RESEARCH ARTICLE

Sequencing and Phylogenetic Analysis of *Helicobacter pylori* through 16S rRNA Gene Isolated from Gastritis Biopsies

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Abstract

Analysis of *16S rRNA* gene sequences is one of the most common methods for investigating the phylogeny and taxonomy of bacteria. The aim of this study is to compare Iraqi *H. pylori* isolates with isolates from different geographical locations. Total of 45 isolates from males and females with gastritis in Samawah and Diwaniya provinces were used to amplify the *16S rRNA* gene by PCR then followed by DNA sequencing. The *H. pylori 16S rRNA* gene was amplified (1471 bp in size) and sequencing. The target sequence has been deposited in the National Center for Biotechnology Information (NCBI) under the accession Number (KP893893). Homology sequence analysis showed high similarity between our sequence with *H. acinonychis* and *H. cetorum* (98%) and the lowest similarity between *H. pylori* and *Microcystis aeruginosa* (51%). The phylogenetic analysis displayed high correlation between *H. pylori* and *H. acinonychis* (a species infected cheetahs and other big cats) at bootstrap value 80%. The cluster containing these two species is nearest from the African out group root *M. aeruginosa*. This study provided an additional insight into the profile of our *16S rRNA* gene and indicates that the Iraqi *H. pylori* isolates are closely related to a South African isolate.

Keywords; *Helicobacter. Pylori*, 16S rRNA, gastritis, phylogenetic, sequencing.

Introduction

Helicobacter pylori are responsible for most duodenal and peptic ulcer and also play an important role in gastric adenocarcinoma (Atherton et al, 2006) (Brenner et al, 2004). The mechanism of *H. pylori* path-ogenic effect is unclear, but it is believed to be related to complex host bacterial interactions triggered by virulence genes (Amieva and El-Omar, 2008) and it is possible that these effects are enhanced by the invasiveness of the bacterium (Nec-chi et al, 2007). On the other hand, *H. pylori* were recently observed within gastric mucosa capillaries, where it appears to establish close association with erythrocytes (Aspholm et al, 2006). DNA sequence analysis of house-keeping and virulence associated genes all have illustrated the unusually high degree of genetic variability in this species (Falush et al, 2003). The molecular tests presently available for diagnosis, including those targeting *16S rRNA* genes, are focused on *H. pylori* and considered as specific targets to confirm *H. pylori* infection, and positive amplification of *H. pylori* specific DNA may be considered as a direct evidence of the presence of the pathogen (Hoshina et al, 1990; Chong et al, 1996). This ribosomal gene is particular in that it is present in all bacteria while, at the same time, it comprises nucleotide sequences that are specific to a given bacterial genus (Smith et al, 2004). Sequence analysis of the *16S rRNA* gene has led to our current understanding of prokaryotic phylogeny and *H. pylori 16S rRNA* gene sequence analysis unambig-uously differentiated the Helicobacter genus from the closely related Campylobacter genus (Gorkie-

wicz et al, 2003) thus allowing creation of the Helicobacter genus. Here, we sequenced the *16S rRNA* genes of *H. pylori* isolated from biopsy samples. By matching this sequence with those available in the National Center for Biotechnology Information (NCBI) nucleotide database, we defined sequences that are homologous among other Helicobacter genus and then study the evolutionary aspects using phylogenetic tree.

Materials and methods

Collection of Samples

A total of 45 biopsy samples were collected from the greater curve of the gastric antrum and duodenum. The samples were collected for the period of October 2013 to March 2014 from Al-Hussien Teaching Hospital in Samawah and Diwaniya provinces. The samples were prepared for bacteriological and molecular conformation at the Biology department/ College of Science/Muthanna University.

Isolation and Identification of *H. p ylori*.

Several different solid media were used for culture of *H. pylori* from biopsies. Among the more common ones are Brain Heart Infusion (BHI) agar, Brucella agar, and human blood agar plates. The media are always supplemented with 3 antibiotics, which are Nystatin, Vancomycin, and Nalidixic acid. The culture incubated at 37°C for 3 to 10 days under a 9% CO2 atmosphere (Saini et al, 2011). The bacterial colonies were identified by visual inspection for grey, translucent, and pinpoint colonies using light microscopic. Subsequent biochemical tests including (oxidase, catalase, and urease) were performed from the primary growth; single colonies were propagated in blood agar for an additional 48hrs (MacFaddin, 2000; Monica, 2006).

Gastric Biopsy Specimens

Gastric biopsy specimens ten were collected from the stomach antrum or corpus. The specimens were used for *H. pylori* extraction of total genomic DNA for PCR purposes.

Bacterial Genomic DNA Extraction and Molecular Detection

Bacterial genomic DNA was extracted from ten stomach biopsy samples (100mg/ml) using the manufactured genomic DNA extraction kit (Geneaid. USA).

The *16S rRNA* gene was amplified by PCR. The gene's primers were designed using NCBI Gene-Bank data base (Table 1).

Primer sequence	
Forward	ATCCTGGCTCAGAGTGAACG
Reverse	GCAGGTTCACCTACGGTTACC

Table 1: *Primers used in this study.*

PCR master mix was prepared according to company instructions using (AccuPower® PCR PreMix Kit). PCR master mix contain DNA template (5µl) at concentrations of (77.5 ng/µl - 163.5 ng/ µl), each of forward and reverse *16S rRNA* gene primer (10pmol) (1.5µl), and PCR water (12µl) for the total of 20µl.

The PCR master mix components that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). All the PCR tubes transferred into Exispin vortex, centrifuge at 3000rpm for 3 minutes, then placed in PCR Thermocycler (Bioneer, Korea). The PCR thermocycler conditions were done by using convention PCR thermocycler system as following; initial denaturation at 95ºC for 5 min repeat 1 cycle ; (denaturation at 95ºC for 30 sec; annealing 58ºC for 30 sec; extension 72ºC for 2 min repeat 30 cycle), final extension 72 for 10 min repeat 1 cycle. The PCR products were analyzed by agarose gel electrophoresis 1.5% at 100 volt for 1hour.

Sequencing Of *H. pylori* 16S rRNA

The amplified *16S rRNA* gene (1471bp in size) was purified from Agarose gel using EZ-10 Spin Column DNA Gel Extraction Kit (Biobasic, CA), and then sequenced using the BigDye Terminator Cycle Sequencing Kit (Bioneer, Korea)

on the ABI PRISM 3100 DNA Sequencer (Bioneer, Korea). The protocol used was essentially identical to these recommended by the kits' manufacturers.

Bioinformatics analysis

Submission of *H. pylori* 16S rRNA Gene Sequence

The *16S rRNA* sequence was submitted in the GenBank nucleotide database for the purpose of registration as a new sequence.

Phylogeny of 16S rRNA

The isolated nucleotide sequence of the *16S rRNA* gene was compared with sequences of other bacterial species deposited on databases using the nucleotide BLAST (Atschul et al, 1997). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using Clustal W2 (McWilliam and Uludag, 2013) by BioEdit software (Hall, 1999). Phylogenetic tree (Maximum Likelihood) of *16S rRNA* genes and their evolutionary relationship with those obtained from database was constructed and phylogenetic tree using Jones-Taylor-Thomton (JTT) model from PHYLIP. The freshwater cyanobacteria (*Microcystis aeruginosa* U40340.2) was used as out-group for the tree. The tree was bootstrap at 1000 replication using SEQBOOT, whereas MEGA4 (Tamura et al, 2007) was used to generate the consensus tree.

Results and Discussion

Molecular Detection of *H. pylori*

The *H. pylori 16S rRNA* gene was amplified and the product size (1471bp in size) was clearly appeared (Figure. 1).

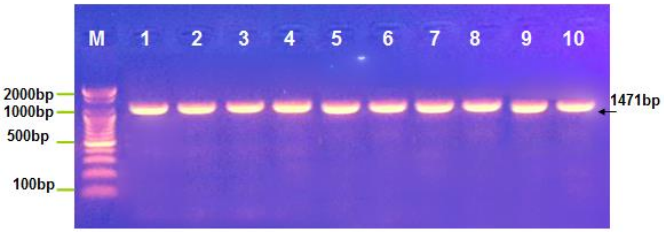

Figure 1: *The PCR product (1471bp in size) of H. pylori 16S rRNA run on 1% Agarose gel for 1 h at 90 V. M; DNA marker (100-2000bp), lane(1-10) positive PCR samples for 16S rRNA.*

Sequencing and Bioinformatics Analysis

The *H. pylori 16S rRNA* gene was sequenced. The cleaned sequenced product was deposited at the NCBI database under the GeneBank accession number KP893893. A total of 17 homologus 16S rRNA sequences were selected based on the degree of sequence similarity with our ribosomal gene (Table 2). The *H .pylori* 16S rRNA sequence was showed a high degree of similarity (98%) with *H. acinonychis*, *H. cetorum* respectively, while the lowest similarity (85%) has been showed with *Microcystis aeruginosa*.

Species scientific name	Sequence ID	Identity
Helicobacter pylori	KP893893	-
Helicobacter acinonychis	NR_074405.1	98%
Helicobacter cetorum	NR_074475.1	98%
Helicobacter bizzozeronii	NR_074392.1	95%
Helicobacter equorum	NR_043707.1	94%
Helicobacter cholecystus	NR_118812.1	94%
Helicobacter cinaedi	NR_074384.1	94%
Helicobacter hepaticus	NR_102911.1	94%
Campylobacter coli	NR_121751.1	86%
Campylobacter lari	NR_074555.1	86%
Campylobacter jejuni	NR_074550.1	86%
Campylobacter concisus	NR_074156.1	86%
Arcobacter butzleri	NR_074573.1	86%
Sulfuricurvum kujiense	NR_074398.1	86%
Sulfurimonas gotlandica	NR_121690.1	85%
Sulfurimonas autotrophica	NR_074451.1	85%
Sulfurospirillum barnesii	NR_102929.1	85%
Microcystis aeruginosa	U40340.2	51%

Table 2: *The homology between H. pylori 16S rRNA and other studied species.*

The MSA showed highly conserved area in all studied species. However there are differences in the type of nucleotides at some places. Our sequence was conserved with *H .acinonchyis*, *H. cetorum. H. bizzozeroni*, and the root *M. aeruginosa* of positions

(125, A1001, T1111, G1307, C1318, and A1433) respectively.

Clearly change was observed at the positions 134 and 406 were nucleotides C and T only located in *H. pylori* 16S rRNA sequence whereas other sequences were highly conserved with C, T and A. The results of our MSA showed that *H. pylori* 16S rRNA sequences was highly conserved with other 17 sequences, indicates that this regions play important role in the function of 16S rRNA.

It is clear that the first four species specially share conserved region with the main out group (*M. aeruginosa*), which is related to the African origin (Moreira et al, 2014). Meanwhile we observed some difference in our H. pylori 16S rRNA sequence at some positions (134 and 406) and this may be indicated special characterization for our sequence.

Maximum Likelihood (ML)

A maximum parsimony (MP) analysis was perfor-med using *Microcystes aeroginosa*, as a root for the tree. Clade I contain bacteria species belong to Helicobacteraceae, while clade II include species related to campylobacteraceae and two clades were cleaved from the main root (Fig. 2).

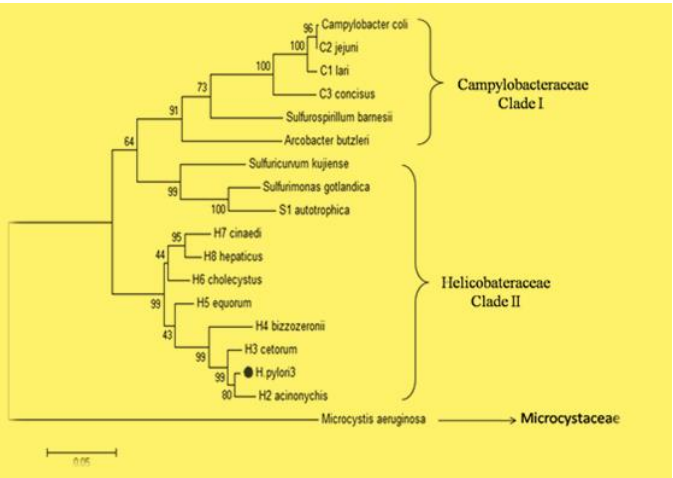

Figure 2: *Phylogenetic analysis of H. pylori 16S rRNA (KP893893). The tree was performed using the maximum likely hood. Bootstrap values are shown above the nodes based on the percentage of occurrences in 1000 replication.*

In clade I, subgroup one Include *H. pylori* species, it was strongly correlated with *H. acinonychis* at bootstrap value 80%. These two species were branched with *H. cetorum* and *H. bizzozeroni at* 99%. The subgroup two contains three species *(H. cholecystus, H. hepaticus, and H. cinadi)* and it was branched with the first subgroup at bootstrap value of 99%.

Clade II include species *(Sulfuricurvum kujiense*, *Sulfurimonas gotlandica*, and *Sulfurimonas autotrophica)* belong to *helicobacteraceae,* it was branched with *campylobacteraceae species (Campylobacter coli, Campylobacter lari, Campylobacter jejuni, Campylobacter concisus, Arcobacter butzleri, and Sulfurospirillum barnesii) at* bootstrap value 64%.

The ribosomal gene *16S rRNA* is particular in that it is present in all bacteria while, at the same time, it comprises nucleotide sequences that are specific to a given bacterial genus (Smith et al, 2004). Sequence analysis of the *16S rRNA* gene has led to our current understanding of prokaryotic phylogeny and *H. pylori 16S rRNA* gene sequence analysis unambiguo-usly differentiated the *Helico-bacter* genus from the closely related *Campylo-bacter* genus (Gorkiewicz et al, 2003) thus allowing creation of the *Helico-bacter* genus.

This work identified two distinct clade based on the phylogenetic analysis of the 16S rRNA. All isolates formed a separate branch with bootstrap value of 99% and 64%, proposed that their novel *Helicobacter* strain may be a new species based on the 16S rRNA sequence analysis.

The 16S rRNA used to confirm generated phylogenetic trees and identify bacteria and closely related species in the clinical setting. The taxonomic resolution of this gene for a number of different bacteria, including H. acinonychis, H. cetorum, H. bizzozeronii, H. equorum, H. cholecystus, H. hepaticus, H. cineadi, s1 autotrophic, S. gotiandica, S. kujiense, A. butzleri, S. barnesii, C. coli, C. jejuni, C. lari, C. concisus and M. aeruginosa. In this study phylogenetic analysis of the larger *16S rRNA* fragment confirmed the separation of *H. pylori* isolates into 2 clades. Intervening sequences of variable lengths and sequences can be found in 16S rRNA of many bacteria. The phylogenetic tree have been described similarity in clade I a bootsrap value of 64% between a number of *Campylobacter*aceae and *Helicobacteraceae* and difference reason back to different in characterization of *16S rRNA* and normal habitat, also we show similarity among (*E. coli*, C. jejuni, C. lari and C. concisus) with a bootsrap value of 96%, 100% and 100% respectively and this result

agreement with results recoded by (Boz˙ena et al, 2006) was a bootstrap value of 100% for *C. coli*, C. jejuni, C. lari, C. concisus using rpoB Gene of *Campylobacter inferred*, while Arcobacter butzleri show bootstrap value of 91% and this result agreement with results recoded by (Karen et al, 2001) was a bootstrap value of 99% for epsilonproteobacterial using *16S rRNA*. Meanwhile, this is supported by a previous study showed highly similarity among these genuses in characterization of *16S rRNA* (Boz˙ena et al, 2006).

The phylogenetic tree have been described a strong branches correlated in clade II among *Helicobacteraceae* genuses with a bootsrap value of 99%.Our data showed a strong relation between H. hepaticus and H. cineadi with a bootsrap value of 95%. This result exceeded the previous result recorded by (Minna and Marja, 2007), which was represent a bootstrap 86% using gyr B gene sequence, which is H. hepaticus infected mice and cause chronic hepatitis (Fox et al, 2010) while *H. cineadi* infected human and cause cellulitis and bacteremia in immune-compromised people (Kiehlbauch et al, 1994). These two genus deterrence from *H. cholecystus* with a bootstrap value of 44%. The result obtain differ from study of (Minna and Marja, 2007) using gyr B gene sequence. This is may be due to the difference of gene or normal environmental to bacteria that infected golden hamster and cause cholangiofibrosis and centrilobular pancreatitis (gallbladder) (Franklin et al, 1996). Also this group was highly clustered with *H. eqourum* with a boot strap value of 99%.

Interestingly, our data also showed at the site 890, the TAA was similar to that in *M. aeruginosa*,, which date back to the African origins (Moreira et al, 2014). This corresponds to the result obtained by (Mona et al, 2015) and support the demonstration of Hushina, (1990) that humans carried *H. pylori* out of Africa 60,000 years ago during their recent global expansions.

Conclusion

It has been concluded that our isolated *H. pylori* could be originated from *H. acinonychis* (cheetahs) that living in Africa region.

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