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Research Article

Epidemiology of *Helicobacter pylori*: Using whole genome sequencing for molecular characterization of two pulsotypes of *cagA* -positive genotype isolated from Suez Canal region

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ABSTRACT

Helicobacter pylori, a bacterium known for its association with stomach ulcers, gastritis, and many gastric malignancies. Cytotoxin-associated gene A (cagA) is a polymorphic strain-specific gene that has a role in the pathogenicity of Helicobacter pylori, strong relation with the most severe clinical symptoms, and represents a fulcrum for defining Helicobacter pylori evolution. In this study, we are presenting a molecular characterization of two pulsotypes of cagA-positive Helicobacter pylori samples isolated from gastric biopsies of Egyptian patients living in the Suez Canal region (Ismailia). We investigated the relation between the presence of cagA gene and the prevalence of other virulence genes like vacuolating cytotoxic gene A (vacA), outer inflammatory protein adhesin gene (oipA), and induced by contact with epithelium gene (iceA). Then, we used Pulsed-field gel electrophoresis (PFGE) using XbaI as a restriction enzyme to select the most dominant pulsotypes for performing a whole-genome sequencing (WGS) analysis. We defined the phylogenic relation of our strain with other strains from different geographical locations. Moreover, detailed virulome and resistome information was obtained by using different bioinformatics pipelines. This data represents an additional brick for full characterization of the evolution of Helicobacter pylori which in return will help in improving diagnosis and treatment strategies.

1. *Introduction*

Helicobacter pylori (*H. pylori*) is a well-known causative agent of many gastric disorders. The severity of the infection ranges from acute infection that triggers gastritis to chronic infection that leads to gastric ulcers, duodenal ulcers and gastric cancer [1, 2]. Therefore, *H. pylori* is considered to be the main risk factor for gastric cancer due to the prolonged chronic atrophic gastritis [3] and many studies showed its relation with other gastric malignancies like mucosa-associated lymphoid tissue carcinoma (MALT) [4].

The pathogenesis of *H. pylori* is a stepwise process. It starts by bacterial adhesins interaction with receptors on host epithelium that leads to persistent infection. Then, toxins causing damage to host tissue are released [5]. In our study, we are concerned with the genetic determinants for these adhesins and toxins which are defined as virulence factors.

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H. pylori is a highly heterogeneous and diverse bacterium that there are different *H. pylori* populations within the same individual host [6]. This heterogenicity differences sound reasonable after investigating the genetic map for different strains isolated from patients who live in different geographical regions throughout the world which proved that there are also host-related factors like host genetics that controls the inflammatory response of the host to the infection, duration of infection, age of the patient, and other environmental factors [5]. Moreover, previous studies have stated that symptoms may vary geographically. For example, the percentage of gastric cancer incidence increases in East Asia, conversely it decreases in Africa and South Asia [7]. In Egypt, the rate of *H. pylori* infection reaches 82.9% in gastritis patients and 71.7% in dyspeptic patients. These facts indicate a strong association between *H. pylori* infection and gastritis in Egypt [8]. Egypt is one of the countries that has a high prevalence of H. *pylori* infection that a previous study on Egyptian patients stated that the frequency of *H. pylori* has reached 72.38% among children and 53.1% among adults. Key risk factors identified included attending schools in socially deprived areas and residing in overcrowded homes. Additionally, infected children exhibited significantly lower body weight and height compared to their non-infected peers, indicating an adverse effect on growth [9]. Similar results were reported in 2005 on dyspeptic patients [10]. Further research highlights that *H. pylori* transmission in Egypt is associated with environmental risk factors such as limited access to clean water and inadequate sanitation practices. These conditions facilitate the spread of the bacterium, especially in densely populated regions [11]. Moreover, studies have documented a high prevalence of *H. pylori* infection among Egyptian children, with rates ranging from 64.6% to 73%. Factors contributing to this high prevalence include residence in Upper Egypt and low socioeconomic status [12]. In a latest review published in April 2020 by Alsulaimany et al [13], it stated that there isn't enough data neither on the epidemiology of the infection in Egyptian patients nor its evolutionary relationship with other strains all over the world. These findings underscore the need for public health interventions in Egypt, focusing on improving living conditions, enhancing sanitation infrastructure, and increasing awareness about *H. pylori* transmission and prevention. Studying *Helicobacter pylori* (*H. pylori*) in the Suez Canal region of Egypt presents unique challenges due to environmental, socioeconomic, and healthcare factors. The high prevalence of *H. pylori* infections in Egypt, coupled with limitations in existing diagnostic approaches, complicates effective management and control of the infection. studies have detected *H. pylori* DNA in Egyptian water systems, suggesting that contaminated drinking water may serve as a transmission route, particularly in densely populated areas [14]. While histological examination via endoscopy is a common diagnostic method, it is invasive and may not be readily available in all healthcare settings [15]. In addition, There is a growing concern about antibiotic resistance in *H. pylori* strains in Egypt, which complicates treatment strategies [16]. Multidrug regimens have been approved as the standard treatment for *H. pylori* infection. In Egypt, many physicians are using quadrable therapy for treating *H. pylori* which includes PPI + bismuth + TWO antibiotics amoxicillin & clarithromycin or Metronidazole & Tetracycline or Amoxicillin & Levofloxacin. They may use three antibiotics in case of unavailability of bismuth. However, resistance against first-line antibiotics, especially clarithromycin, tetracycline, and metronidazole is starting to emerge [17]. Because of the previous mentioned fact that *H. pylori* is an heterogenous bacterium [6] [5], the investigation of virulence genes alone away from the genetic fingerprint won't be representative for the epidemiology and the genetic evolution of the infection. For that reason, we decided in a previous review [18] using advanced techniques like next generation sequencing (NGS) and don't just depend on traditional PCR techniques for the detection of *H. pylori* genomic virulence factors, resistance determinants, or epidemiological markers. We concentrated our study on cytotoxin-associated gene A (*cagA*) gene which is an essential virulence factor mostly reported to be responsible for disease development in cases of mucosal inflammation and gastric cancer due to its role in increased IL8 production and nuclear factor-kB activation [19]. Other genetic factors correlated with *H. pylori*-associated diseases are vacuolating cytotoxic gene A (*vacA*), outer inflammatory protein adhesin (*oipA*), and induced by contact with epithelium gene (*iceA*). These factors with *cagA* gene are closely related and not independent [20]. We gave most of our attention to *H. pylori* samples isolated from Suez Canal region as it is an important privileged geographical area that affected trade, culture, and human evolution all through the last two centuries and we need to compare the genomic fingerprint of *H. pylori* isolated from this region with other international fingerprinting database. Through whole genome sequencing , we can acquire a complete assembled genome, annotate all the genomic factors in a rapid and cost-effective process and state the phylogenic relation of our isolated strain with other strains in the geographical genetic map of *H. pylori* which in return help in the understanding and the explanation of clinical manifestation and will help in the design of the most effective treatment regimen [21].

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2-Materials and methods:

2.1. Patients' selection:

Patients who attended the endoscopy unit of Suez Canal University educational hospital, Ismailia, Egypt in the period from December 2016 to April 2019 for gastrointestinal endoscopy, were recruited for this study. Samples were taken only after written acceptance consents were approved from patients, and this research was approved by the research ethics committee of faculty of pharmacy Suez Canal university under registration No.PM-HR1-MD-17. The patients' confidentiality was maintained along all steps of the research.

The study includes 284 patients (175 males and 109 females) with the age of (40±15) years old who are attending to the endoscopy unit suffering from different gastroduodenal disorders such as peptic ulcer, duodenal ulcer, gastritis, gastro-duodenitis, and gastro-esophageal reflux disease (GERD) and who were suspected that *H. Pylori* was the cause of their clinical symptoms. The diagnosis was done by specialized staff members of the endoscopy unit. Patients who have been on antibiotics or *H. pylori* eradication therapy for the last 15 days before sample collection were excluded because the over consumption of antibiotics reduce the viability of *H. pylori* for the culture.

2.2. Sample collection:

Two specimens were taken from each patient suspected to be infected with *H. pylori*: one for rapid urease test and one for culture. Gastric biopsies were taken from special spots next to the visualized trauma (inflammation or ulcers). Positive samples for rapid urease test were immediately placed in 1.5 ml brain heart infusion broth (BHI broth, Oxoid™, United Kingdom) supplemented with Oxoid *Helicobacter Pylori* Selective Supplement (Dent) supplement for *H. pylori* (SR0147E Oxoid™, United Kingdom) then transported to bacteriology lab within 4 hours in 4°C adjusted icebox.

2.3. Bacterial isolation and growth conditions:

Urease positive samples were cultured at 37°C on 5% blood agar plates supplemented by DENT *H. pylori* selective supplement (SR0147E Oxoid™, United Kingdom) in microaerophilic conditions (5%CO2, 5%H2, 85%N2 and 95% humidity) using Campygen gas kit (CN0035A Oxoid™, United Kingdom).

Confirmed *H. pylori* colonies were identified as translucent, small, round, Gram-negative, and positive for catalase, oxidase, and urease tests. Then they were frozen in brain heart infusion (BHI) broth (CM1135 Oxoid™, United Kingdom) containing 20% glycerol and stored at − 80 °C for future use.

2.4. Chromosomal DNA extraction:

DNA extraction was done using Genetix minikit nucleopore (61307, Genetix biotech™) according to the manufacturer's instruction. Five µl aliquot from each sample was analyzed by Nanodrop then another 5µl was used for PCR. The residual DNA samples were inoculated in a DNA stable tube kit for room temperature preservation of DNA (93021-001-1Kt, Sigma-Aldrich).

2.5. PCR primer design and virulence genes amplification:

Primers for *ureA, cagA, vacA, oipA,* and *iceA* were designed using primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) using the available published data on NCBI for different *H. pylori* strains from different geographical regions (25 different strains at least). All 25µl PCR reactions contained 12.5µl Master mix (MyTaq® Red Mix, Bioline™), 9.5µl purified molecular grade water, 1µl of each primer (Sigma Scientific Services Co.) and 1µl of genomic DNA. The amplification reaction was performed by T100 Thermal cycler (Bio-Rad™, Singapore). The amplified PCR products were purified and visualized using 1.5% agarose gel electrophoresis (Merck, SA) referring to the basic protocol mentioned earlier.[22] Primers and the conditions used for the amplification are shown in **Table 1**.

2.6. DNA preparation and restriction endonuclease digestion:

PFGE analysis was performed in total of 22 samples. Twenty samples that were positive for *cagA* gene and additional two samples that were negative for all genes. DNA preparation was done according to the previously described procedures with some modifications [23-25]. The concentration of pulsed field high quality agarose gel (Bio-Rad ™) was 1.5% and lysis buffer incubation period was extended to 4 days. The *XbaI* restriction enzyme (Promega™) was used for restriction endonuclease digestion and restriction incubation period was 24hrs at 37°C for of each of the tested genotypes.

2.7. PFGE:

Genomic DNA was separated by PFGE using (CHEF DR III contour clamped homogenous electric field, Bio-Rad) in 1.5% LMP agarose gel at 14°C under the following conditions: initial switch time= 4s, final switch time= 50s, Voltage= 6V, Angel= 120 degree, and run time at 22 hrs. Lambda phage ladder (NBE ™) was run alongside as a genetic marker.

2.8. PFGE fingerprint analysis:

After electrophoresis, gels were stained with ethidium bromide and the fragments were imaged by a gel documentation system (G: BOX, Ireland). Bionumeric® software, version 8.0 (Applied Maths, Belgium) was used for the analysis of PFGE Fingerprint banding patterns. The

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dendrogram was processed using the band-based similarity coefficient (number of different bands) with 1% band optimization and 1% band tolerance. Error flags were calculated, and branch quality was determined by cophenetic correlation.

Table 1 Designed primers and amplification protocols for ureA, cagA, vacA, oipA, and iceA.

Gene	Primer	Amplification protocol	Predicted Product size	Reference for Amplification protocol
ureA	5′-CGTTGTCTGCTTGCCTATCA-3′ 5′-CGGCTCACACTTCCATTTCT-3′	30 sec denaturation at 94°C, 30 sec annealing at 55°C, 1 min elongation at 72°C & All steps repeated for 35 cycles	197 bp	[60]
cagA	5′-AAT ACA CCA ACG CCT CCA-3′ 5′- ACAATCCTTTAGCCCACA-3′	45 sec denaturation at 94 °C, 30 sec annealing at 55 °C, 1 min elongation at 72 °C & All steps repeated for 40 cycles	550 bp	[13]
vacA	5′- ACACACCGCAAAATCAAT -3′ 5′-AGCCCAAATTCAAAAACACT-3′	30 sec denaturation at 94°C, 30 sec annealing at 52°C, 1 min elongation at 72°C & All steps repeated for 35 cycles	260-285 bp	[61]
oipA	5′-CACGCTGAAAGGAATGGA-3′ 5′-TTTATACCCAAAGCTGAGTTC-3′	30 sec denaturation at 94°C, 30 sec annealing at 52°C, 1 min elongation at 72°C & All steps repeated for 35 cycles	240-250 bp	[60]
IceA	5′-GTTGGGTAAGCGTTACAGAATTT-3′ 5′-TCATTGTATATCCTATCATTACAAG-3′	1 min Denaturation at 95°C, 1 min annealing at 57°C, 1 min elongation at72°C & All steps repeated for 35 cycles	560 bp	[13]

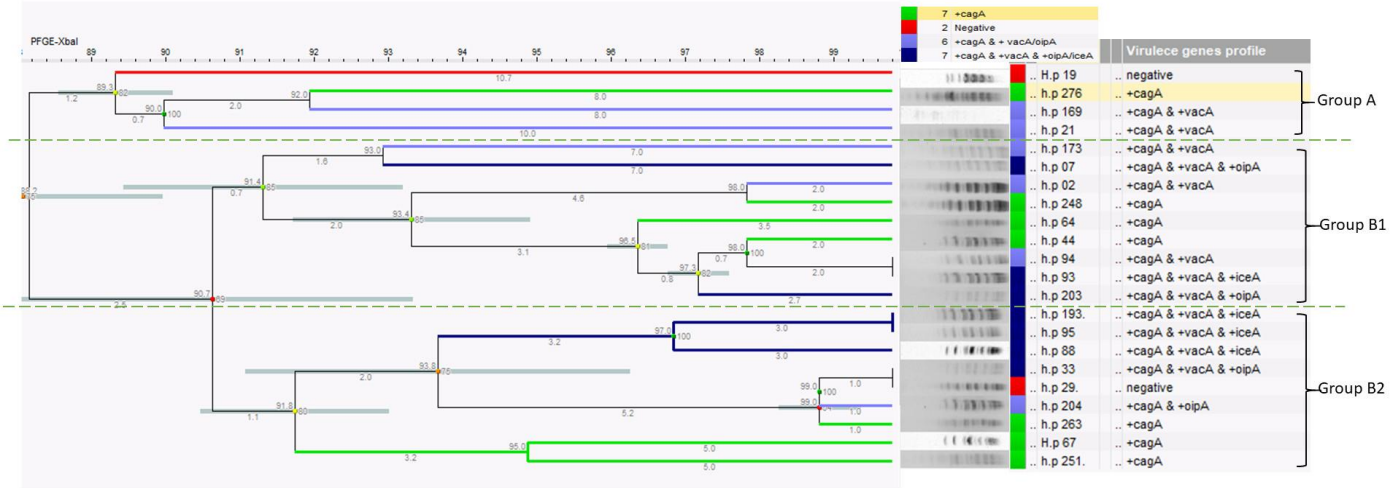

Figure 1 Dendrogram for 22 H. pylori samples based on PFGE fingerprinting in 20 cagA positive samples and 2 -negative for all genes- samples. Each isolate is related to its fingerprint and its virulence gene profile. Samples were classified according to virulence gene profile into four different groups represented with different colors on the dendrogram: positive for cagA gene alone, positive for cagA and other virulence gene vacA/oipA, positive for both cagA, vacA and iceA/oipA, and negative for all genes. They were given the colors green, light blue, dark blue, and red, respectively.

Table 2 A comparison between the resultant contigs from PATRIC, Velvet, and SPAdes assembly algorithms

	PATRIC		Velvet		SPAdes	
Sample No	M1	M2	M1	M2	M1	M2
Contigs	213	95	61	43	1112	67
GC Content %	45.05	57.65	43.03	57.65	49.76	57.64
Contig L50	3	5	2	5	7	5
Genome Length	2,292,779 bp	2,283,947 bp	1,998,096 bp	2,277,070 bp	3,401,732 bp	2,293,823 bp
Contig N50	313,559	209,115	566,293	283,761	68,483	268,873
Plasmids	0	0	0	0	0	0

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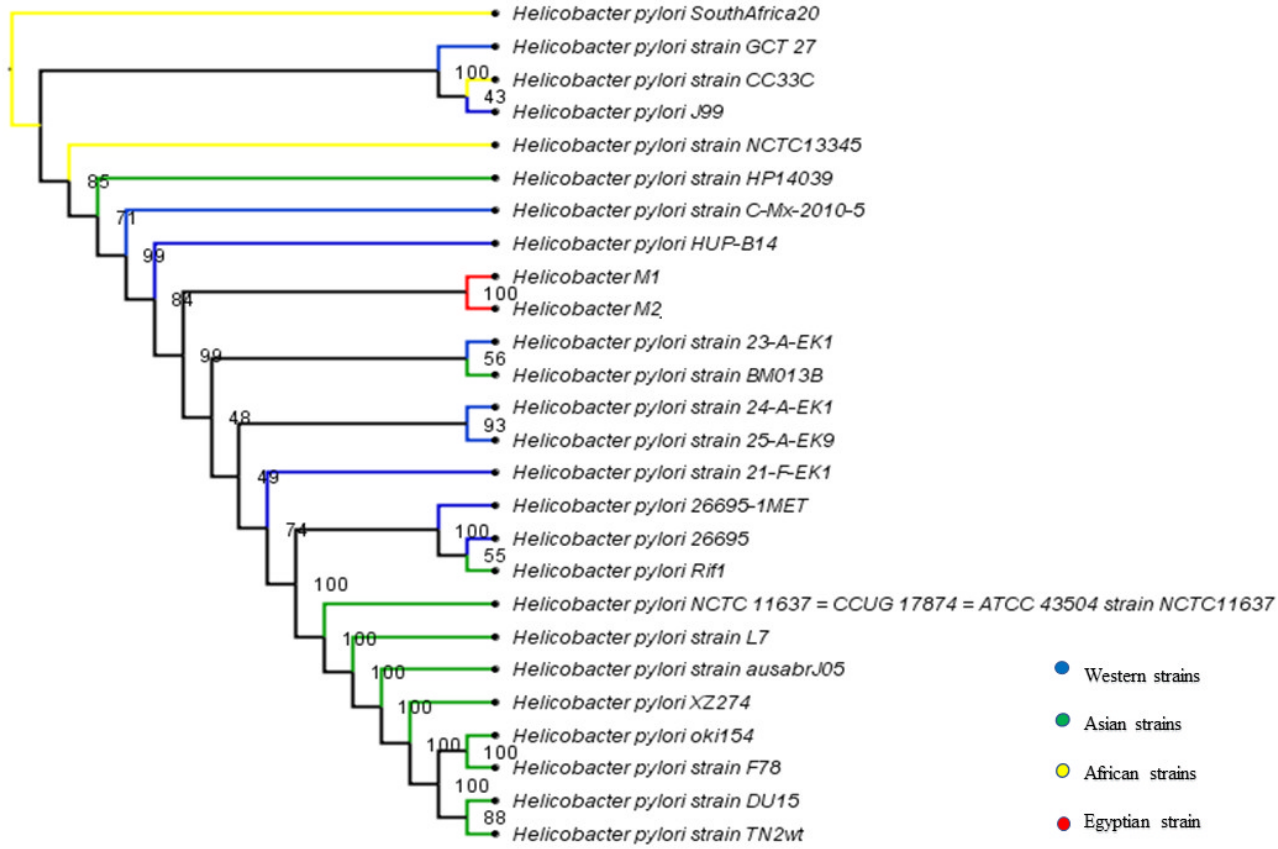

Figure 2. Genomic phylogenic tree built from samples M1 and M2 with 24 other H. pylori strains isolated from different geographic locations around the world. It showed that our samples represented with red branches, have a closer relation to western strains, represented with blue branches, than Asian strains, represented by green branches and African strains represented in yellow branches.

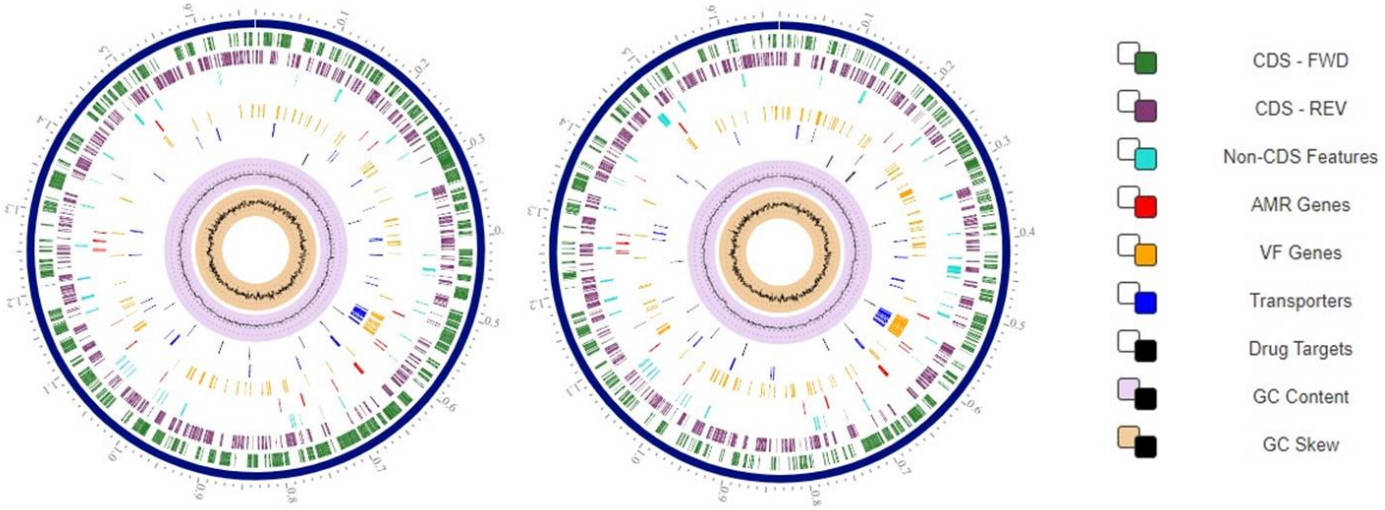

Figure 3. Circo plot shows H. pylori complete genome annotation for samples M1 (left) and M2 (right). The figure shows the annotation for CDS genes on forward strand (CDS-FWD), CDS genes on reverse strand (CDS-REV), non-CDS features, antimicrobial resistance genes (AMR Genes), Virulence genes (VF Genes), Transporters genes, Drug targets, GC content, and GC skew.

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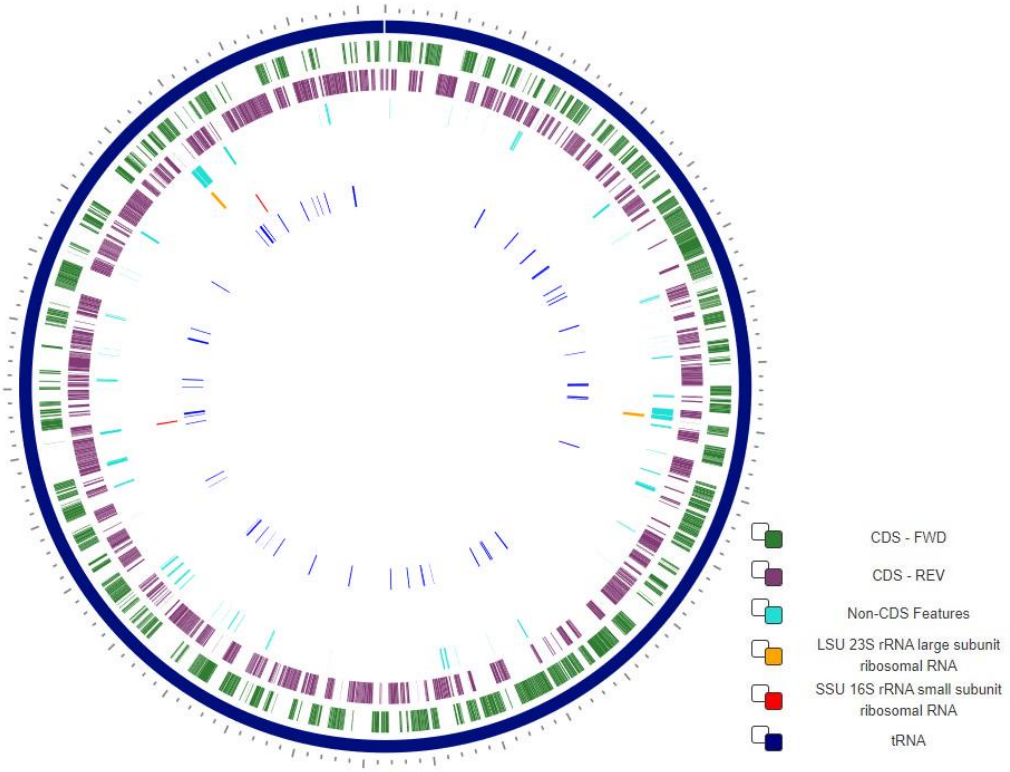

Figure 4. Circo plot representing the position of tRNA, 16S rRNA, 23S rRNA, CDS and non-CDS features.

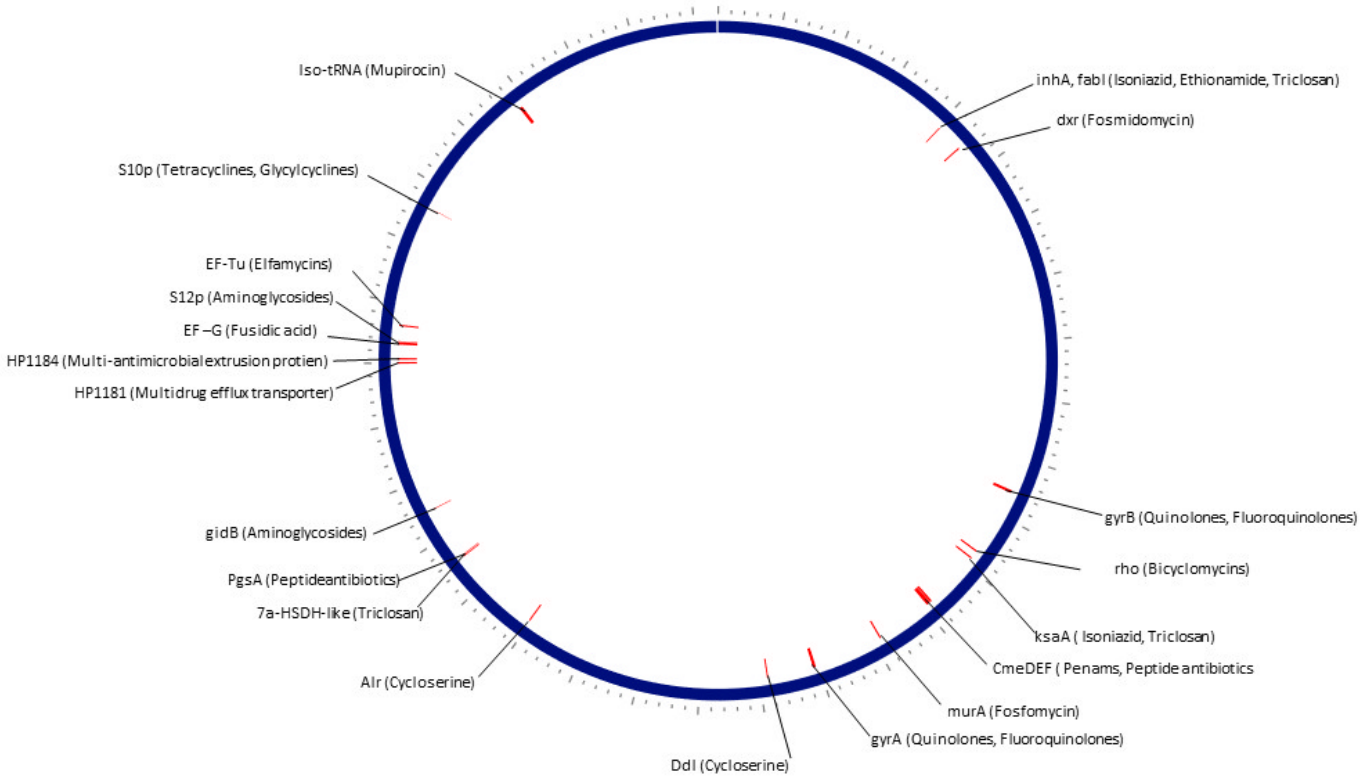

Figure 5. Circo plot shows an overview of H. pylori resistome. Genome annotation for resistance genes of samples M1 and M2 provided from PATRIC annotation service.

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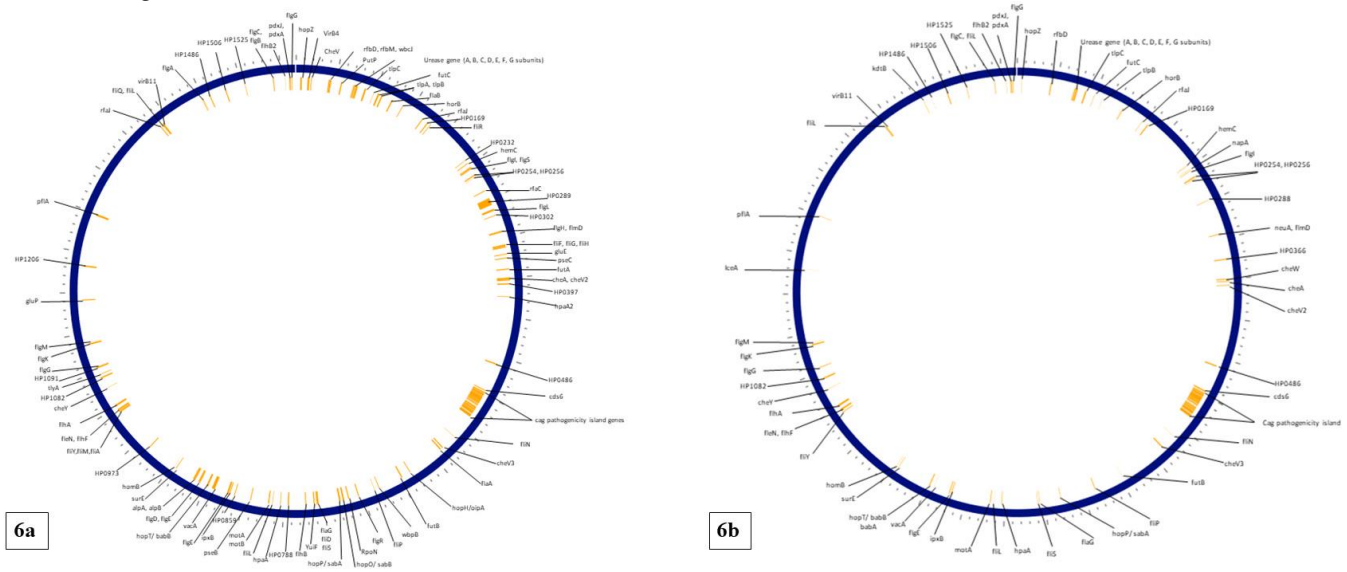

Figure 6. Circo plot shows H. pylori virulome in samples M1 (6a) and M2 (6b). Genome annotation for virulence genes in sample M1 on the left (6a) and sample M2 on the right (6b) provided from PATRIC annotation service.

2.9. Genome sequencing and assembly:

Two samples, 7 and 193, which are *cagA*- positive, showed two different PFGE fingerprinting patterns (two different pulsotypes groups) and had the most severe clinical manifestations; erosive gastritis with melena and gastric ulcer with hyperemia, respectively, were sent in DNAstable tubes for Illumina sequencing and given the codes M1 and M2, respectively. For library preparation, (Ovation® Ultralow V2 DNA-Seq Library Preparation) kit (Tecan, Männedorf, Switzerland) has been used following the manufacturer's instructions. Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and quality tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent technologies, Santa Clara, CA, USA).

Libraries were then prepared for sequencing and sequenced on Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) in paired-end 150 mode. Then, the primary bioinformatics analysis was conducted which includes base calling and demultiplexing. Processing raw data for both format conversion and de-multiplexing by Bcl2Fastq 2.20 version of the Illumina pipeline were done.[26] Adapter sequences are masked with Cutadapt v1.11 from raw fastq data using the following parameters: --anywhere --overlap 5 --times 2 --minimum-length 35 --mask-adapter.

All sequences were checked for quality by using FastQC[27] and raw data were submitted to GeneBank databases under BioProject accession number PRJNA689250, BioSample accession number SAMN17204307 and SAMN17204307, and SRA accession number SRR13343600 and SRR1334360. Addresses as follows:

https://www.ncbi.nlm.nih.gov/bioproject/PRJNA689250

Sequences of low quality were trimmed using Geneious Prime 2021.0.3-BBDuk plugin with an error probability limit of 0.05 for both 5′ and 3′ ends. Then, sequences were error corrected and normalized using Geneious Prime 2021.0.3-BBNorm plugin with 50x as minimal depth and target coverage level of 80%.[28] We used three different tools for the De-Novo-assembly of the provided fastq data of samples M1 and M2: the web-based bacterial bioinformatics resource provided by the Patho-systems Resource Integration Center (PATRIC)[29], Velvet assembler[30], and SPAdes assembler [31]. Parallel to the De-Novo-assembly process fastq data was mapped to reference genome *H. pylori* 26695 using Bowtie2 alignment algorithm [32].

2.10. Phylogenic relation determination and geographical revolution study:

The resultant contigs from bowtie2 alignment were tested against KmerFinder [33-35] and Multi Locus Sequence Typing (MLST) [36]. These tools are provided by the Center for Genomic Epidemiology (CGE) for strain identification. Moreover, PATRIC phylogenic codon tree plotter was used for geographical revolution study and phylogenic relation comparison between Egyptian strain and other 24 strains isolated from different geographical locations from around the world with a bootstrap value of 100. These strains are provided in supplementary materials (**Table S1**). Then, the (.nwk) formatted file was used as an input file for FigTree (v1.4.4) software to build the phylogenetic tree [37].

2.11. Genome annotation:

The annotation for the resultant contigs from the Map to reference processes (Bowtie2 alignment) was done by using Prokka tool kit with default settings[38] and RAST

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tool kit (RASTtk) provided by Patho-systems Resource Integration Center (PATRIC)[29] with Pacon and Pilon iteration of 2, Min Contig length = 300, Min Contig coverage =5, and referenced to Helicobacter Taxid 209.

Under the recommendations of [39], two different databases were used to detect antimicrobial resistance genes like The Comprehensive Antibiotic Resistance Database (CARD)[40], ResFinder tool provided by CGE [41] and from specific genes tab at PATRIC annotation and prediction service. We used PointFinder tool to search for point mutations that are known to cause resistance as well [42]. Virulence genes detection was done using PATRIC annotation and prediction service as well [29]. For the investigation of integrons, plasmids, and transposons, we used INTEGRALL[43], PlasmidFinder[44], and PHAST[45], respectively.

2.12. Statistical analysis:

Data were then processed using SPSS statistics subscription (IBM SPSS 30.0.0.- USA). The correlation between the different virulence genes and PFGE clusters with virulence factors profiles was done by Fisher's exact test and chi-square test with a significant p-value lower than 0.05.

3-Results:

3.1. Strain Isolation and virulence genes PCR amplification:

H. pylori was identified morphologically as small transparent colonies that showed positive reaction for catalase, oxidase, and urease tests. Out of 284 patients, *H. pylori* was isolated as pure culture from 47 (16.5%) of biopsy samples. From the clinical manifestation history of the 47 cases, it has been found that 55.5% were diagnosed as gastritis and gastro-duodenitis and 31.3% as peptic ulcer and duodenal ulcer. Only 13.3 % of samples were diagnosed with GERD. Forty-seven out of 284 (16.5%) were positive when samples were checked for *ureA* gene. These 47 ureA positive strains were then tested for four virulence factors regulating genes *cagA*, *vacA*, *oipA*, and *iceA*. Gene *cagA* was detected in 44.4 % (20 samples) of positive cultures and was the most abundant among other virulence genes. While *vacA*, *oipA* and, *iceA* were present in 31% (15) , 20% (10) , and 11.1% (5), respectively. The abundance of each virulence gene in different clinical diagnostic cases is demonstrated in **Figure S1** in supplementary materials. There was a significant correlation between *cagA* and *vacA* (p=0.001) and *vacA* with *iceA* (p=0.002). We accepted the null hypothesis for the relation of *cagA* with *iceA* (p=0.15). *oipA* gene had no significant correlation with neither *cagA*, *vacA*, nor *iceA* (p=1, p=0.68, and p=0.56, respectively).

3.2. PFGE fingerprint analysis:

The DNA of 22 samples of *H. Pylori* experimented for PFGE fingerprinting gave acceptable patterns with *XbaI* restriction enzyme. Analysis of the resultant patterns from *XbaI* digestion showed two distinct clusters A and B (**Figure 1**). Isolates' similarity ranged between 100% for identical isolates and 88.2% for isolation of different pulsotypes. Four isolates out of 22 are in cluster A. Cluster B is the dominant cluster, and it is further divided into two clusters: 9 samples in group B1 and 9 samples in group B2. Within cluster groups, the isolates are categorized according to their virulence genes profile into four groups and given for different colors as indicated in **Figure 1**. The same results were represented in **Figure S2** in supplementary materials which showed the genetic fingerprint of 22 samples in comparison to each other in the form of heatmaps.

3.3. Genome content:

We used three different tools for the De-Novo-assembly of the provided fastq data of samples M1 and M2: the web-based bacterial bioinformatics resource provided by the Patho-systems Resource Integration Center (PATRIC), Velvet assembler, and SPAdes assembler. The assembly options were set into the default assembly options. Both sequenced samples M1 and M2 were of good quality and were classified in the super-kingdom level taxonomy as follows: cellular organisms > Bacteria > Proteobacteria > delta/epsilon subdivisions > Epsilonproteobacteria > Campylobacterales > Helicobacteraceae > Helicobacter. A comparison between the results of the three assembly algorithms is shown in **Table 2**.

When samples were mapped to *H. pylori* 26695, they gave two consensus sequences, one for each sample, where the gap between contigs were replaced with Ns. These consensus sequences were further used for phylogenic determination, genome annotation, resistance genes, and virulence genes investigation.

3.4. Phylogenic relation determination and geographical revolution study:

When the resultant consensus sequences from bowtie2 alignment were tested against KmerFinder tool, it showed direct relation with six *H. pylori* strains: 25-A-EK9, 23-A-EK1, HP14039, Rif1, BM013B, and 24-A-EK1 for sample M1 and strains: 21-F-EK1 and 24-A-EK1 for sample M2. For the MLST, both samples belong to *Helicobacter pylori* ST181. The phylogenic analysis was done using PATRIC phylogenic tree plotter. It showed a closer relation to western strains than Asian and African strains **Figure 2**

3.5. Genome annotation:

Through Prokka and RASTtk annotation services, the annotated genomes for both samples showed similar

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characters in terms of protein features and coding genes (CDS) content except for some differences in virulence genes characters as shown in **Figure 3**. Our genomes revealed 1680 predicted genes, 1225 coding genes (CDSs), and 455 non-CDS features. Moreover, there were thirty-four transfer RNAs (tRNAs), as well as two separate sets for both 23S and 16S ribosomal RNAs (rRNAs). The number and position of tRNA, 16S rRNA, 23S rRNA, CDS features, and non-CDS features for both samples on the genome is represented in **Figure 4**.

3.6. Antimicrobial resistance genes annotation (Resistome):

Both samples had identical antibiotic resistance characters (resistome) when tested against CARD, ResFinder, PointFinder and PATRIC annotation and prediction service. PATRIC and CARD databases detected antimicrobial resistance genes *gyrA* and *gyrB* which are encoding for resistance against fluoroquinolones antibiotics but in PointFinder database there were no detectable mutations in the quinolone resistance-determining regions (QRDRs). Same results for gene hp1181 which is responsible for efflux pump conferring antibiotic resistance against tetracycline antibiotic. The gene was detected in the PATRIC and CARD database but in ResFinder tool there is no resistance against tetracycline antibiotic. Besides, there were no point mutations detected in rpoB gene responsible for synthesis of beta-subunit of RNA polymerase in bacteria in both samples. Other antibiotic resistance genes were predicted with PATRIC service and were presented on the circular plot of the resistome of both samples in **Figure 5**. Resistance genes detected in both PATRIC annotation service and ResFinder/PointFinder are listed in **Table S2** and **Table S3** in supplementary materials.

3.7. Virulence genes annotation (Virulome):

Our genomes were screened for virulence genes using PATRIC annotation and prediction service. The two consensus sequences M1 and M2 showed different characters regarding virulence genes. Sample M1 showed more predicted virulence factors (170 genes) in comparison to (91) predicted virulence genes in sample M2 which encode proteins related to flagellar motility, colonization, adherence, toxins, and pore formation, acid resistance, chemotaxis proteins, and pro-inflammatory effect. In both samples, we identified cagPAI (cag pathogenicity island) at the position 547,328 bp to the position 579,921 bp with a total of 22 CDS genes including *cag-alpha*, *cag1*, *cag3*, *cagA*, *cagC*, *cagE-I*, *cagL-N*, *cagQ*, *cagS-Z* that represents the major of virulence genes. Moreover, our samples were recognized to be from the type *s1*/*m1vacA* allelic variant which starts at 938,415bp and ends at 942,287bp and has more cytotoxic action for gastric cells. On the other hand, *iceA* gene was found only in sample M2 at the position 1.286,191 bp and ends at 1,286,709 bp. Another group of gene's type to be identified was outer membrane proteins (OMP) which are very important for adhesion. In sample M1, *hopZ, sabA, sabB, babB, oipA, alpA, alpB* were detected at different positions on the genome **Error! Reference source not found.a**. In contrast, sample M2 showed *babA, babB, sabA, hopZ* and lacks *oipA, alpA, alpB,* and *sabB* genes **Figure 6b**. Furthermore, a cluster of 9 genes encoding for urease enzyme (*ureA-I*) was detected in both samples at position 72,021bp which plays an important role in colonization and acid resistance of *H. pylori* in the gastric environment, and it ends at 72,818 bp. Other genes like *cheV1, cheV2, cheW, cheY, tlpB,* and *tlbC* were detected which are responsible for chemotaxis mechanisms, and napA which is used by the bacterium for DNA protection during starvation and harsh conditions. Finally, our genomes were equipped with various types of genes that are responsible for flagellar motility like *flaA/B, flaA-I, flgK, flgL, flgM, flgS, fliD-H, fliL-N, fliP-S, fliY, flhA/B, flhF, fleN, motA/B,* and lipopolysaccharides synthesis like *wbcJ, rfbD, rfbM, ipxB, rfaJ, rfaC*. A summary for virulence genes for both genomes is shown in **Figure 6** and a detailed list of virulence genes with its function is mentioned in **Table S4** and **Table S5** in supplementary materials.

3.8. Plasmids, integrons and transposons annotation (Mobilome):

Interestingly, after investigation of the consensus sequences against INTEGRALL, PlasmidFinder and PHAST, there were neither detectable plasmids nor prophages in our tested samples.

4-Discussion:

H. pylori infection is one of the most prevalent microbial infections. It has been extensively studied in different parts of the world due to its determinant role in developing many gastric malignancies. [4]. However, little is known about *H. pylori* strains in Egypt [46]. To our knowledge, all typing studies conducted on the Egyptian *H. pylori* strains concentrated mainly on different methods of diagnosis, its association with gastric and extra gastric diseases, virulence genes profiling, antibiotic resistance studies (antibiogram), and the best treatment regimen that suits this fast-emerging resistance [47]. On contrary, this study aimed at putting the fingerprinting of genomic features under investigation especially in an extinguished geographical region like Suez Canal region.

Several factors have been known to affect successful culture and isolation of *H. pylori* from biopsy specimens like the quality of the clinical specimen, occurrence of other commensal flora in specimens, time interval between sampling and culture, and inappropriate transport conditions [48].

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In this study a total of 284 biopsy samples were collected. Only 16.5% were positive for *H. pylori* with a percentage lower than that detected in previous studies [9, 49, 50]. This observation has been noted before in Nigeria, Thailand, Indonesia, Iran, and South Africa. Previous reports attributed this result due to the non-viability of some strains of *H. pylori* to grow on blood agar medium because they are deeply embedded in gastric mucosa and due to its conversion to coccoid form which is unculturable. In this case, it can be only detected in IgG serum test and not by culture methods.[51] Hence, culturing alone cannot be approved as the sole diagnostic method and it is usually advised to use more than one identification tool for *H. pylori*. However, it is still an important tool for studying resistance patterns. Several virulence factors have been identified in our study. Cytotoxin-associated gene A (*cagA*) is an important gene in terms of H. *pylori* pathogenicity. It is responsible for disease development due to its role with increased IL8 production and nuclear factor-kB activation [19]. Especially, in cases of gastritis and gastroduodenitis. On the level of clinical symptoms, *cagA* gene was the most abundant gene in each clinical manifestation category as shown in **Figure S1** in supplementary materials. That result has been previously reported as well [19, 52, 53]. It is also a polymorph strain-specific gene that many reports have used for the evolution studies of *H. pylori* [54, 55]. For this reason, *cagA* positive samples were chosen for correlation with other virulence genes and for further PFGE fingerprinting studies and whole genome study.

Our results concerning the relation between *cagA* and *vacA* agreed with the previous studies done [56]. For *iceA* gene, this study results were similar to what was previously mentioned regarding its relation to *vacA* gene and it irrelevant relation to the clinical outcome [57]. Besides, we could not find a significant relation between *cagA* and *iceA* like what has been reported earlier [56]. Moreover, *oipA* gene had no significant correlation with *cagA*, *vacA*, and *iceA* (p=1, p=0.68, and p=0.56, respectively) and this was acceptable and described in previous study [58]. Pulsed-field gel electrophoresis was the gold standard for typing of bacterial species but in comparison to whole-genome sequencing, the latter is more advantageous and reliable for genomic characterization. We used PFGE to choose the most dominant pulsotypes for the WGS analysis. Since our samples were isolated in the form of small sets from patients attending the endoscopy unit in Suez Canal University teaching hospital, Ismailia, therefore, this fact suggests that they are epidemiologically related as suggested before by [59]. This appears in the analysis of the resultant patterns with *XbaI* that showed two distinct clusters A and B (**Figure 1**). Isolate similarity ranged between 100% for identical isolates and 88.2% for isolation of different pulsotypes. Therefore, we confirmed that PFGE was the best selection tool for choosing samples for further investigation by WGS to decrease the cost and the time of analysis process. We chose two isolates that belong to the most dominant pulsotypes on PFGE (group B pulsotypes) as appeared in **Figure 1** and had two different clinical manifestations and different virulence genes profile for the whole genome sequencing to have a closer look at differences in the genomic characters. We used three different tools for the De-Novo-assembly of the provided fastq data of samples M1 and M2 to assure the quality of the results obtained. Velvet assembly algorithm showed the best result among the three assemblers with the lowest L50 = 2, 5, and higher N50 = 566,293 and 283,761 for samples M1 and M2, respectively. Consensus sequences resultant from mapping to reference genome *H. pylori* 26695 gave the best results for phylogenic determination, genome annotation, resistance genes, and virulence genes investigation as it eliminates all possible contaminants or errors in the reads that couldn't be eliminated by error correction and normalization tool. As shown in **Figure 2**, our samples showed a closer relation towards western strains than Asian and African strains. This sounds reasonable when explained in terms of human migration history. There are seven *H. pylori* population types that are known based on geographical associations and human migration. hpEurope includes almost all *H. pylori* strains isolated from ethnic Europeans, including people from countries colonized by Europeans. Our theory suggests that Egypt was one of the countries that have been colonized by European soldiers during world war for more than 74 years, especially Ismailia that lies in a special geographical location on Suez Canal and from which samples under investigation were isolated. This period was sufficient to transfer hpEurope strain of *H. pylori* to Egypt [60]. We screened the virulome and the resistome of the two *H. pylori* isolated samples. They were of the type ST181. Furthermore, the detection of *cagPAI* genes specially *cagA* and *cagE* along with the *m1s1vacA* allele and *babA* genes which are a more cytotoxic strain than other allelic variants explain the severe inflammation. Genetic and protein features of the virulome of both samples illustrated the potentials for successful persistent infection, colonization, and disease pathogenesis and clinical manifestation during infection which appeared as erosive gastritis with melena and gastric ulcer with hyperemia [61, 62]**.** These clinical manifestations were also augmented with other genes like *cheV1*, *cheV2*, *cheW*, *cheY*, *tlpB*, and *tlbC* in both samples and *iceA* gene in sample M2. (**Figure 6**). Currently, the antimicrobial therapy of *H. pylori* solely depends on the phenotypic antimicrobial sensitivity testing which is starting to be undependable. However, whole-genome sequencing analysis gives us a more comprehensive analysis of antimicrobial resistance

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in terms of genes annotations and enables us to build the complete resistome of any microbial cell, not only *H. pylori*. Whole-genome sequencing helps us to detect mutations as well [63]. *H. pylori* develop drug resistance to single antibiotics; thus, combination therapy of several antibiotics is recommended. In Egypt, many physicians are using the quadrable therapy for treating *H. Pylori* together with probiotics [64, 65]. However, resistance against first-line antibiotics, especially clarithromycin, tetracycline, and metronidazole is starting to emerge [48]. Regarding our samples, we detected resistance genes against aminoglycosides, tetracyclines, and quinolones which are important members of the quadrable therapy used in Egypt (**Figure 5**). However, further investigation in point mutations in these genes gave negative results for mutations in *gyrA, gyrB* and *rpoB* genes. In addition, when we tested consensus sequences of our strains against ResFinder tool, we detected no resistance against any known antibiotic. These facts indicate the significance of using multi databases for searching for resistance genes [39] and that the description of mutations in existing genes is more consistent regarding the description of *H. pylori* resistome than the presence/absence of the gene itself .

In conclusion, this study used PFGE for the epidemiological fingerprint typing of *H. pylori* isolated from Egyptian patients. Through the resultant PFGE fingerprints, we were able to classify isolates of the same clinical manifestation and virulence gene profiles (*cagA* positive isolates) into further subgroups (A and B) based on dendrogram. In addition, it reported the genomic features including virulome and resistome of *cagA*-positive strains that were isolated from a special geographic region, and we proved the advantage of NGS technique as a typing method over PFGE for studying the genomic fingerprint of heterogenous bacteria like *H. pylori*. However, further studies should be conducted for the full characterization of *H. pylori* in that area.

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Conflict of interest

The authors declare that no competing financial interests exist.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Mennatallah Abdelkader. The first draft of the manuscript was written by Mennatallah Abdelkader and Shymaa Enany. All authors read and approved of the final manuscript.

Compliance with Ethical Standards

This study was approved by Suez Canal University, Egypt's ethical board under registration No.PM-HR1-MD-17. Permission and informed consent to collect biopsy samples were obtained from patients attending the endoscopy unit, Suez Canal University hospital, Ismailia, Egypt.

Ethical consideration: All the participants in this study gave their informed permission.

Supplementary Information

https://odr.journals.ekb.eg/jufile?ar_sfile=1713045

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