



ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF *H. PYLORI* FROM CLINICAL SAMPLES IN BABYLON CITY, IRAQ

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Abstract

Helicobacter pylori is Gram-negative bacterium with spiral-shaped, the bacterium has bluntly rounded ends and measures 2 to 4 μm long with a diameter of 0.5 to 1 μm . It is a curved microaerophilic and it colonizes the mucus layer of the gastric epithelium of humans the bacterium has 2-6 monopolar flagella. Because there was a problems in treatment of this bacteria in old few years and emergence of high degree of resistance for most antibiotic there for the aim of this study was to isolation, identification and study of antimicrobial susceptibility test for clinical samples of *H. pylori* in Babylon city. One hundred twenty (120) patients were analyzed in this study, who were suffered from abdominal problems, the complaining of suggestive symptoms of peptic ulcer disease such as upper abdominal pain, acidity, nausea and vomiting were reported from these patients through the endoscopic process in AL Imam AL Sadiq Hospital and Marjan Medical City, GIT unit through December 2018 to April 2019. After preserved of biopsy for 24 hours in brain heart infusion broth for activation of bacteria, culturing was done on two different selective media. Modified blood agar and skirrow agar medium and incubation for 3-4 days on 37°C. Identification was occur by morphology, microscopic feature, biochemical test and molecular methods. Antimicrobial susceptibility test was done by disc diffusion method on modified Molar Hinton agar for seven antibiotic belonged to five class. The results were show high degree of resistant for most antibiotics. The ratio of resistant to amoxicillin was 59.3%, Doxycycline 66.7%, metronidazole 100%, ciprofloxacin 55.6%, rifampicin 70.4%, clarithromycin which is the most antibiotic used to eradication of *H. pylori* in the world today, show 66.7% resistant and 33.3% sensitive, while the most effective antibiotic according to the result of this study was levofloxacin which is recorded higher ratio of sensitivity (88.9%). The current study was also show (11.11%) of isolates which appear multidrug resistant (MDR) for all these antibiotic and (29.62%) was appears MDR to all used antibiotic except levofloxacin.

Key words: clinical samples; environment; Antimicrobial

Introduction

Helicobacter pylori is Gram-negative bacterium with spiral-shaped, the bacterium has bluntly rounded ends and measures 2 to 4 μm long with a diameter of 0.5 to 1 μm . It is a curved microaerophilic and it colonizes the mucus layer of the gastric epithelium of humans the bacterium has 2-6 lophotrichous (monopolar bundle) flagella (Falush *et al.*, 2003).

A key feature of *H. pylori* is its microaerophilicity, it requires O₂ levels of 2 to 5% and the additional need of 5

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to 10% CO₂ and high humidity for optimal growth. There is no need for H₂. Many laboratories utilize standard microaerobic conditions of 85% N₂, 10% CO₂ and 5% O₂ for *H. pylori* culture with the optimum temperature of 37°C, also it has ability to grow at 34 to 40°C (Kusters *et al.*, 2006). Although its natural habitat is the gastric mucosa, *H. pylori* is considered to be a neutralophile. The bacterium will survive brief exposure to pH of <4, but growth occurs only at the relatively narrow pH range of 5.5 to 8.0, with optimal growth at neutral pH (Kusters *et al.*, 2006).

H. pylori is a fastidious microorganism and requires complex growth media. The solid media for isolation and culture the bacterium usually consist of Columbia or Brucella agar supplemented with either (lysed) horse or sheep blood, for optimal growth may supplemented with additional sources of nutrients and possibly also protect against the toxic effects of long-chain fatty acids (Kusters *et al.*, 2006). However, for isolation and culture *H. pylori*, it favorite for media contain selective antibiotic mixtures containing vancomycin, trimethoprim, cefsoludin and amphotericin B. (Dent *et al.*, 1988).

The treatment of *H. pylori* infection continues to evolve and remains a topical global research interest (O'Connor *et al.*, 2017). Triple therapy has been modified in that it is now recommended to use double-dose (80 mg) proton-pump inhibitor (PPI), quadruple dose (2g) amoxicillin, and clarithromycin (1 g) for at least 10 days, and preferably 14 days (Malfertheiner *et al.*, 2017; Gisbert and McNicholl, 2017). The substitution of vonoprazan, a novel potassium-competitive acid blocker that provides reversible acid suppression by preventing K⁺ from binding to gastric H⁺/K⁺-ATPase, for PPIs has shown promising results, however remains to be tested outside Asia (Ozaki *et al.*, 2018; Tanabe *et al.*, 2017).

Materials and Methods

Preparation of culture media

Modified blood agar base (selective media)

Forty five (45) gram of blood agar base media were taken and suspended in one liter of D.W. and sterilized in autoclave (121°C for 20 min), and after that left to cool to 45°C and the following antibiotics were added to it.

Amphotericin B 2.5 mg/ ml

Trimethoprim 5 mg/ml

Vancomycin 10 mg/ml

And 10% of human blood was added , mixed well then poured in petri dish (25 ml/one petri dish), leaving the media to cool and then saved in refrigerator for its use.

Skirrow media

This media consist of the following :

- Brain heart infusion agar
- Human blood (5%)
- Polymixin B
- Trimethoprim
- Vancomycin

Urea agar base

It was prepared by dissolving 6 gm of urea agar base in 100 ml of D.W., sterilized by autoclave (121°C for 20 min), left to cool and one vial of urea supplement (5 ml) was added to it, mix well and poured in sterile plain tube (5 ml/tube) and saved in refrigerator until its used. This media was used to test the ability of bacteria to produce urease enzyme.

Brain heart infusion broth

This media was prepared by dissolving 3.7 gm of brain heart infusion broth in 100 ml of D.W, sterilized by autoclave (121°C for 20 min), left to cool, mix well and poured in sterile plain tube (5 ml/tube) and saved in refrigerator until its used. This media was used to activation of bacteria as well as preserved of biopsy through taken in endoscopic unit for culturing in microbiology lab.

Preserved media

This media was prepared in the same way of brain heart infusion broth except adding of glycerol (15-20%) and then poured in sterile plain tube (5 ml/tube) and saved in refrigerator until its used. This media was used to preserved of bacteria for long time.

Antibiotics

All antibiotics used in this study are listed in table 1.

Table 1: Types of antibiotics.

Break-point	Concentration	Name	Purpose	Group
≥21<16	10 µg	Amoxillin	Antibiotic susceptibility test	1
≥23<21	15 µg	Clarithromycin		
≥24<17	5 µg	Metronidazole		
≥19<17	5 µg	Levofloxacin		
≥22<17	5 µg	Rifampicin		
≥19<17	30 µg	Doxycycline		
≥25<20	30 µg	Ciprofloxacin		
	2.5 µg	Polymyxin B	Antibiotic for selective Media	2
	5 µg	Trimethoprim		
	10 µg	Vancomycin		

Culturing of *H. pylori*

The antral biopsy specimens were transported to the microbiology laboratory immediately within less than two hour in 2-2.5 mL brain heart infusion broth as a transport medium. Incubated at 37°C for 24 hours then subcultured on modified blood agar base and skirrow agar for 3-5 days

Morphological identification of *H. pylori*

This is depending on the morphological properties of colonies such as color, size of colony, shape, condition of growth, edge and elevation of texture part, also

microscopic examination as Gram staining, shape and size of bacterial cell and arrangement of these cell.

Biochemical diagnostic test of *H. pylori*

1- Gram stain

Smear was taken from bacteria and Gram stain was applied on the smear.

2- Oxidase test

A small drop of reagent place on filter paper and then small portion of bacterial colonies was spread on this paper by wooden stick, positive result appeared as color change to purple, while negative result appeared as no color change. This test depend on the presence of certain bacterial oxidase that would catalyze the transport of electrons between electron donors in bacteria and a redox dye (Tetramethyl-p-phenylene- diaminedihydrochloride) (Forbes *et al.*, 2007).

3- Catalase test

The isolates were tested for catalase activity by using slide method in which the sterile wire loop is stacked on the surface of the pure colony and transferred to a microscope slide and one or two drop of 3% H₂O₂ were added onto the organism on the slide and observed for immediate oxygen bubble formation (Forbes *et al.*, 2007).

4- Urease Test

The urease test was applied according to MacFaddin (2000). The pure isolates were inoculated heavily on the entire surface of urea agar and stab with loop wire. The tubes were inoculated at 37°C in the incubator. The formation of purple color was examined after 4h.

Molecular detection of *H. pylori* and virulence factors

PCR assay was performed to detection of *H. pylori* based on 16S rRNA gene as well as detection of most important virulence factors, genomic DNA was extracted directly from bacteria and using FAVORGEN genomic DNA extraction kit as described by manufactured.

Table 2: Sequence of primers and amplicon size.

Reference	Product size (bp)	5'-Sequence-3'	Target Gene	
Ho <i>et al.</i> , 1991	110	CTG GAG AGA CTAAGC CCT	F	16S
		ATT ACT GAC G CT GATTGT GC	R	rRNA

Table 3: Thermal cycling conditions.

No of cycles	Final extension	Extension	Annealing	Denaturation	Initial denaturation	Genes
35	72 C°/5min	72 C°/1min	55 C°/1min	95 C°/30 sec	95 C°/10 min	Hp1

A. Polymerase chain reaction

primer pairs used in this study were dissolved using nuclease free water, firstly the primer stock prepared as 100 pmol and then the working primer would prepared from primer stock tube. According to the instruction provided by manufacture (Bioneer/Korea) nuclease free water was added to get 100 Pmol/μl as a stock solution. Then making dilution to get 10 Pmol/μl as working solution.

Primers were used in this study are illustrated in table 2.

B. Thermal cycles condition

Conventional PCR was used to amplify the target DNA using specific primers. It include three consecutive steps that repeated for specific number of cycles to get amplified PCR product, thermal cycling conditions were listed in table 3.

C. The PCR mixture

Amplification of DNA was carried out in a final volume of 25 μl reaction mixture as illustrated in table 4.

Table 4: Content of PCR reaction mixture.

Volume	Content of PCR Reaction Mixture	No
12.5 μl	Master mix	1-
2 μl	Forward primer	2-
2 μl	Reverse primer	3-
5 μl	Template DNA	4-
3.5 μl	Nuclease free water	5-
25 μl	Total volume	

D. PCR product analysis

Agarose gel electrophoresis analyzed the PCR products as in the following steps:

1- A 1.5 % agarose gel was prepared by using 1x TBE buffer and dissolving in Macrowave, after that left to cool to 50°C.

2- A 5 μl of red safe stain was added in to agarose gel solution and mix well.

3- Agarose gel solution was poured in to the tray after fixing the comb in the proper position, then left to solidify at room temperature.

4- The comb was remove gently from the tray and then PCR product was loaded in the comb well and also 10 μl of DND marker (100 bp ladder) was loaded in first lane.

5- The gel tray was fixed in the electrophoresis chamber and filled by 0.5X TBE buffer and electric was performed at 100 volts and 70 AM for one hour.

6- After finished of electrophoresis, PCR product was visualization by using UV transilluminator.

E. DNA sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both (forward and reverse) termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystem) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of the investigated samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

Results and Discussion

One hundred twenty (120) patients were analyzed in this study, who were suffered from abdominal problems, the complaining of suggestive symptoms of peptic ulcer disease such as upper abdominal pain, acidity, nausea, and vomiting were reported from these patients through the endoscopic process units in AL Imam AL Sadiq Hospital and Marjan Medical City, GIT unit through December 2018 to April 2019. There were 67 (55.8%) male and 53 (44.2%) female patients, from each patient we collected two different samples of gastric biopsy in tube which containing brain heart infusion broth and transported directly by cool box through two to three hours to the microbiology lab for other processing.

Culturing of *H. pylori*

A total of 48 (70.5%) of gastric biopsy was given positive result for *H. pylori* culture. The process of culture was include activated media (brain heart infusion broth BHI) that was used to preserved and incubation of biopsy at 37°C for 24 hours, it is well know that *H. pylori* is very fastidious bacteria that required special culture media and also incubation condition, Brain Heart Infusion Broth (BHI) media contains protease peptone and infusions from calf brain and beef heart which serve as sources of

carbon, nitrogen, essential growth factors, amino acids and vitamins. Dextrose is used as a source of energy. Disodium phosphate helps in maintaining the buffering action of the medium whereas sodium chloride maintains the osmotic equilibrium of the medium. Sub culture was doing on modified blood agar base and Skirrow media then incubated in anaerobic condition for 3-6 days at 37°C. The main superiority of bacterial culture for *H. pylori* is the possibility of antibiotic susceptibility tests to choose proper antibiotics in the treatment of subjects and avoiding a new generation of antibiotic resistance among the symptomatic patients (Ohara, 2009). *H. pylori* can grow slowly on many solid media under microaerophilic condition. As a general rule, *H. pylori* needs blood or lysed blood supplements to grow optimally on agar plates. Currently, Wilkins Chalgren agar, Brain heart agar, Columbia and Brucella agars are most used base media to propagate *H. pylori* culture in routine diagnosis (Megraud and Lehours, 2007).

Characteristic of *H. pylori* in culture

A. Morphological characteristic :

Bacterial colonies was appear as small, entired white to creamy colonies on both modified blood agar and skirrow agar as show in Fig. 1A. These selective media was supported with specific suppliment to prevent growth of others microbes such as vancomycin against Gram positive bacteria and polymixin against fungi. Microscopically *H.pylori* appear as Gram negative, elongated with different form that varies from rode, spiral, filamentous and coccoid shaped as show in Fig. 1B. Most bacteria possess a cell wall that, due to its covalently closed, net-like structure, maintains a specific shape and thus also imposes it on cells, *H. pylori* have peptidoglycan modifying enzyme such as Csd1- and, 3 endopeptidases, Csd6 carboxypeptidase and Csd4 carboxypeptidase and other regulatory protein such as CcmA and Csd5 that play important role in the maintenance the morphology , motility, pathogenicity as well as ability to colonized the host (Sycuro *et al.*, 2012, Sycuro *et al.*, 2013).

B: Biochemical Characteristic :

Confirmed biochemical tests were include positive

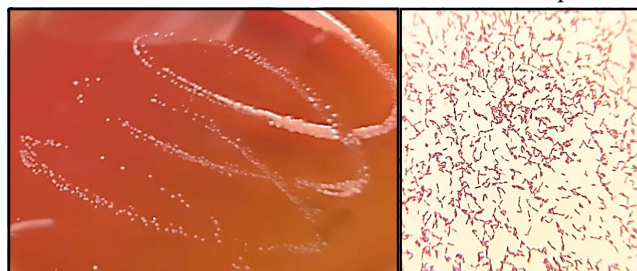


Fig. 1: *H. pylori* characterization

results for catalase, oxidase and strongly positive for oxidase, as show in Fig. 2. Urease test is very important biocemical test for this bacteria, its principle depended on the hydrolysis of urea to ammonia that increase the pH of medium and converted to rosy color in the presence of phenol red as indicator. Similar results to this study was documented for many authers such as (Al-Sulami

et al., 2008).

C. Molecular detection of *H. pylori*

A total of 48 (70.5%) of bacterial culture of gastric biopsy was given positive result for specific 16S rRNA of *H. pylori* culture , the sensitivity and specificity was 100% as show in table 5. 16S rRNA is useful for primary detection of *H. pylori* use Hp1, Hp2 primers with sensitivity up to 100%, this primer widely used and has been proven to have a high sensitivity as show in Fig. 3 (Ho *et al.*, 1991).

Genetic test based of PCR enable the specific detection of nucleic acid and have been used for diagnosis of *H. pylori* in clinical samples. Because its high sensitivity PCR is suitable for diagnosis when an organism present in small number, slow growing or difficult to identify. In the same time, the process is very susceptible to inhibitors by contaminant present in the clinical samples, thus may be given false results (Widjoatmodjo *et al.*, 1992). The PCR technique represent more developed process and the most accurate one for the diagnosis of *H. pylori* which depend on the DNA of the bacteria (De Boer, 1996). So that, Final diagnosis of *H. pylori* was depened on genitic test, ampilification of 16S RNA and alignment of DNA sequencing with gene bank on NCBI.

D. Sequencing:

Sequencing of the 1493 bp region within the 16S rRNA gene within the *Helicobacter pylori*

Within this locus, 1 sample was included in the present study. This sample was screened to amplify 16S rRNA genetic sequences in the targeted organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Concerning the supposed 1493 bp amplicons, NCBI BLASTn engine

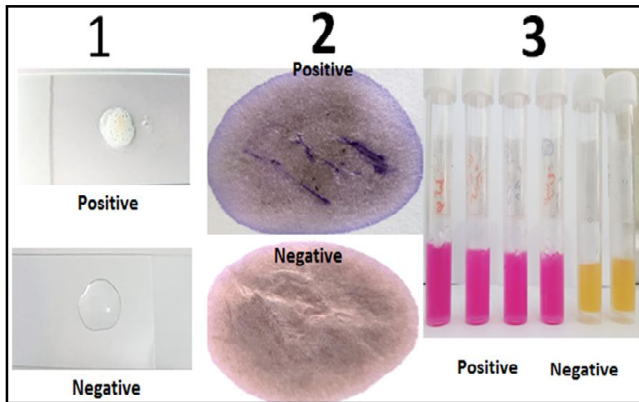


Fig. 2: Main Biochemical Tests of *H.pylori*.

1- Catalase test. Positive: air bubbles formation. Negative: no bubbles. 2- Oxidase test. Positive: ink color formation. Negative : no color. 3- Urease test. Positive: Rose color formation. Negative : yellow color.

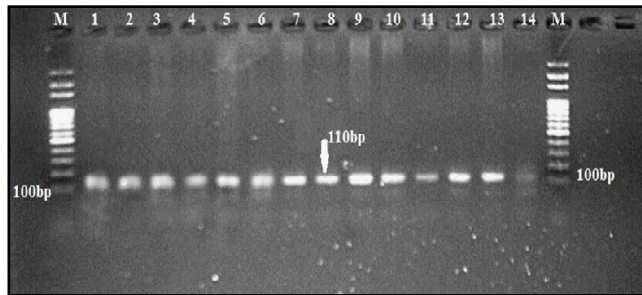


Fig. 3: 1.5% Agarose Gel Electrophoresis image showing the PCR product analysis of 16S rRNA of *H. pylori*. Lane M:Marker (100-1500 bp) Lane 1-14: bacterial samples with a PCR product 110 bp.

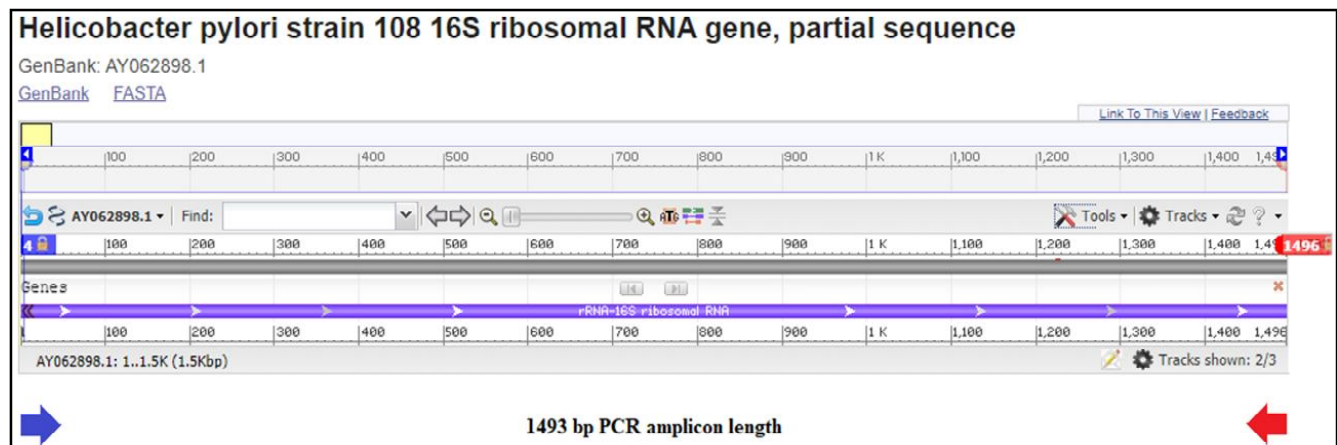


Fig. 4: The exact position of the retrieved 1493 bp amplicon that partially covered a portion of the 16S rRNA gene within *Helicobacter pylori* (Gen Bank acc no. AY062898.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its end point.

Table 6: The position and length of the 1493 bp PCR amplicons used to amplify a portion of the 16S rRNA gene within *Helicobacter pylori* (GenBank acc. no. AY062898.1). The gray colored sequences referred to the position of the reverse and forward primers, respectively.

Amplicon	Referring locus sequences (52-32)	length
DNA sequences within the 16S rRNA gene	<p>*GTTTGATCCTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAG TCGAACGATGAAGCTTCTAGCTTGCTAGAATGCTGATTAGTGCGCACGGGT GAGTAACGCATAGGTCATGTGCCTCTTAGTTTGGGATAGCCATTGGAACGA TGATTAATACCAGATACTCCCTACGGGGGAAAGATTTATCGCTAAGAGATCA GCCTATGTCCTATCAGCTTGTGGTAAGGTAATGGCTTACCAAGGCTATGAC GGGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAAGTGAACACGGTCC AGACTCCTACGGGAGGCAGCAGTAAGGAATATTGCTCAATGGGGGAAACCCT GAAGCAGCAACGCCGCTGGAGGATGAAGGTTTTAGGATTGTAACTCCTTT TGTTAGAGAAGATAATGACGGTATCTAACGAATAAGCACCGGCTAACTCCGT GCCAGCAGCCGCGTAATACGGAGGGTGAAGCGTTACTCGGAATCACTGGG CGTAAAGAGCGCTAGGCGGGATAGTCAGTCAGGTGTGAAATCCTATGGCTT AACCATAGAAGTGCATTTGAACTACTATTCTAGAGTGTGGGAGAGGTAGGT GGAATTCTTGGTGTAGGGGTAATAATCCGTAGAGATCAAGAGGAATACTCATT GCGAAGGCGACCTGCTGGAACATTACTGACGCTGATTGCGCGAAAGCGTGGG GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGGATGCT AGTTGTTGGAGGGCTTAGTCTCTCCAGTAATGCAGCTAACGCATTAAGCATC CCGCCTGGGGAGTACGGTTCGAAGATTAATACTCAAAGGAATAGACGGGGAC CCGCACAAGCGGTGGAGCATGTGGTTAATTGATTCTACACGAAGAACCCT ACCTAGGCTTGACATTGAGAGAATCCGCTAGAAATAGTGGAGTGTCTGGCTT GCCAGACCTGAAAACAGGTGCTGCACGGCTGTCGTCAGCTCGTGTCTGAG ATGTTGGGTTAAGTCCCACAACGAGCGCAACCCCTTTTCTTAGTTGCTAACA GGTTATGCTGAGAACTCTAAGGATACTGCCTCCGTAAGGAGGAGGAAGGTGG GGACGACGTCAAGTCATCATGGCCCTTACGCCTAGGGCTACACACGTGCTAC AATGGGGTGCACAAAGAGAAGCAATACTGCGAAGTGGAGCCAATCTTCAAAA CACCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTGCATGAAGCTGGAATC GCTAGTAATCGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGT ACTCACGCCCGTACACCATGGGAGTTGTGTTTGCCTTAAGTCAGGATGCT AAATTGGCTACTGCCACGGCACACAGCGACTGGGGTGAAGTCGTAACAA GGTAACCGTAGGTGAACCTGCGGCTGGATCACCTCCT**</p>	1493bp

* refers to the reverse primer sequences (placed in a forward direction)

** refers to the forward primer sequences (placed in a reverse direction)

shown 100% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. AY062898.1), the approximate positions and other details of the retrieved PCR fragments were identified Fig. 4.

After positioning the 1493 bp amplicons' sequences within *Helicobacter pylori*, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 1493 bp amplified amplicon table 6.

The alignment results of the 1493 bp samples revealed the presence of no mutation in the analyzed sample in comparison with the referring reference DNA sequences Fig. 5.

A comprehensive tree was constructed. The sequences of the presently investigated isolates were aligned side by side with other related sequences within the tree to provide an inclusive description of the precise phylogenetic relationships among the investigated organisms. The currently constructed comprehensive tree indicated the presence of only one genus all over the scanned of 16S rRNA sequence, namely *Helicobacter* spp. thus, the currently used 16S rRNA-specific primers were proved their ability to confirm the currently investigated organisms. This power of high discrimination was added to the original 16S rRNA-based identity detection ability of such comprehensive tree to accurately identify the precise phylogenetic position of these investigated bacterial species. This, in turns, gives a further indication of the power of the currently utilized

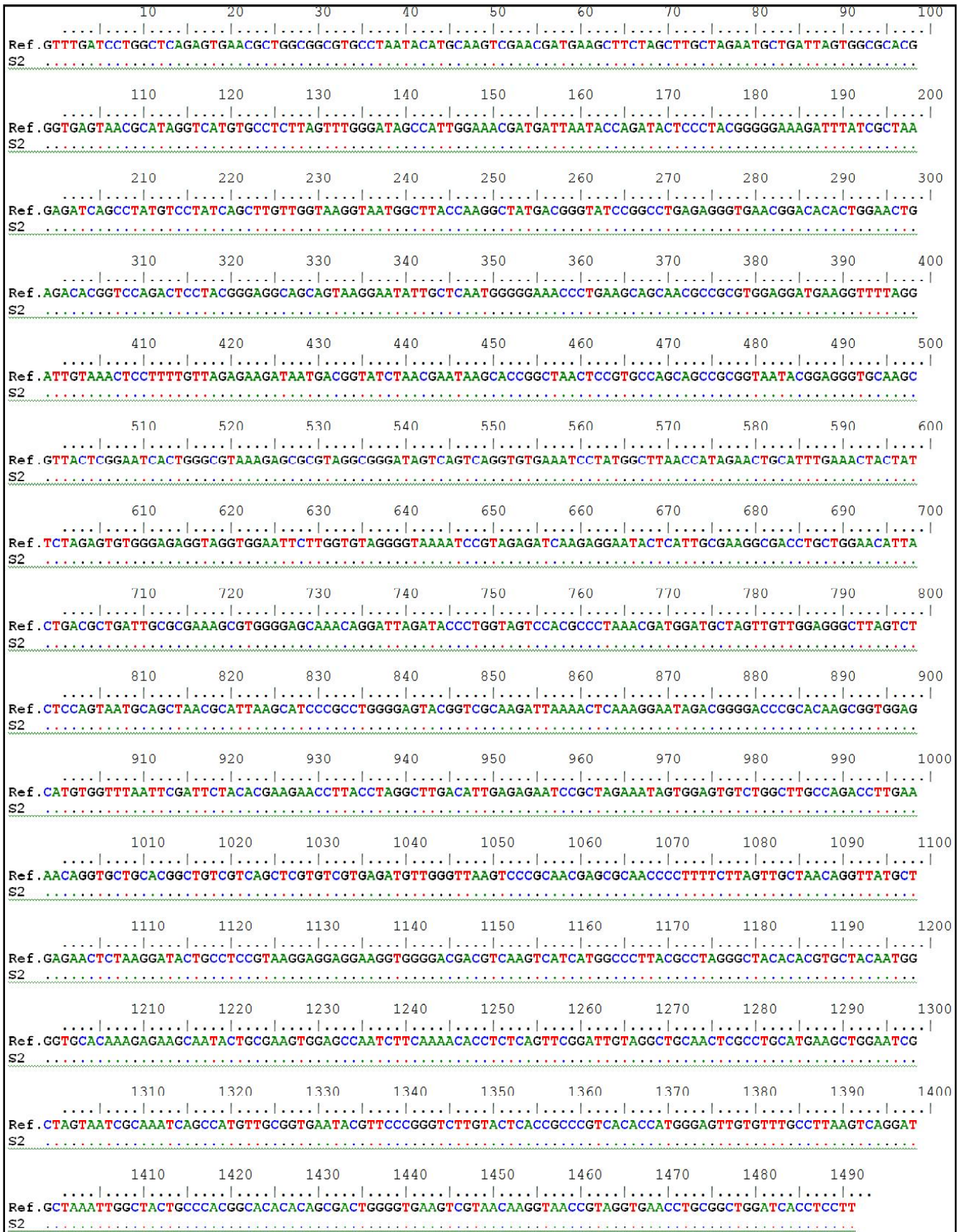


Fig. 5: DNA sequences alignment of 1 sample with its corresponding reference sequences of the 1493 bp amplicons of the *16S rRNA* genetic DNA sequences. The symbol “ref” refers to the NCBI referring sequence, “S2” refers to the sample no. 2.

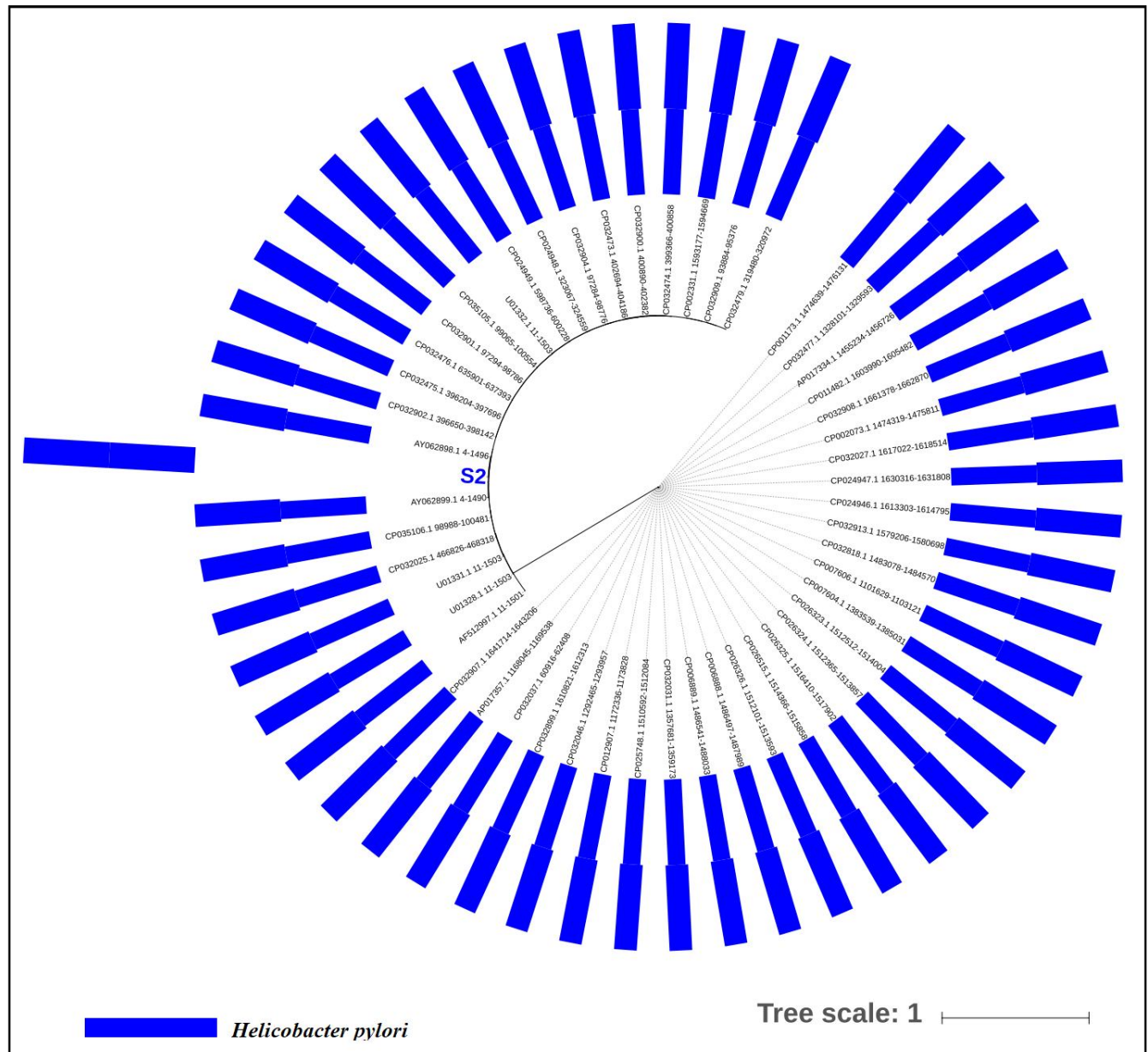


Fig. 6: The comprehensive phylogenetic tree of genetic variants of the 16S rRNA fragment for one local bacterial isolate of *Helicobacter pylori*. All the mentioned numbers referred to Genbank acc. no. of each referring bacterial organisms. The number “1.0” at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. Different species were highlighted in different colors. The highlighted symbol “S2” refers to the studied *Helicobacter pylori*.

16S rRNA based primers to confirmation of *Helicobacter* species. Moreover, the presented tree has added another layer of confirmation about the assured identity of this isolates. Furthermore, more details have been given to insure their accurate phylogenetic positions in the presently constructed comprehensive tree.

S2 was suited beside two bacterial Genbank accession numbers belonged to two strains of *Helicobacter pylori*, namely AF512997.1 and AY062898.1, which they belonged to the Netherland 181 and the Canadian 108 strains, respectively Fig. 6.

Antimicrobial susceptibility

In this study the susceptibility of 48 *H. pylori* clinical isolates was evaluated against 7 antibiotics belonged to five class . The results were show high degree of resistant for most antibiotics, as show in Fig. (4.8) the ratio of resistant to amoxicillin was 59.3%, Doxycycline 66.7%, metronidazole 100%, ciprofloxacin 55.6%, rifampicin 70.4%, clarithromycin which is the most antibiotic used to eradication of *H. pylori* in the world today, show 66.7% resistant and 33.3% sensitive, while the most effective antibiotic according to the result of this study

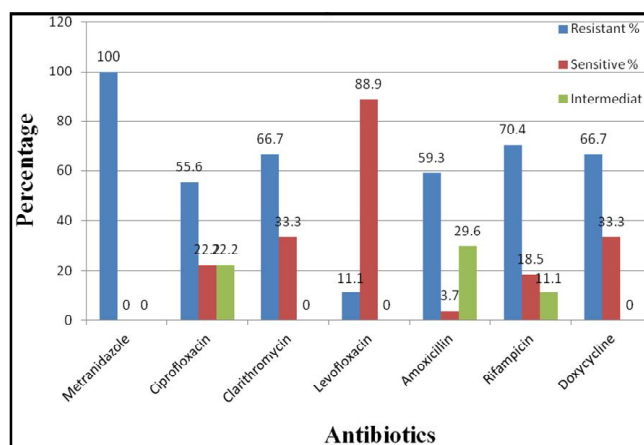


Fig. 7: Antimicrobial susceptibility test of *H. pylori*.

was levofloxacin which is recorded higher ratio of sensitivity (88.9%) as illustrated in the following Fig. 7.

The current study was also show (11.11%) of isolates which appear multidrug resistant (MDR) for all these antibiotic and (29.62%) was appears MDR to all used antibiotic except levofloxacin.

The decrease in the efficiency of these antibiotic may be due to increase exposure to these antibiotics in the past decade which lead to overexpression of multidrug resistant (MDR) efflux pump.

The treatment of *H. pylori* infection continues to evolve and remains a topical global research interest (O'Connor *et al.*, 2017). Triple therapy has been modified in that it is now recommended to use double-dose (80 mg) proton-pump inhibitor (PPI), quadruple dose (2g) amoxicillin and clarithromycin (1g) for at least 10 days, and preferably 14 days (Gisbert & McNicholl 2017).

The substitution of vonoprazan, a novel potassium-competitive acid blocker that provides reversible acid suppression by preventing K^+ from binding to gastric H^+ / K^+ -ATPase, for PPIs has shown promising results, however remains to be tested outside Asia (Ozaki *et al.*, 2017). Levofloxacin remains one of the most favored second-line therapies, however, bismuth, when available, is an increasingly successful option. Sequential therapy remains in use in areas of high resistance, but may prove challenging in terms of compliance and is no longer recommended (Gatta *et al.*, 2018) Three-in-one formulations of bismuth quadruple therapy (BQT) may improve compliance (Miehke *et al.*, 2017).

Several studies in different countries was show high resistant of *H. pylori* to most antibiotics, for example a study from Syria highlighted insufficient responses to both clarithromycin and levofloxacin-based triple therapies with eradication rates of 35.1% and 29.7% (Cheha *et al.*, 2018). Resistance to clarithromycin may be because

higher frequency of CLA usage in developed countries, another cause by specific point mutations in the 23S rRNA gene, particularly at positions 2143 and 2142 of domain V. Studies that have evaluated the presence of these mutations in clinical isolates from the United States in comparison to susceptibility determined using the gold standard agar dilution method are lacking (Chey *et al.*, 2017). Rifampicin resistance in *H. pylori* has been associated with an exchanges in the rifampicin resistance-determining region (RRDR) of *rpoB*, mainly at codons 525 to 545, 547 and 586 (positions according to *H. pylori* reference strain 26695; corresponds to codons 512 to 573 in *Escherichia coli*), the mechanism of resistance to metronidazole is less clear. Frame shift mutations and truncations in genes that encode electron transfer proteins, such as the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-flavin nitroreductase (*frxA*) and oxygen-insensitive NADPH nitroreductase (*rdxA*), have been implicated in elevated MIC values to metronidazole (Hays *et al.*, 2018). The result of this study was similar to (Mabeku *et al.*, 2019) in regard to metronidazole resistant, in which (97.85%) of clinical isolates were resistant to that antibiotics, in the same way amoxicillin resistant was very high (59.3%) which also homologous to previous study (97.14%), over consumption of these two antibiotic in dental infection may be interpret these result.

The result of this study was also similar to (Chen *et al.*, 2017) that was found high rate of clarithromycin resistance (70.4%) and increase of metronidazole and ciprofloxacin MICs In contrast, most isolates had a low amoxicillin and tetracycline MIC (Shiota *et al.*, 2015).

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