Detection of *Helicobacter equorum* in Equine in Egypt

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1. Abstract

Helicobacter pylori, previously known as *Campylobacter pylori*, is a gram-negative, microaerophilic, spiral (helical), multi-flagellated bacterium usually found in the stomach, horses, calves, pigs, rabbits, and chickens were evaluated for *Helicobacter* presence, and the pathogenetic effect on their gastric mucosa. Therefore in our study, fifty gastric lavage and fecal samples were collected from fifty diseased horses and application of Lateral Flow and PCR assays for detection of *Helicobacter* species. Positive results confirmed only by PCR which revealed that twelve samples were positive among the examined samples (24%), sequencing of *16s rRNA* gene amplicon shared 100% identity with *H.equorum* on GenBank, young foals show higher prevalence than adult horses. Occasionaly, *H.equorum* act as a potential zoonotic risk hazard thus, further studies are needed to investigate the zoonotic pathway to the human contacts.

Key words: *Helicobacter* species, horse, gastritis, PCR, 16S rRNA sequencing, *H.equorum*

2. Introduction

Equine gastric ulcer syndrome (EGUS) is Characterized by ulceration in the terminal esophagus, proximal (squamous) stomach, distal (glandular) stomach, and proximal duodenum [1, 2]. *Helicobacter* spp (other than *H. pylori*) have been isolated from humans and a variety of animals suffering from gastric ulcers and gastritis [3].

The incidence of gastric ulcers in racehorses in active training has been shown to exceed 90% in some circumstances, surprisingly, in horses, where gastritis and gastric ulcers are very common, there exist very few reports of Helicobacter infection in the gastric mucosa [4].

The genus *Helicobacter* belongs to the Ɛ-subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. This family also includes the genera *Wolinella*, *Flexispira*, *Sulfurimonas*, *Thiomicrospira*, and *Thiovulum* [5].

Several non-*pylori helicobacter* species have been isolated from diverse animal's species such as *H. marmotae* in Prairie dogs [6] *H. cinaedi* and *H. macacae* in Rhesus monkeys which associated with intestinal adenocarcinoma [7]**,** *Helicobacter magdeburgensis* in mice [8], *H. suis* in pig's stomach [9].

The potential zoonotic pathway for the transmission of *Helicobacter* spp. and epidemiology of this genus, deserve more attention to these emerging pathogens [5]. One of the theories is transmission via raw uncooked milk from animals to humans that **Turutoglu*et al.*,** have investigated the presence of *H. pylori* in sheep milk [10].

Recently, in **2015, Abdel-Moein *et al.*,** suggested that the occurrence of *H. pylori* in fish may make such species a direct

source for human infection through handling during cleaning of fish (hand to mouth transmission), a matter which was supported by the results that (61.1%) were positive from 18 examined fish handlers [11].

Next to the previous study**, Aya *et al.*,** reported that sheep were proved to be significant reservoir host for multiple species of *Helicobacter* such as *H.canis, H.winghamensis, H.canadensis, H.equorum and H.bovis* and sheep could be considered a potential source of human infection with these micro-organism, in addition,*H.canis* and *H.bovis* were detected in goats and cattles respectively [12].

Both invasive and non-invasive tests are available for diagnosis of *Helicobacter*. Invasive tests which require endoscopy for diagnostic or therapeutic evaluation and include: gastric biopsies for culture, Gram stain, histology or rapid urease test (RUT). While the noninvasive tests available in clinical practice include serologic tests, ^{13}C-Urea Testing, and stool antigen tests (HpSA) [13].

Molecular diagnosis of *H. pylori* is achieved mainly by PCR, The choice of a target is important in designing the primers which must be specific for *H. pylori* but conserved in all strains of the species [14]**.**

Although several risk factors for the development of gastric ulcers have been widely studied, investigation of microbiological factors has been limited.

H. equorum was isolated from faecal samples of two clinically healthy horses. The organism is a Gram-negative, slightly curved rod which is positive for oxidase, catalase and alkaline phosphatase activity, but negative for urease, indoxylacetate esterase and glutamyltranspeptidase activity. Cells carry a single, unipolar sheathed flagellum [15].

Although the *16S rRNA*gene sequence is an essential part of the description of a novel organism, for many noncultured bacteria it may be the only taxonomic description [16]. So that, many researchers have tended to use genetic criteria including virulence gene and *16S rRNA*gene sequencing for distinguishing it from other curved Gram-negative rods [17].

Aim of the study: Detection of *Helicobacter* spp. in gastritis cases in horses in Egypt, identification to the species level.

3. Materials and Methods

3.1 Samples

This study was carried out on fifty diseased horses (30 foals and 20 adults) suffering from various degrees of abdominal colicky pain and samples were taken during episodes of colic. These horses were selected from the clinic of the Faculty of Veterinary Medicine, Cairo University and also from different farms for breeding Arabian horses in Cairo.

3.1.1. Gastric lavage samples

Horses are unable to vomit due to anatomy of gastrointestinal tract [18], hence nasogastric intubation was performed in all horses taking part in the study to obtain gastric content, refluxes obtained was put in sterile containers.

3.1.2. Fecal samples

Fecal samples were collected directly from rectum from each horse in clean, dry, waterproof containers containing no detergents, preservatives or transport media [19].

All samples were transferred directly in ice box to the laboratory.

3.2. Lateral flow immunoassay

Lateral flow immunoassay (LF) was used as a screening test for detection of *H. pylori* antigen in gastric lavage and fecal samples by using monoclonal anti-bodies dependant kit. This test was previously recommended by many researchers and showed high sensitivities and specificities [20, 21].Antigen of H. pylori was extracted from all collected fecal samples using specific extraction buffer (supplied with the used kit). The extracts then were screened for the

presence of *H.pylori* antigen using Onsite *H. pylori* antigen rapid test-cassette (Biotest, Germany). The procedure was carried out according to the manufacturer directions.

3.3 Polymerase chain reaction (PCR)

3.3.1. DNA extraction

DNA was extracted from 200 mg of each fecal sample using QIAamp DNA Stool Mini Kit (Qiagen, Germany) While DNA was extracted from 200 µl of each lavage sample using QIAamp DNA Mini Kit for body fluids (Qiagen, Germany) relying on the kits instructions, DNA extractions were frozen at-20^{0}C until further analysis[19].

3.3.2. Primers and PCR amplification condition

According to **Moyaert*et al.,*** the PCR reaction was conducted using *Helicobacter*species specific primer for *16S rRNA* gene [22] (table.1).

All PCR reactions were performed in a 25 µl volume, the reaction mixture contained 12.5 µl Master Mix (Takara Biotechnology, Japan), 10 pmol/µl of each forward and reverse primers, 5.5 µl nuclease free water, and 5 µl of extracted DNA under the conditions for amplification as following: one initial denaturation cycle at 95ºC for 5 min; 45 cycles of 30second denaturation at 95ºC, 30 second annealing at 65ºC, and30 second elongation at 72ºC; and a final extension cycle of 10 min at 72ºC. All PCR products were subjected to electrophoresis in an agarose gel and visualized [23]. Bands were scored as positive when they had the diagnostic size of 764bp. *H. pylori* (ATCC #51110) used as a positive controls and DNase/RNase-free water as negative controls.

3.4. Sequencing

One amplicon for *16S rRNA* gene (764bp) was chosen for sequencing and purified using QIAquick gel extraction kit (Qiagen, Valencia).

A purified PCR product was sequenced in the forward and reverse directions on an automated DNA sequencer (Applied Biosystems 3130 genetic analyzer, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City), then purification of the sequence reaction using Centrisep (spin column) ,all the previous reactions was done according to the instructions of the manufacture, after that The purified sample was loaded in the sequencer machine [24].

3.5. Phylogenetic analysis:

A BLAST® (Basic Local Alignment Search Tool) analysis was initially performed to establish sequence identity to GenBank accessions [25].

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by [26] and Phylogenetic analyses were done using maximum likelihood, neighbour joining and maximum parsimony in MEGA X software [27].

4. Results

4.1. Lateral flow immunoassay:

Unexpectedly, all (LF) tests were negative for both samples gastric lavages and faeces.

4.2. Polymerase chain reaction (PCR):

Out of the 50 examined fecal samples, 12 (24%) were positive for *Helicobacter*species and all were detected in foal's samples (table.2 and fig.1).The results were obtained by PCR after using *16S rRNA* gene (fig.2).

4.3. Sequencing and Phylogenetic analysis**:**

The sequence shared 100% identity to *Helicobacter equorum* (fig.3). The *16S rRNA* gene sequence were deposited in GenBank under the accession numbers (MT007125).

5. Discussion

Approximately half of the world's population is infected with *H. pylori*, and the majority of colonized individuals develop coexisting chronic inflammation. In most persons, *H. pylori* colonization does not cause any symptoms [28] that discovery increased interest to other spiral bacteria that had been seen in many animal species most of these bacteria belong to the genus *Helicobacter* [29].

In the present 12 samples were positive for *Helicobacter* spp. from total 50 examined horses by PCR;making positivity (24%) all of these horses were suffering from gastrointestinal diseases. This confirms the colonization of *Helicobacter* spp. in horse's gastric mucosa [30, 31] and after sequencing of 16S rRNA gene (764bp) amplicon shared 100% identity with *H.equorum.*

Gastric ulcers are common in horses resulting in decreased performance and economic losses to the industry and caused by many factors, infection with *Helicobacter* species is one of them [32] but the clinic relevance of this genus on EGUS has not yet been demonstrated [33].

Although lateral flow test is specific for detection of *H.pylori* [34] all samples were negative, this may be due to absence of that species of genus *Helicobacter* from the examined horse's samples.

But in our study, our positive results were obtained by PCR technique through detection of 16S rRNA specific gene for *Helicobacter* species. Therefore, the use of PCR has an optimal sensitivity and specificity (100%) [35 and 36] when compared to Lateral Flow test for the detection of *Helicobacter* spp.in clinical samples in horse

Unfortunately, in this study, no positive results obtained from gastric lavage samples neither by LF test for Ag detection nor by PCR, indicating that the biopsies in the present study contained no bacteria, the possible reasons for that may be due to, that *Helicobater* spp. colonizes the gastric antrum in particular [36], where few acid-secretory parietal cells are present thus the used technique could not reach to that region of stomach.

Or may be due to the theory that, after *H. pylori* enters the host stomach, and utilizes its urease activity to neutralize the hostile acidic condition ,it moves through flagella toward host gastric epithelium cells, followed by specific interactions between bacterial adhesins with host cell receptors, thus leads to successful colonization and persistent infection [37],so taking tissue sample from the tip of the epithelium of the basal membrane of gastric sample or from the ulcers is recommended through biopsy or necropsy [38] **.**

In view of the faecal origin and the lack of urease activity, one can assume that *H. equorum* is associated with the intestines rather than with the stomach [19] further research is needed to determine the natural habitat and the clinical significance of *H. equorum.*

Amazingly, in the current study, foals showed higher prevalence than adult horses in detection of *Helicobacter* spp. (table.1).The cause of This may be due to the active and mature immunity in adults which can overcome the infection, thus they acquired the pathogen and persists lifelong in most infected subjects and so harbour low and subdetectable levels of helicobacters in their gut which shed in low numbers and may be missed [39].

The *16S rRNA* gene is universal in bacteria, and relationships can be measured among all bacteria thus, the comparison of the *16S rRNA* gene sequences allows differentiation between organisms at the genus level, in addition to classifying strains at multiple levels, including the species and subspecies level**s** [40] and also provides accurate, more rapid, unambiguous identification of the difficult bacterial isolates than did convential methods that can clarify their clinical importance [41].

Our finding may spotlight the potential zoonotic pathway for *Helicobacter* among horses and humans as it can affect equines

and this has been reported in many previous articles [19, 38, 42, and 43] as they could find *H.equorum* DNA in either privately owned, riding school or hospitalized horses. On the other hand, *H.equorum* like bacterium was reported in Japan [44] in a human with X-linkedagammaglobulinemia who had refractory chronic pleurisy indicating that the pathogen can infect humans and act as possible zoonotic pathogen.

Clinical symptoms associated with non-*pylori* helicobacters in humans can be characterized by atypical complaints such as acute or chronic epigastric pain and nausea. Other aspecific symptoms include hematemesis, recurrent dyspepsia, irregular defecation frequency and consistency, vomiting, heartburn, and dysphagia, often accompanied by a decreased appetite [33, 45, and 46] some people infected with non-*pylori* helicobacters do not present obvious clinical signs [47].

Inspection of the gastric mucosa of people infected with long spiral bacteria through endoscopy reveals a variety of lesions, ranging from a normal to slightly hyperemic mucosa to mucosal edema and to multiple erosions and ulcerations in the antrum or in the duodenum [48, 49].

6. Conclusion

It is noteworthy that our findings may have useful public health implications, as the occurrence of *Helicobacter* in horse may make such species a direct source for human infection.

7. References

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Table 1
Primers for PCR detection [22]

Primer name and direction	Nucleotide sequence (5" to 3")	Amplicon (bp)
16S rRNA for *Helicobacter*spp		
Forward	5-GGCTATGACGGGTATCCGGC-3	764
Reverse	5-GCCGTGCAGCACCTGTTTTC-3	

Table 2
Occurrence of *Helicobacter* spp. in the examined fecal samples by PCR

Horses	Number of horses	Number of positive
Adult horses	20	0
Foals	30	12
Total	50	12

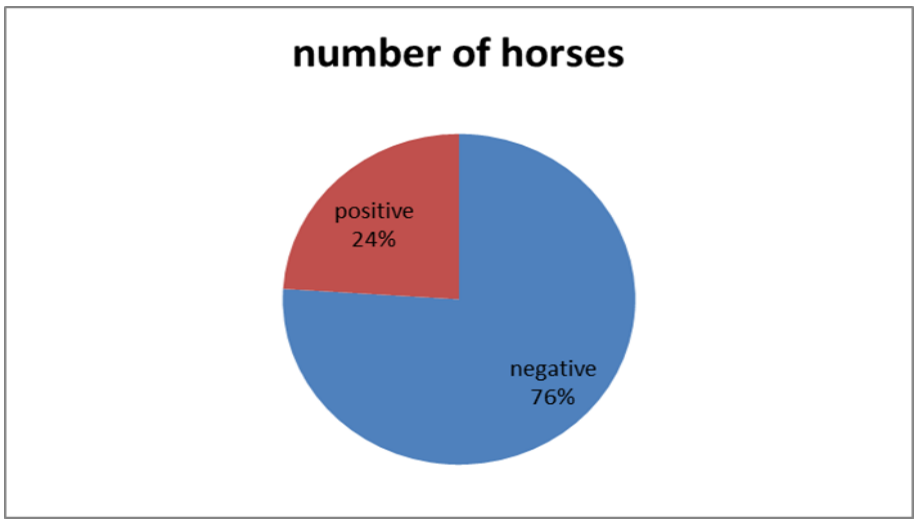

Fig .1. Rate of positive and negative PCR results.

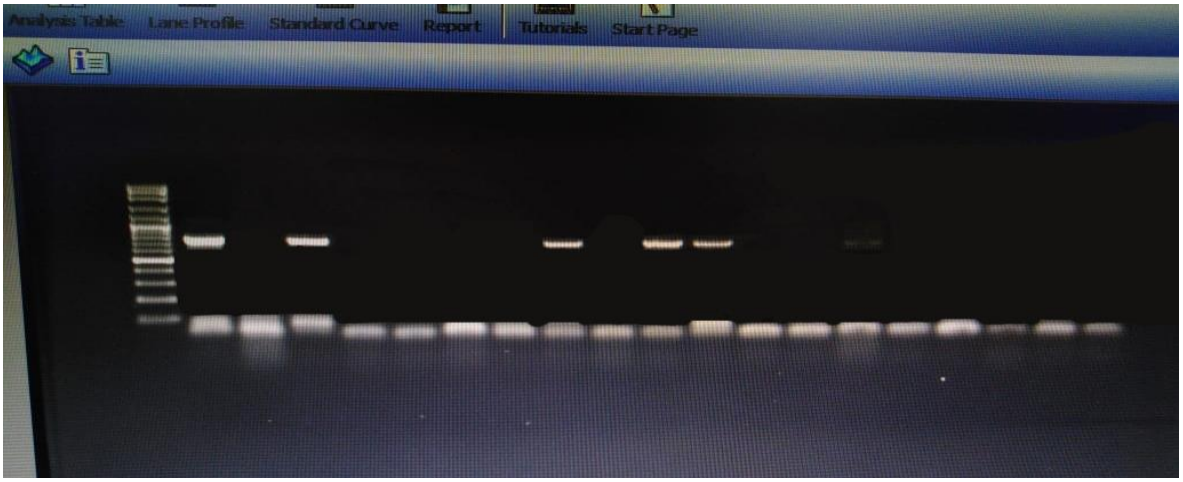

Fig.2. Electrophoretic profile of PCR for *Helicobacter* species. Lane 1:3000 bp marker, lane 2: positive control, lane 3: negative control, lane 4, 9, 11, 12, 15: positive sample show specific band at 764 bp, the other lanes: negative samples.

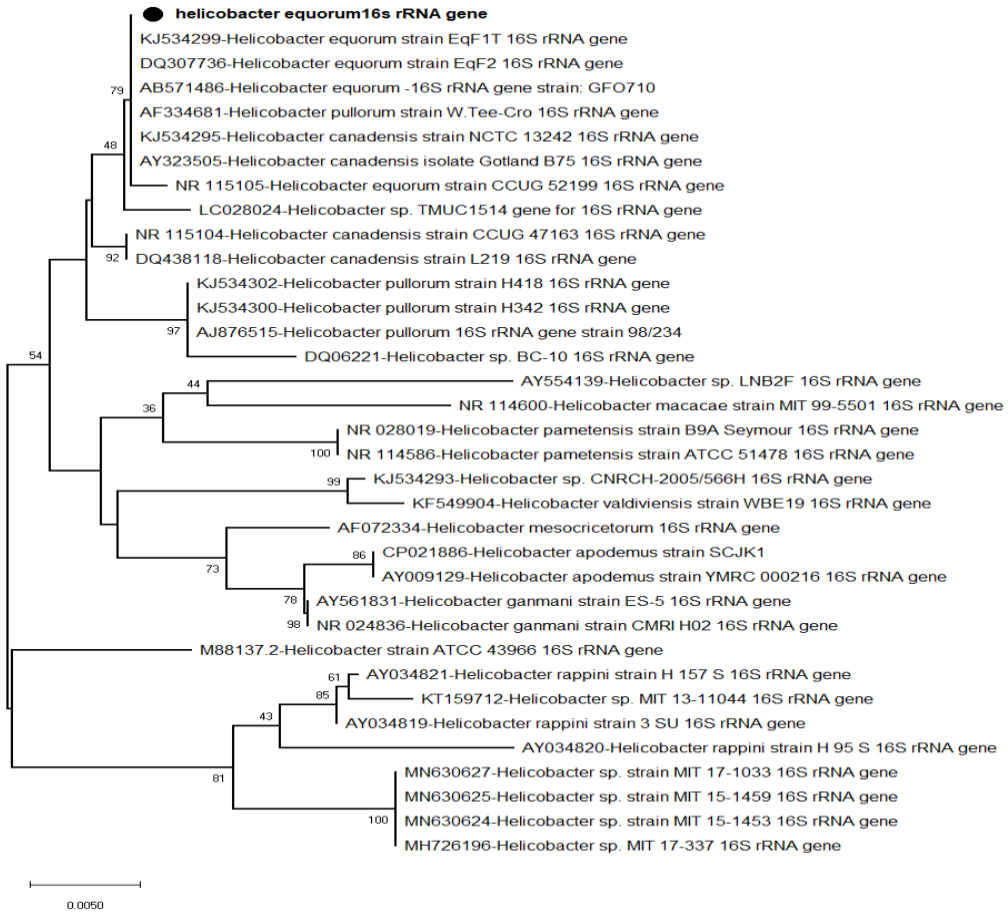

Fig.3. Phylogenetic tree for partial sequence of *16s rRNA* gene for *Helicobacter* species which was done by MEGA 6 software.