Molecular Characterization of Helicobacter Pylori in Human and Pet Animal in Egypt (Ismailia Governorate)

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Abstract

The genus Helicobacter contains more than 35 species, Gram-negative microaerophilic bacteria, which colonizes the gastrointestinal tracts of humans and different animals. aim of work transfer to end of introduction to determine the susceptibility of *H. pylori* isolates to several antimicrobial agents to overcome the *H. pylori* treatment failure. For this purpose, 25stool sample from dog, 20 stool samples from cat and 75 stool samples from their human owners were collected for *H. pylori* infection. *H. pylori* was detected by culture in 36%, 20% and 41.3% of dog, Cat and human samples, respectively. Antimicrobial resistance of *H. pylori* in human, dog and cat to ceftazidime is100% .to amoxicillin is 76%, 100% and 100% respectively, to bacitracin is 88%, 100% and 100% respectively, to erythromycin is 80%, 100% and 100% respectively, to trimethoprim sulphate is 48%, 50% and 50% respectively, to doxycycline is 40%, 50% and 50% respectively, to levofloxacin is 24%, 50% and 50% respectively, to nitrofurantoin is 44%, 50% and 50% respectively. 9 stool samples were collected from human, dog and cat examined for presence of specific16s rRNA gene for *H. pylori* by nested PCR. The positive samples were subjected to Helicobacter genus- specific 16s r RNA gene sequencing.

Keywords: *H. pylori*, Antimicrobial resistance, 16s r RNA sequencing

Introduction:

Helicobacter pylori is motile, Gram- negative spiral shaped bacteria, and has (4-6) Sheathed flagella which allow high motility and has smooth surface [1,2]. *H.*

pylori is urease, oxidase, and catalase positive, it needs special cultural and environmental conditions (10% CO2, 5% O2) so, it is fastidious, microaerophilic microorganism, it grow well on moist heated blood agar, appears gray, translucent colonies after 3-5day incubation and may extend to 7 days [3,4].

H. pylori is the most common bacterial infection of human stomach and its cover approximately 50% of the world population with a higher percentage of *H. pylori* infection in developing countries especially in adults [5].

H. pylori infection is a major cause of more than 80% of chronic active gastritis and other gastroduodenal diseases as gastritis, (MALT)lymphoma, or gastric adenocarcinoma [6]. This bacterium involved in the pathogenesis of several extra gastric diseases, as gastroesophageal reflux disease, anemia, skin disease and rheumatologic condition [7].

There are several diagnostic methods for *H. pylori* detection as PCR, urease test, cytology, histopathology, electron microscopy [8], but the most Specific diagnostic method is the microbiological cultural method [9,10].

There is a relationship between the antimicrobial resistance and the treatment failure for *H. pylori* infection, so the different susceptibility test methods have been used to determine the antimicrobial resistance for H. pylori to overcome the treatment failure [11,12,13].

Materials and methods:

1. Samples:

A total number of 75 stool samples from human with male to female ratio (44 male and 31female) with age 7-56 years' old who suffering from diarrhea and healthy patients as well as 25 from dogs and 20 from cats in AL-ghareeb pets in Ismailia government. All stool samples were collected in clean, sterile and dry plastic bags and immediately transferred to the laboratory with ice box within 2 hrs.

2. Isolation of bacterium:

A part of collected stool samples was cultured into sterile plastic tubes contain enriched transport medium to support the bacterial growth. The enriched sample was added to Columbia agar medium in sterile petri dishes, were incubated at 37ºC for 3-7 days under microaerophilic conditions (5% O2, 13% CO2, and 85%N) with an anaerobic jar system with high degree of humidity was obtained by placing a wet paper towel [14,15]. The growing colonies was identified from the third day, appear as small (1 mm or less in diameter), clear, translucent colonies [16]. The

suspected growing colonies were tested for *H. pylori* based on the colony morphology and positive biochemical tests of urease, oxidase and catalase with negative Gram-stain [17.18].

3. Antibiotic sensitivity test:

In this study, eleven Antibiotic discs were used as shown in table 4. The antibiotics are amoxicillin/ clavulanic acid, trimethoprim sulphamethe, ceftazidime, ofloxacin, doxycycline, tobramycin, nitrofurantoin, levofloxacin, impenem, bacitracin and erythromycin

The test performed on Mueller Hinton agar media (Oxoid) [19]

The bacteria were structed on Mueller-Hinton agar using sterilized cotton swabs and the disc were spread over the agar surface with sterile forceps. The discs were pressed gently into the Muller Hinton agar to exactly complete contact with the agar. The results were recorded by measuring the clear zone of inhibition after incubation at 28°c for 18-24h.and the results were interpreted according to (CLSI 2007) [20].

4. Molecular identification by PCR: DNA extraction.:

DNA extraction was done by using the QI Amp DNA Mini kit. 200 µl of the sample suspension was incubated with 10 µl proteinase K and 200 µl lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 mmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

5. Sequencing of H. pylori genus –specific 16S rRNA gene:

PCR products were purified using QIA quick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST (Basic Local Alignment Search Tool) **[21]** was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the Meg 6 module **[22]** and Phylogenetic analyses was performed using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 [23].

Table (1): *Number of stool samples from pet animal's owners from different ages and sexes (healthy and induced diarrhea):*

Human	Age	Induced diarrhea	Healthy	Total
Man	27-56	15	25	40
Women	Over40	8	19	27
Children	7-14	3	5	8
Total		26	49	75

Table (2): *Number of stool samples from different cases of dogs' breed:*

Dogs Breed	*Cases*	*Age (year)*
-German	8	1-6
-Husky	7	1-4
-Golden	7	2-5
-Gerson	3	1-7
Total	**25**	

Table (3): *Number of stool samples from different cases of cat's breed:*

Cats Breed	Cases	Age (year)
-Egyptian Mau	10	1-8
-Siames	4	1-3
-Cherazy	6	1-5
Total	20	

Table (4): *antibiotic discs used in antibiotic sensitivity test:*

Antibiotic	Antibiotic group	Symbol
Amoxicillin / Clavulanic acid	Penicillin	AMC (30Mg)
Trimethoprim sulphamethe	Diamino pyrimidine	SXT (25mg)
Ceftazidime	Cephalosporins	CAZ (30mg)
Ofloxacin	Quinolones	OFX (5Mg)
Doxycycline	Tetracyclines	DO (30Mg)
Tobramycin	Aminoglycosides	TOB (10Mg)
Nitrofurantoin	Nitrofurans	F (300Mg)
Levofloxacin	Fluoroquinolones	Lev (5mg)
Imipenem	B-lactam	IPM (10mg)
Bacitracin	Cyclic peptides	BA (0.04IU)
Erythromycin	Macrolides	E (15Mg)

Results:

The suspected growing colonies was identified as *H. pylori* were based on the colony morphology (small, flat whitish gray translucent colonies) Gram negative, and positive catalase, oxidase, and urease test.

In this study, A total of 49 stool samples from the related healthy person(25 men, 19 women, and 5 children) were positive *H. pylori* 14 with incidence 28.5% (8 men, 5 women, and 1 children).

A total of 26 stool samples from related persons with induced diarrhea (15 men, 8 women, and 3 children) were positive *H. pylori* 17 with incidence 65.3% (10 men, 6 women, and 1 children).

A total number of 75 stool samples from the healthy and diseased related persons (40 men, 27 women, and 8 children) were positive *H. pylori* 31(18 men, 11 women, and 2 children) with incidence 41.3% (45% men, 40.74%women, and 25% children) (table 5).

Table 6 show that a total of 25 stool samples from dogs from different breeds were positive *H. pylori* 9 with incidence of 36%.

Table 7 show that a total of 20 stool samples from cats from different breeds were positive *H. pylori* 4 with incidence of 20%.

Antibiotic sensitivity test:

The determination of antibiotic sensitivity was measured by the clear zone of inhibition which produced by the antimicrobial diffusion from the discs into the surrounding medium [24].

This study revealed the antimicrobial sensitivity rate of *H. pylori* in human, dog, and cat. The most sensitive antibiotic is imipenem were recorded 100% sensitive in all. tobramycin was recorded 92%, 100%, and 100% respectively. ofloxacin were recoded 68%, 67%, and 100% respectively. trimethoprim sulphate was recorded 32%, 67%, and 50% respectively. levofloxacin was recorded 76%, 0%, and 50% respectively as shown in (table 8).

PCR results:

In this study *H. pylori* was detected by 16S rRNA gene of *H. pylori* is 100%.

The nucleotide sequences of *H. pylori* targeting16S r RNA from the human isolates. The sequence indicated that the isolate from human was similar to species Helicobacter pylori strain LVRN-53 strain with accession number MT477178 by percentage 100 %. The Gen Bank data base was deposited the isolate by Helicobacter pylori H7-MHSH with accession number (MW839587). The phylogenetic relationship for this experimental isolate and the closely related relatives were analyzed as shown in Figure (5).

The sequence indicated that the isolate from dog was similar to species Helicobacter pylori strain LVM-53strain with accession number MT477177 by percentage 99.71%. The Gen Bank data base was named isolate by *Helicobacter pylori* H4-MHSH with accession number (MW839451). The phylogenetic relationship for this experimental isolate and the closely related relatives were analyzed as shown in Figure (6).

Figure (1): *H. pylori* colonies on Columbia blood agar (pinhead-sized, whitish grey, and small translucent.

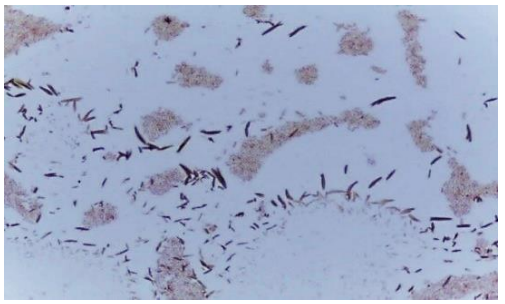

Figure *(2):* *Gram stain (H. pylori is Gram negative bacterium which show spiral shaped bacilli).*

Table (5): *Frequency of H. pylori among studied human cases using culture (healthy and induced diarrhea cases):*

Human cases	Healthy	Induced Diarrhea	Total	Results		
				***H. pylori* isolates from stool**		
				Healthy	Induced diarrhea	Total
Men	**25**	**15**	**40**	**8**	**10**	**18(45%)**
Women	**19**	**8**	**27**	**5**	**6**	**11(40.74%)**
Children	**5**	**3**	**8**	**1**	**1**	**2(25%)**
Total	**49**	**26**	**75**	**14(28.5%)**	**17(65.3%)**	**31(41.3%)**

Table (6): *Frequency of H. pylori isolates among dog breed cases using culture:*

Dogs Breed	Cases	*H. pylori* isolates from stool
-German	**8**	**2**
-Husky	**7**	**4**
-Golden	**7**	**2**
-Gerson	**3**	**1**
Total	**25**	**9(36%)**

Table (7): *Frequency of H. pylori isolates among cat breed cases using culture:*

Cat Breeds	Cases	*H. pylori* isolates from stool
-Egyptian Mau	**10**	**3**
-Siames	**4**	**1**
-Cherazy	**6**	**-**
Total	**20**	**4 (20%)**

Table (8): *Frequency of sensitivity rate of antibiotics in human, dog, and cat:*

Sensitivity Rate			
Antibiotics	**Human**	**Dog**	**Cat**
Tobramycin (10mcg)	92%	100%	100%
Trimethoprim SXT (25mg)	32%	67%	50%
Ceftazidime CZ (30mg)	0%	0%	0%
Doxycycline DO (10Mg)	32%	33%	0%
Amoxicillin (clavulanic cid) AMC (30Mg)	12%	0%	0%
Ofloxacin OFT(5Mg)	68%	67%	100%
Nitrofurantoin (300Mg)	56%	33%	50%
Levofloxacin Lev (5mg)	76%	0%	50%
Imipenem IPM (10mg)	100%	100%	100%
Bacitracin BA(0.04IU)	8%	0%	0%
Erythromycin E(15Mg)	0%	0%	0%

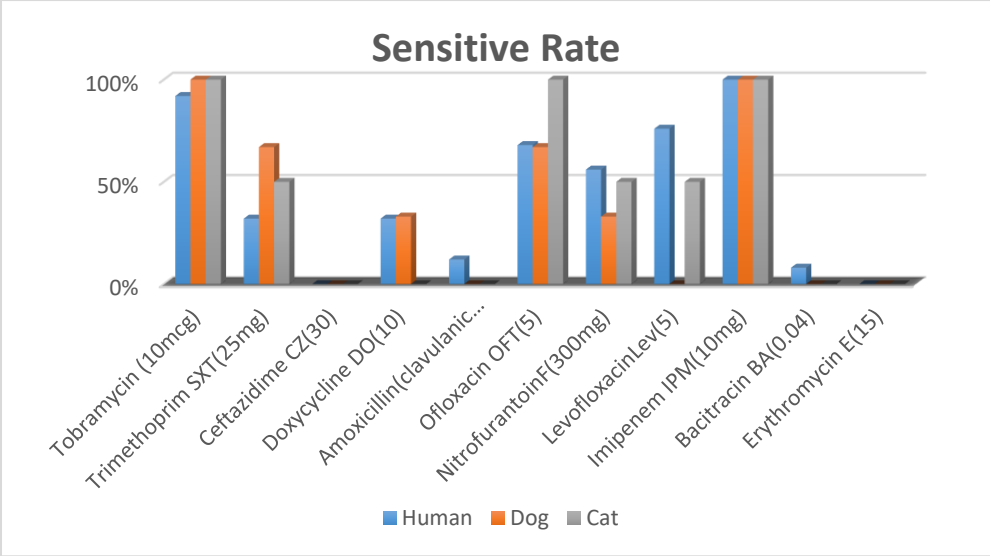

Figure (3): Frequency of sensitivity rate of antibiotics in human, dog, and cat:

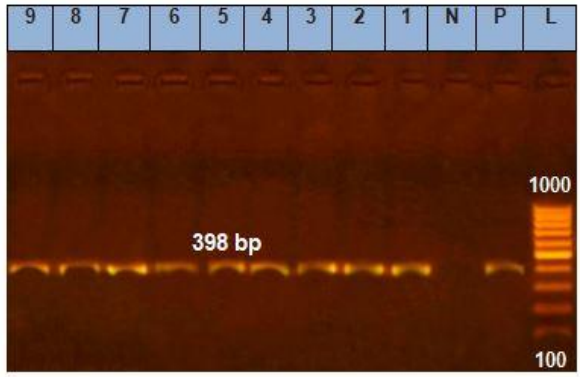

Figure (4): 16S rRNA nucleotide sequence obtained from Helicobacter pylori H7-MHSH

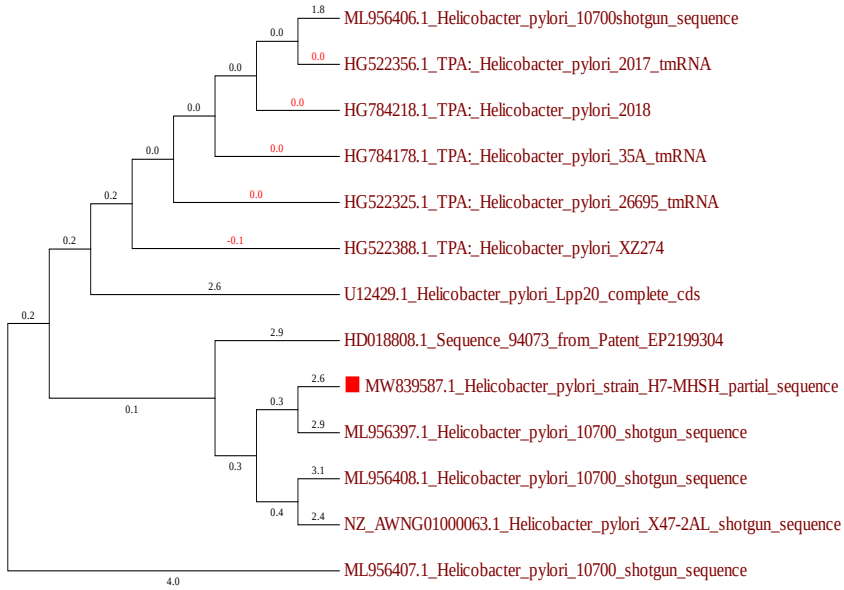

Figure (5): The Phylogenetic tree of Helicobacter pylori H7-MHSH based on partial sequencing of 16S rRNA

Figure (6): The Phylogenetic tree of *Helicobacter pylori H4-MHSH* based on partial sequencing of 16s

Discussion:

This study revealed that dogs may play an important role in *H. pylori* transmission to humans [25,26]. The prevalence rate of *H. pylori* infection is higher in developing countries (50-70%) than in developed countries (30%) [27]. Poor hygienic conditions and close contact with stray animals may increase the prevalence of *H. pylori* infection [28].

In the present study, among 75 stool specimens were collected

from human suffering from hematemesis, dyspepsia, vomiting, nausea, and abdominal pain, *H. pylori* were diagnosed in 31 cases (41.3%) by culture. This result agrees with previous studies of [29]; who found *H. pylori* were positive in 53.1%; and disagrees with [30] who recorded 22%. A higher incidence rate of 70% [31].

In the present study, among 25 stool sample of dog, the results were found positive to *H. pylori* in 9 cases (36%). This result disagreed with the previous result of [32] who recorded that the prevalence of *H. pylori* infection in dog is high and reach 100% [33]; [34] revealed that dogs may play an important role in *H. pylori* transmission to humans.

The result in table showed that antibiotic susceptibility of *H. pylori* isolates to different antimicrobial discs are tobramycin, trimethoprim sulphate, ceftazidime, doxycycline, amoxicillin, ofloxacin, nitrofurantoin, levofloxacin, imipenem, bacitracin, and erythromycin.

In this study, the average rate of resistance to levofloxacin in both human, dog and cat was 41.3%. This result is disagreed with the previous result of [35] who recorded 16.7%. This result is agreed with the previous result of [36] who recorded 32.7%.

In the obtained data, the average rate of resistance to amoxicillin which is a proton pump inhibitor (PPIs) was 92% This result is agreed with the previous result of [37] and not agreed with [38] who recorded 33.3%. Also, the average rate of resistance to doxycycline is 46.6%. This result is disagreed with the previous result of [39] who recoded 9.20%.

In this study, the higher prevalence of *H. pylori* infection is 100% by PCR. this result is agreed with [40] who

showed that higher rate of *H. pylori* infection by using PCR (76.6%).

In this study, showed that imipenem, tobramycin, and ofloxacin are the best commonly used antimicrobial agents for *H. pylori* where, the average sensitivity rate in each human, cat, and dog are 100%, 97.3%, and 78.3% respectively. This result is disagreed with [41] who is showed that amoxicillin is the one of the most commonly used antimicrobial agents, although a higher percentage of isolates was resistant to amoxicillin.

In conclusion, *H. pylori* can be detected by high prevalence among human stool samples suffering from GIT disturbances especially, human males than females and increase with age. PCR is highly sensitive technique for *H. pylori* detection and

recorded higher detection rates of *H. pylori*.

Author contribution

Most authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Heba Ibrahim, Shymaa Enany, and Mahmoud Kelany. The main manuscript was written by Heba Ibrahim and Shymaa Enany and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

This study was approved by the Suez Canal University, Egypt ethical board. Permission and informed consent to collect stool samples were obtained from dog and cat from Al- ghareeb pets and their human owners in Ismailia Governorate, Egypt. Informed consent was obtained from all individual participants included in the study. All procedures performed in the study involving animals were in accordance with the ethical standard of the Suez Canal University, Egypt ethical board.

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التوصيف الجزيئي للميكروب الحلزوني في الحيوانات المنزليه والانسان في مصر- في محافظه الاسماعيلية

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الملخص العربي

فى هذه الدراسه: تم عزل الميكروب الحلزونى من 75 عينه من البراز من الانسان و25 عينه من الكلاب و20 عينه من القطط بمحافظه الاسماعيليه وقد تم فحص العينات لمعرفه وتشخيص تواجد الميكروب الحلزونى باستخدام العزل وطرق التصنيف البيوكيميائيه وقد اظهرت النتائج وجود الميكروب الحلزونى بنسبه (41.3%) من الانسان و (36.00%) من الكلاب و (20.00%)من القطط

وقد اثبتت اختبار الحساسيه للمضادات الحيويه المختلفه والذي اجري عمليا علي ميكروب الحلزونيه البوابيه في الانسان والكلاب والقطط ان نسبه المقاومه للسفتاذيديم (100%و0100%و 100%) و الباكيتراسين (88%و100%و100%) و الاريثرومايسن (80%و100%و100%) و الاموكسيسيلين (76%و100%و100%) و الترايمثوبريم 48%و50%و50%) والنيتروفورانتوين (44%و50%و50%) والدوكسيسيكلين (40%و50%و50%) و الليفوفلوكساسين (24%و50%و50%) و الافلوكساسين (16%و0%و0%) و التوبراميسين (8%و0%و0%) و الامبينيم (0% و0%و0%) علي التوالي وقد وجد ان الامبيينم والتوبراميسين واالافلوكساسين من افضل المضادات الحيويه المستخدمه بشكل شائع لعلاج الميكروب الحلزونى . تم اجراء اختبار تفاعل البلمره المتسلسل للتاكد من تواجد الحمض النووى الخاص بالميكروب الحلزونى فى تسعه معزولات المفحوصه باستخدام جين (16S rRNA) وقد تم تواجده بنسبه (100%) فى جميع المعزولات وايضا تم اختبار التتابع الجينى للجينات فى عينتين من العطرات وقد تم مطابقه العينه الاولى ومسجله سابقا ومشابهه للعطره (LVRN-53) برقم (MT477178) بنسبه(100%) وتم تسجيلها ببنك الجينات برقم (MW839587) والعينه الاخرى تم مطابقتها بالعطره (LVM-53) برقم (MT477177) بنسبه (99.71%) وتم تسجيلها ببنك الجينات برقم (MW839451).