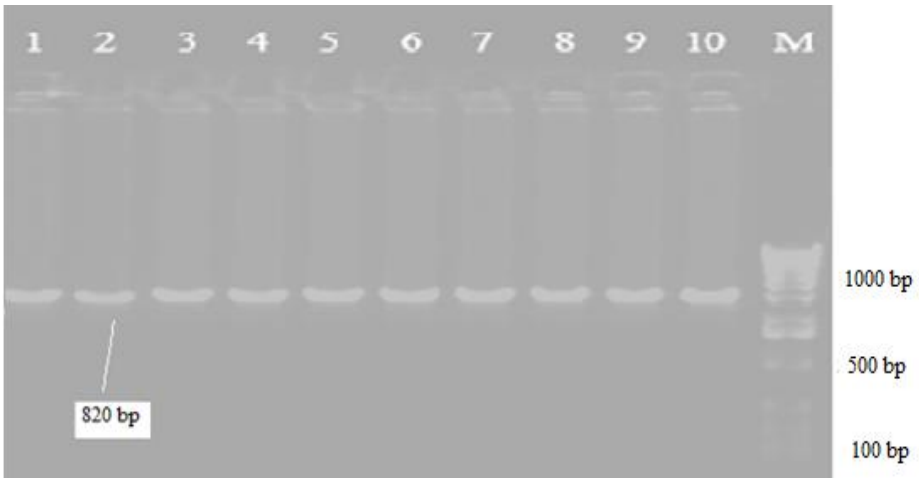

Figure 6 Gel photo showing specific detection of H. pylori UreC gene by PCR method which indicate amplify product of 820bp

Table 5 Results of PCR in terms of predicted value

Value	CagA	VacA	UreA	UreC
Positive predicted Value	65.0% (52/80)	48.7% (39/80)	43.7% (35/80)	22.5% (18/80)
Negative predicted value	35% (28/80)	51.2% (41/80)	56.2% (45/80)	77.5% (62/80)

Table 6 Endoscopic findings in relation with PCR.

Diseases	Total patients 80	PCR result		P value
		Positive (60)	Negative (20)	
Gastric ulcer	27	19 (70.3%)	8 (40%)	0.45
Duodenal ulcer	12	10 (83.3%)	2 (10%)	
Gastritis	21	14 (66.6%)	7 (35%)	

Stomach cancer	20	17 (85%)	3 (15%)	

Table 7 Detection and Distribution of cagA, vacA, ureA and ureC according to the main pathologies of the patients

Virulence genes	Total number of Virulence genes	Endoscopic findings 60 of *H. pylori* infected patient				P Value
		Gastric ulcer 19	Duodenal ulcer 10	Gastritis 14	Gastric Cancer 17	
CagA	52	14	9	12	17	0.13
VacA	39	15	5	8	11	0.39
UreA	35	11	8	10	6	0.08
UreC	18	9	2	5	2	0.1

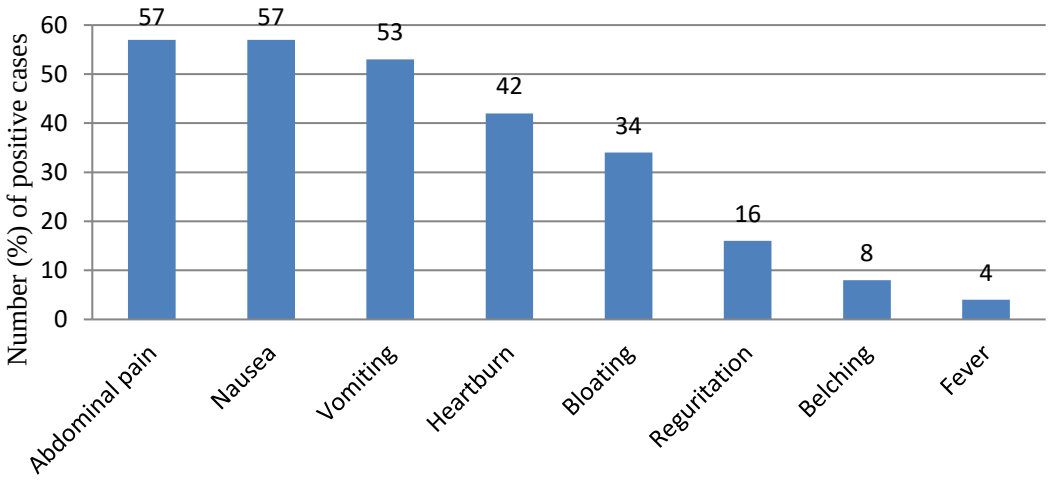

Figure 7Diagrammatic Representations of Clinical Symptoms

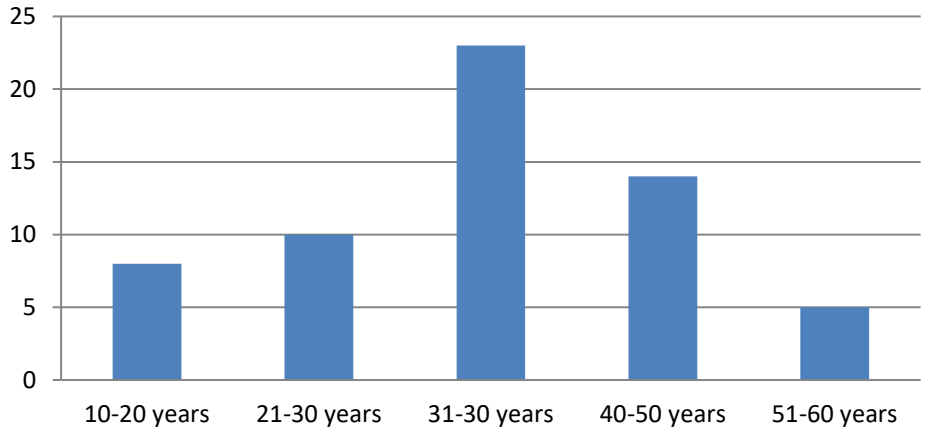

Figure 8 Diagrammatic Representations of H. pylori infection with respect to age

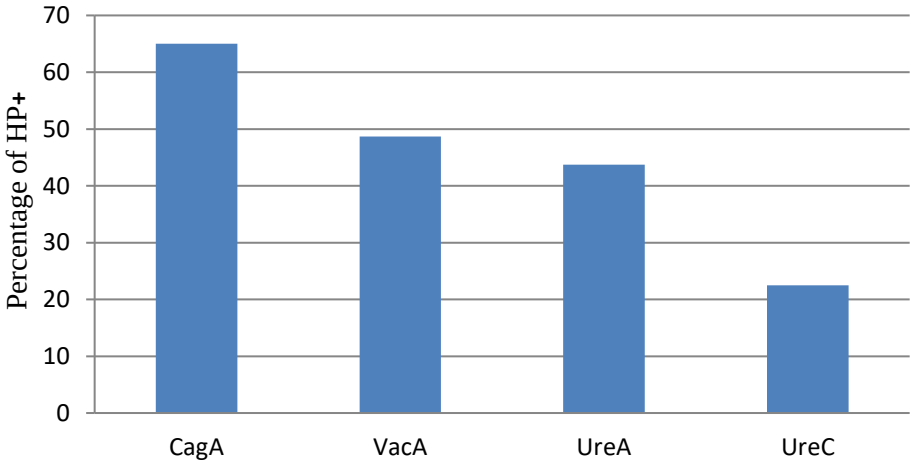

Figure 9 Diagrammatic Representations of H. pylori prevalence by PCR method

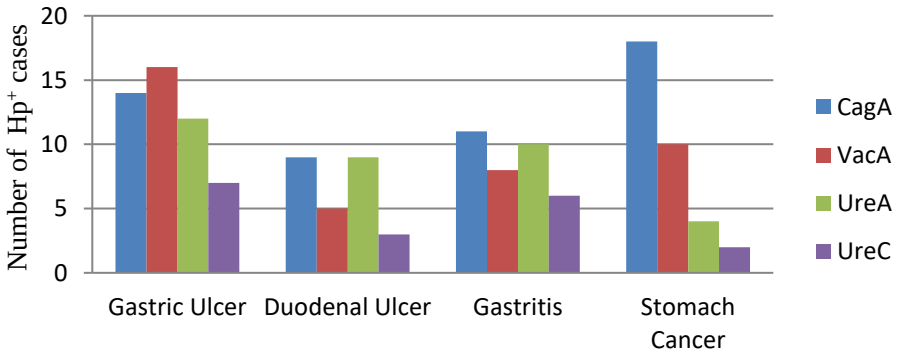

Figure 10 Diagrammatic Representation shows virulence genes positivity with gastric diseases

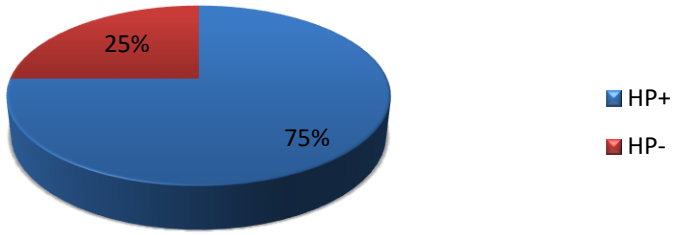

Figure 11 Diagrammatic representations show Prevalence of H. pylori in Peshawar

4. DISCUSSION

H. pylori is well-known bacteria that cause infectious disease in people. It causes and determined gastritis that is interconnected to gastric ulcer and gastric adenocarcinoma [11]. *H. pylori* colonize the gastric mucosa and produce inflammation in gastric mucosa of gastritis in both experimentally infected animals and human beings. Such circumstances can be continues for various periods in the lack of action against microorganism. Gastritis is one of the earliest recognizable modifications in a development of tissues abnormalities that can lead to stomach cancer [12, 13]. This bacterium infection is spread through family members and acquired during early middle age, in the absence of sufficient requirements and treatment affect the stomach for prolonged existence of *H. pylori* colonization [14]. There is a strong relationship between the gastric cancer and *H. pylori* that lead to its categorization as definite carcinogen type 1 by WHO [15]. In this study, we found existence of major *H. pylori* virulence genes which isolated from various disorders of gastric ulcer, gastric cancer, duodenal ulcer and gastritis among dyspepsia patient. The cagA gene was detected in 65% (52/80), vacA in 48.7% (39/80), ureA in 43.7% (35/80) and ureC in 22.5% (18/80) strains.

In the current study, *H. pylori* was isolated and detected or confirmed by using a polymerase chain reaction (PCR) assay and a culture technique, respectively [16]. The biopsy sample has been cultured and each sample show positive result for urease, catalase and oxidase test. Although culture isolation has been biological method for organism detection. Catalase and oxidase test, while the rapid urease test may not appeared to be a specific test since other pathogenic bacteria

producing urease enzyme which similar to the morphology of *H. pylori* might exist in the stomach. PCR is easy, accurate, quick, automated, and very effective for detection of *H. pylori* [17,18]. The selected specific primers were used, A proper gene selection and accurate primers design are essential for successful PCR reactions [19]. The Thermal cycler PCR was amplified *H. pylori* genome by using genes like ureA, ureC, cagA, and vacA with the specific primers [20,21]. The prevalence of cagA positive result is 65% in our study are similar to those obtain in Morocco and Tunisia where 61.2% and 61.6% cagA were reported respectively [22,23], which is associated with the frequent occurrence of stomach cancer in that country. Greater risk of gastric cancer is associated with infections of *H. pylori* strains that are cagA positive than with cagA negative strains [24]. Additionally, cagA-positive bacteria were detected in the stomach ulcer and chronic gastritis patient isolates [25]. Major virulence factors in *H. pylori* that cause gastric disease include the cagA and vacA genes. This toxin is linked to stomach cancer and gastric ulcers and can cause severe gastric mucosal inflammation [26]. Weakly cytotoxic strains were widely prevalent, which may contribute for the lower chance of stomach cancer [27]. The housekeeping genes (ureC and ureA) were found, with prevalence rates of ureA (glmM gene) of 43.7% and ureC (glmM) of 22.5%, which are comparable to those of earlier studies from Karachi, Pakistan, which showed positive results of ureA (50%) and ureC (20%) [28]. It shows that the ureA gene has greater clinical applicable sensitivity than the ureC gene. Even though without significant difference ($p=0.1$), it was noted that most of the patients in the current study were in their third and fourth decade of life. This figure is similar with the findings observed for those between the ages of 31 and 50 years [29]. A decrease in the immune response to *H. pylori* or a drop in the quantity of the microorganism in people may cause a decrease in the incidence of this bacterium in the gastric mucosa of adult people [30]. In this investigation, the prevalence of *H. pylori* infection between male and female patients was not significantly different ($p=0.6$), a finding that is consistent with those of Brown's studies [31]. If the P-value is greater than 0.05, the null hypothesis was accepted and the alternative hypothesis was rejected. These variations might be attributable to *H. pylori*-related diseases, such as infected individuals in our research having poor living circumstances (nutrition and cleanliness). Males appear to have a greater frequency of *H. pylori* infection than females [32].

5. CONCLUSION AND RECOMMENDATIONS

From the present study it was concluded that *H. pylori* infection has a significant role in the development of dyspepsia cases in district Peshawar. Thermal cycler PCR was used and find out existence of CagA, VacA, UreA and UreC genotypes in gastric biopsy samples. The prevalence rate of *H. pylori* infection among dyspeptic patients was reported 75% in shaukat khanam hospital Peshawar. The main virulence genes combinations in the dyspepsia patients were; cagA/vacA/ureC/ureA mainly in gastric cancer gastric ulcer, duodenal ulcer and gastritis. The most virulent gene cagA and less virulent gene ureC gene were found in dyspepsia patients. In this study, a statistical significant difference was not found between the investigated genes and various gastric disorders such as stomach cancer, gastric ulcer, duodenal ulcer, and gastritis ($P>0.05$) which indicates strong evidence for the null hypothesis. Molecular diagnostics provides proof and confirmation of the presence of *H. pylori* in gastric biopsy samples, as well as a clear image of the identification of virulent genes, which is linked to more serious disorders in Pakistan.

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