A DNA Sequence Analysis of Helicobacter pylori in Jeddah City, Western Saudi Arabia

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ABSTRACT
Helicobacter pylori are the type of Gram-negative bacteria which colonize the mucous lining of the human stomach. These bacteria have two major virulence factors: (vacuolating cytotoxin A gene) and (cytotoxin-associated A gene). This study aimed to provide data to determine the prevalent virulence factors (vacA and cagA genes) in Jeddah city, western Saudi Arabia, by sequence analysis. This study included 60 patients with symptoms similar to H. pylori infection. H. pylori were identified by using the 16s rRNA sequence. Then, the screening for specific genes in H. pylori (vacA and cagA) was done by using automated DNA sequencing analysis, and the DNA sequences were compared by BLAST and sequence alignment of the vacA nucleotides that are present in all H. pylori strains using those already reported in GeneBank from various studies. Results indicated that H. pylori infection was detected in 13.3%, while 86.7% were negative samples in our study patients. Interestingly, the vacA gene was found in 8.3%, while the cagA gene was not appear in patient. Also, the female prevalence rate was higher than males (11.7% female versus 1.7% males), and the highest infection was between age 40-49 by 6.7%. In conclusion, this study revealed that the vacA gene was spread in the patients infected with H. pylori in Jeddah, while the cagA gene was not appear in any isolate.

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INTRODUCTION

H. pylori were considered one of the most types of pathogenic bacteria in humans and are the major cause of most diseases of the digestive system (Hanafi and Mohamed, 2005; Zardast et al., 2016). The infection with H. pylori can cause gastrointestinal diseases like ulcer diseases, gastritis, and stomach cancer (Dunn et al., 2000; Kadi et al., 2014). There is a variation in the spread of bacterial infection among countries, races, and socioeconomic status (Khan and Ghazi, 2007). Although H. pylori is not as lethal as some bacteria, it is considered to be one of the most successful pathogens in human history because it affects 50% of the world population (Tanih et al., 2011; Bakri, 2013). This type of bacteria has been classified in humans (1994) because 1% of those infected with the bacteria develop stomach cancer (Karlik et al., 2009; Saber et al., 2015). H. pylori are Gram-negative bacteria, and the cellular morphology for these bacteria may be either curved, spiral, or fusiform. H. pylori are microaerophilic but require oxygen at a lower concentration in order to survive (Oskouei et al., 2010; Akram et al., 2011). Warren and Marshall (1983) discovered H. pylori and since that time it has been considered one of the pathogen bacteria in the world (Al-Shraim et al., 2015; Ermis and Tasci, 2015; Marie, 2012).
H. pylori possess two virulence factors: (the vacuolating cytotoxin A gene - vacA gene) and (cytotoxin-associated gene A - cagA gene) (Karlik et al., 2009; Alvi et al., 2014). The vacA gene stimulates formatting in anion channels and pores in epithelial cell membranes. Gene vacA is already found in every strain of H. pylori around the world but is expressed differently. This gene includes several polymorphisms: signal region (s), the intermediate region (i)/middle (m) region. Each of these polymorphic regions have two major types/alleles (Gusmão et al., 2000). Also, in genome of H. pylori, the cagA gene is one of several genes in cag-PAI. This gene encodes a protein that causes many cellular changes, and cagA gene is not found in all H. pylori. The risk of developing duodenal ulcers, stomach cancer, and severe inflammation of the mucosa in the stomach mayoping duodenal ulcers, stomach cancer, and severe inflammation of the mucosa in the stomach may increase with positive strains of cag-PAI (Saber et al., 2015; Cellini and Donelli, 2017). Further, the cagA gene is associated with severe clinical outcomes due to being strong connection with expression of the vacuolating cytotoxin. Moreover, most of the strains that possess cagA have a more virulent vacuolating form, vacA (Saber et al., 2015; Zhang et al., 2002). The H. pylori genotypes differ in the acuteness of disease in different geographic regions (Alvi et al., 2014). Most infected individuals present no symptoms (about 85%) at all stages of life (Gusmão et al., 2000; Akeel et al., 2018). The rapid and correct diagnosis of H. pylori helps to choose the right treatment and to prevent potential complications (Abo-Shadi et al., 2013). The most common treatment is triple therapy, which entails two antibiotics and a proton pump inhibitor (PPI) (Smith, 2015). Globally, the percentage of infection with H. pylori among developed countries was 25% and in developing countries was 80% (Jaber, 2006). Numerous studies have proven the spread of H. pylori in Saudi Arabia. Hence, the aim of this study was to estimate prevalence of H. pylori among a group of patients and determine prevalent virulence factors (vacA and cagA genes) in Jeddah city by sequence analysis.

MATERIALS AND METHODS

Sample Collection

Biopsy samples were collected through a period of four months from patients selected for endoscopy in the stomach area in King Fahad hospital in Jeddah city, Saudi Arabia. Sixty patients with symptoms similar to H. pylori infection were selected, aged between 19 and 69 years old. One sample was taken (biopsy sample) from each patient by an endoscope and then placed in transport media; then, it was cultured in the laboratory. To reduce the negative outcomes induced by using antibiotics, all individuals who took antibiotics within two weeks before to the test were excluded.

Gastric Biopsy Culture

The bacteria were cultured on H. pylori Selective Supplement (DENT) medium and then incubated (three days) under microaerophilic conditions using CampyGen CN025 (Atmosphere Generation Systems) at 37 °C[9].

A Rapid Urease Test for H. pylori

A single colony of H. pylori colonies was taken by sterile loop and then placed into rapid urease tests for H. pylori secretes a urease enzyme that hydrolyses urea to ammonia, that raises the pH, thus changing the sample color from (yellow - negative) to (pink - positive) (Uotani and Graham, 2015).

Genomic DNA Extraction

After the H. pylori culture from the biopsy samples, DNA extracted according to manufacturer’s protocol for the Gene JET Genomic DNA Purification Kit. Bacteria obtained from the Technolab solutions company. Extracted samples of DNA were stored at −20 °C until used.

Amplification of vacA and cagA Genes

DNA was used from all positive samples of H. pylori for detecting cagA and vacA genes responsible for formation of CagA and VacA proteins using a primer and product size (bp) (Table 1) (Marie, 2012; Essawi et al., 2013). The PCR tubes contain 12 µL of the master mix, 2 µL of the forward primer and reverse primer of the vacA and cagA genes, and 50 ng/µL of the DNA sample (9 µL).

The standard PCR protocol of vacA and cagA genes included an initial denaturation step at 94 °C for 5 min and then 35 cycles followed by 1 min at 94 °C, 1 min / 58 °C, and at 72 °C /1 min. Last step done for 10 min / 72 °C. A PCR reaction without a DNA template was used as a negative control during each run. The samples were placed in a thermal cycler and were run for 2 h. The resulting products were separated on an agarose gel (Figure 1).

![Figure 1: PCR amplification of the patient’s samples of vacA gene. Lanes 1–5 represent the specific DNA fragments of the vacA gene at approximately 678 bp.](image-url)
Table 1: PCR primers for cagA and vacA sequences

| Gene | Primer | Primer Sequence (5’→3’) | PCR Product Size (bp) |
|------|--------|--------------------------|-----------------------|
| CagA | CagA-F | F: 5’-GATAACAGGCAAGCTTTTGAGG-3’ | 349 |
|      | CagA-R | R: 5’-CTGCAAAGATTGTTTGGCAGA-3’ |          |
| VacA | VacA-F | F: 5’-GCCGATATGCAAATGAGCCGC-3’ | 678 |
|      | VacA-R | R: 5’-CAATCGTGTTGGGTTCTGGAG-3’ |          |

Table 2: H. pylori infection rate.

| Characteristics | H. pylori Infection |
|-----------------|---------------------|
|                 | Positive | Negative |
| Age groups      |          |          |
| 19–29 Years     | 3 (5.0%) | 14 (23.3%) |
| 30–37 Years     | 0 (0.0%) | 12 (20.0%) |
| 40–49 Years     | 4 (6.7%) | 9 (15.0%)  |
| 50–59 Years     | 1 (1.7%) | 12 (20.0%) |
| 60–69 Years     | 0 (0.0%) | 5 (8.3%)   |
| Total           | 8 (13.3%) | 52 (86.7%) |
| Nationality     |          |          |
| Saudi           | 7 (11.7%) | 45 (75.0%) |
| Non-Saudi       | 1 (1.7%)  | 7 (11.7%)  |
| Total           | 8 (13.3%) | 52 (86.7%) |
| Gender          |          |          |
| Male            | 1 (1.7%)  | 18 (30.0%) |
| Female          | 7 (11.7%) | 34 (56.7%) |
| Total           | 8 (13.3%) | 52 (86.7%) |

Table 3: Phylogenetic identification of vacA gene of H. pylori.

| No. | Samples | Closest Matches Identification         | Sequence Identity |
|-----|---------|----------------------------------------|-------------------|
| 1   | VacA-22 gene | VacA gene-isolate: BH70 | 96.8%               |
| 2   | VacA-27 gene | VacA gene-isolate: M18660 | 93.6%               |
| 3   | VacA-29 gene | VacA gene-isolate: BH111 | 96%                 |
| 4   | VacA-35 gene | VacA gene-isolate: AFN4124 | 97.7%               |
| 5   | VacA-47 gene | VacA gene-isolate: MV040A | 96.9%               |

DNA Sequencing Protocol

The sequencing analysis involved two main steps,

1) purification of the PCR products and cycle sequencing
2) purification of cycle sequencing products and drying and denaturing.

The purified sequencing samples were then loaded into a 96-Well microtiter sequencer plate and run on a genetic analyzer for data interpretation (3500). The nucleotide sequence data were examined for comparison with a reference sequence retrieved from the National Center for Biotechnology Information and the Basic Local Alignment Search Tool database.

Statistical Analysis

By using SPSS version, 24.0, data were entered and analyzed. Also, a bivariate analysis performed for statistically differences among (H. pylori-positive or negative) and (vacA/cagA positive or negative genes) with individuals in relation to the specific variables.

RESULTS

Prevalence of cagA and vacA Genes

H. pylori infection was diagnosed in 13.3%. Further, the female spread rate was higher than that of males, and the highest infection rate was between ages 40 and 49 (6.7%). Those aged between 19 and 29 had a
5.0% prevalence rate and those between 50 and 59 had a rate of 1.7%, as shown in Table 2. The vacA gene was obtained in 8.3%, and 91.7% were negative samples, as shown in Figure 2, while cagA gene was not detected in any isolate.

**DNA Sequencing and Phylogenetic Analysis**

DNA sequencing for all positive samples of vacA gene done and compared to the DNA sequences
through BLAST and the alignments of the vacA nucleotides that were present in the H. pylori strain already present in GenBank. The percentage of sequence identity of the vacA was also determined (Table 3). The phylogenetic relationships of the experimental isolates (vacA gene) and closely related species were analyzed by using MEGA7, and results were presented in phylogenetic trees, as shown in (Figures 3, 4, 5, 6 and 7).

**DISCUSSION**

H. pylori infection occurs worldwide, but its spread among countries is very different and also differs among ethnic groups in the same country and age groups (Hanafi and Mohamed, 2005). Infection with H. pylori leads to ulcers and develop to stomach cancer, which contributes to a large number of cancer related deaths worldwide. In the late 1980s, Saudi Arabia began to study gastritis after the discovery of this pathogen (H. pylori), and several studies have been carried out to determine the effectiveness of different methods of detecting and isolating H. pylori. Further, several studies since 1980 have aimed to understand the cause of this pathogen. Moreover, Saudi Arabia has taken the lead among the countries of the Middle East in the study of H. pylori's pathogenesis, the sequence of the whole genome, and molecular characterization, in order to understand more about its methods of transmission, determine the different strains of H. pylori bacteria in Middle East, and elucidate interaction among the host and pathogen (Somily and Morshed, 2015).

H. pylori infection is widespread in Saudi Arabia among men and women, as well as children. Hence, in our study, to estimate the spread of H. pylori among a group of patients and to determine its prevalent virulence factor (vacA and cagA genes) in Jeddah city by sequence analysis we used 60 patients with similar symptoms of H. pylori infection. One sample was taken (biopsy sample) from each patient by an endoscope, and age (19: 69) years old.

H. pylori were identified by using a 16s rRNA sequence, and H. pylori infection found in 13.3%, while 86.7% were negative isolates. This result is similar to that of another study conducted by Jaber in 2006, where the spread of H. pylori in patients was about 23.6%. However, the number of samples in this study was bigger than that in our study (Jaber, 2006). Furthermore, the incidence rate with H. pylori among Saudi patients was higher than that among non-Saudi patients, and the highest infection of H. pylori was between the ages of 40 and 49 years. According to our study, it seems that gender does not affect the acquisition of H. pylori. Moreover, the infection rate in females was higher than that among males. However, Abo-Shadi et al. found, a higher risk of infection among males than among females (Abo-Shadi et al., 2013).

The study of H. pylori genome focused on trying to understand disease and its ability to cause illness (Karlik et al., 2009). Growing evidence to genetic variation of H. pylori could be clinical significance (Marie, 2012). Also, the clinical results of infection with the bacteria are linked with the virulence factors of H. pylori (Saber et al., 2015). Virulence factors of H. pylori are responsible for many illnesses of the stomach. The prevalence of genes (cagA and vacA) are significantly different between countries (Essawi et al., 2013). There is usually variation in the DNA sequence between the isolates, even in essential genes, by a rate of 3% to 5%. This diversity may reflect a set of factors, such as recombination between different strains, mutation, and transport between members of the family and people in close contact with them. There is a difference in certain traits from person to person, like the strength of the immune system, specificity, which leads to variations between H. pylori strains (Tanih et al., 2011). Interestingly, the vacA gene was present in 8.3%, while the cagA gene was not detected in any isolate.

DNA sequencing for all our positive samples for the vacA gene was done and compared to the published vacA gene DNA sequences from H. pylori through BLAST. The percentage of sequence identity of vacA gene was 96.8% for vacA-22, 93.6% for vacA-27, 96% for vacA-29, 97.7% for vacA-35, and 96.9% for vacA-47, with the closest matches strains. The geographical distribution of the distinct genotypes of H. pylori and the extent of spread of virulent genotypes is still unknown in several regions of Saudi Arabia (Zhang et al., 2002). This study suggests the need for a larger and more comprehensive study of adults and children to detect the virulence factors in patients infected with H. pylori in the Saudi population in Jeddah city.

**CONCLUSIONS**

This study detects that the vacA gene was distributed in patients hadH. Pylori (Jeddah/ Saudi Arabia), while the cagA gene was not detected in any isolate A large national epidemiological study is needed to determine prevalence of bacteria. This will open many new avenues for many studies about the virulence factors of H. pylori (vacA and cagA) genes. According to our results, there is a need for a larger and more comprehensive study of adults and
children to detect the virulence factors in patients infected with *H. pylori* in Jeddah city.

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**Conflicts of Interest**

The authors declare no conflict of interest.

**Ethical approval**

This study was approved by Directorate of Health Affairs-Jeddah (protocol number: A00548).

**Author Contributions**

A.A.A.; sample collection, methodology, analysis, writing, and F.A.A.; review and editing. All authors have read and agreed to publish version of manuscript.

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