

Molecular Identification Of 16s Ribosomal RNA Gene of *Helicobacter pylori* Isolated from Gastric Biopsies in Sudan

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Abstract *H. pylori* are a ubiquitous microorganism infecting up to half of the world's population. A total of 81 gastric biopsies taken from patients complaining of gastric disorders in Khartoum state, Sudan screened for *H. pylori*. Eighteen samples (22.2%) yielded positive culture results. The majority of them were males. Also results indicated higher prevalence of *H. pylori* in patients with gastritis. Further identification performed using PCR targeted a region of 16S ribosomal RNA gene of *H. pylori* and gene amplified on 12 samples. Six of isolated sequences subjected to BLAST analysis that showed high similarity to GenBank strains of *H. pylori*. Multiple sequence alignments were performed between isolated 16S rRNA gene sequences and most related *H. pylori* strains deposited on GenBank. One isolate differed on one base-pair substitution (G-A) from other isolates and selected reference *H. pylori* strains. Phylogenetic analysis based on 16S rRNA gene sequences reflects that *H. pylori* could be originated from Africa.

Keywords: *Helicobacter pylori*, BLAST, Phylogenetic, 16s rRNA, Sudan

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1. Introduction

Helicobacter pylori, a Gram-negative bacterium found on the luminal surface of the gastric epithelium, was first isolated by Warren and Marshall in 1983 [1]. It is an etiologic agent of peptic ulcer disease, primary gastritis, gastric mucosa-associated lymphoid-tissue lymphoma, and gastric adenocarcinoma [2]. The prevalence of *Helicobacter pylori* infection worldwide is approximately 50% [3]. The infection is usually contracted in the first few years of life and tends to persist indefinitely unless treated [4]. Its prevalence increases with older age and with lower socioeconomic status during childhood and thus varies markedly around the world [5]. Approximately 20% of persons infected with *H. pylori* develop related gastroduodenal disorders during their lifetime [3], but only 10%–20% of infected persons become symptomatic [6]. *H. pylori* seem to be transmitted in various ways, including oral–oral and faecal–oral routes [7,8]. Documented risk factors include low socioeconomic status, overcrowding, poor sanitation or hygiene, and living in a developing country [2]. *H. pylori* is well known for being highly diverse and recombining frequently [9,10]. Genetic studies

have established that diversity is geographically and ethnically structured [9,11,12,13]. The biogeographic relationships within *H. pylori* are likely a result of intra-familial transmission combined with recycling within local communities [9,14]. DNA sequence analysis of house-keeping and virulence associated genes all have illustrated the unusually high degree of genetic variability in this species [9,10]. 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 [15,16,17]. 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 [18]. Sequence analysis of the 16S rRNA gene has led to our current understanding of prokaryotic phylogeny and *H. pylori* 16S rRNA gene sequence analysis unambiguously differentiated the *Helicobacter* genus from the closely related *Campylobacter* genus [19] thus allowing creation of the *Helicobacter* genus. Ten strains of *H. pylori* examined for contribution of point mutation and conservative nature of their sequences of

16S rRNA gene fragments and findings revealed few differences and weak relation with diversity [20].

2. Materials and Methods

2.1. Study Area

This study was carried out mainly in Khartoum state, in the major hospitals, including Khartoum teaching hospitals, Omdurman teaching hospital, Ibn-Sina hospital and El-Neelien Medical Centre. All the procedures were carried out in National Centre for Research, Department of Microbiology and Central Lab, Departments of Virology and Molecular Biology.

2.2. Study Subjects

Eligible subjects are patients who were referred to hospitals for upper gastrointestinal tract endoscopy and suspected to be infected with *H.pylori* according to endoscopy findings.

2.3. Clinical Samples

A total of eighty one gastric biopsies were collected from eligible patients who had Gastrosopy and tentatively diagnosed by the physician as having a helicobacter infection. They were of different sex and ages. The samples were labeled and transferred immediately to the laboratory for bacteriological examinations.

2.4. Isolation and Identification

All gastric biopsies were cultured on helicobacter pylori selective medium (Columbia blood agar base, Oxoid) mixed with selective supplement (DENT, Oxoid) and supplemented with horse blood. Inoculated plates incubated at 37°C under microaerobic conditions using CampyGen paper sachet (Oxoid, UK) for five days. They were identified by colony morphology, microscopic appearance, Oxidase, Catalase and Urease reactions.

2.5. DNA Extraction and PCR Amplification of 16S rRNA Gene

DNA was extracted from biopsies using QIAamp DNA Micro Kit (Qiagen, Germany) and performed according to the instructions of the manufacturing company. A single PCR assay targeting helicobacter pylori species specific 16S rRNA gene was carried out using the following primers:

(F:5'-GCGCAATCAGCGTCAGGTAATG-3')
(R:5'GCTAAGAGAGCAGCCTATGTCC-3').

Primers and template DNA added to PCR master mix (GoTaq, Promega, USA) following the manufacture guide. 30 cycles were performed in a thermocycler, Each cycle has three steps of denaturation (94°C for 1 min), annealing (55°C for 1 min), extension (72° C for 3 min) and final extension time of 72°C for 5 min. The amplified products of 502 bp were detected by electrophoresis.

2.6. Molecular Analysis

Standard sequencing was performed to the 16s rRNA of twelve isolates. The PCR products of the 16s rRNA gene

were prepared according to the instructions of the sequencing company (Macrogen Inc. Seoul, Korea).

Purification performed using mixture of 5 µ l of a post-PCR reaction product with 2 µ l of ExoSAP-IT PCR Clean-up Kit. A combined 7 µ l reaction volume incubated at 37°C for 15 min to degrade remaining primers and nucleotides and later incubated at 80°C for 15 min to inactivate ExoSAP-IT.

Sequencing reactions were performed using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with the BigDye XTerminator Purification Kit (Applied Biosystems). The samples were injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

2.7. Bioinformatic Analysis

The isolated nucleotide sequences of the 16s rRNA genes were compared with sequences of related strains deposited on databases using the nucleotide BLAST [21] (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software [22]. Phylogenetic tree of 16s rRNA genes and their evolutionary relationship with those obtained from database was done online by Clustal W2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) [23] and phylogenetic tree was constructed using One click mode from Phylogeny.fr.home (<http://www.phylogeny.fr.home>) [24-29].

Table 1. BLAST result of 16S rRNA gene sequence identity between the Sudanese *H. pylori* isolates and some GenBank sequences

Strain Name	GenBank Accession Number	Identity
Helicobacter pylori oki828	CP006826.1	99%
Helicobacter pylori Rif2	CP003906.1	99%
Helicobacter pylori Shi112	CP003474.1	99%
Helicobacter pylori Puno135	CP002982.1	99%
Helicobacter pylori PeCan18	CP003475.1	99%
Helicobacter pylori SNT49	CP002983.1	99%
Helicobacter pylori 52	CP001680.1	99%
Helicobacter pylori G27	CP001173.1	99%
Helicobacter pylori Gambia94/24	CP002332.1	99%
Helicobacter pylori SouthAfrica7	CP002336.1	99%

3. Results

Eighteen biopsies (22.2 %) yielded positive cultures for *H. pylori*. Most positive cases (61.1%) were male and aged between 15-30. Positive cultures for *H. pylori* in relation to the endoscopy findings differed significantly as resulted by chi square test (P value: 0.036). Out of the 18 DNA extracts isolated from gastric biopsies, a region of 16S rRNA gene of *H. pylori* was amplified by PCR in 12 (67%) samples. Sequence analysis confirmed the identification of *H. pylori*. BLAST showed 99% homology

between isolates and revealed several accessions of *H. pylori* 16s rRNA sequences obtained from Genbank (Table 1). The alignment of isolated sequences showed that our isolates exhibited the same sequence except one (isolate 21) that differed on one base-pair substitution (G-A) (Figure 1(A,B)). Multiple sequence alignment of the

isolates with *H. pylori* Genbank strains approved the occurrence of that variation from isolates and from selected published nucleotide sequences (Figure 1.C). Phylogenetic analysis done and a cladogram (Figure 2) revealed that our strains are so closed to South African strains.

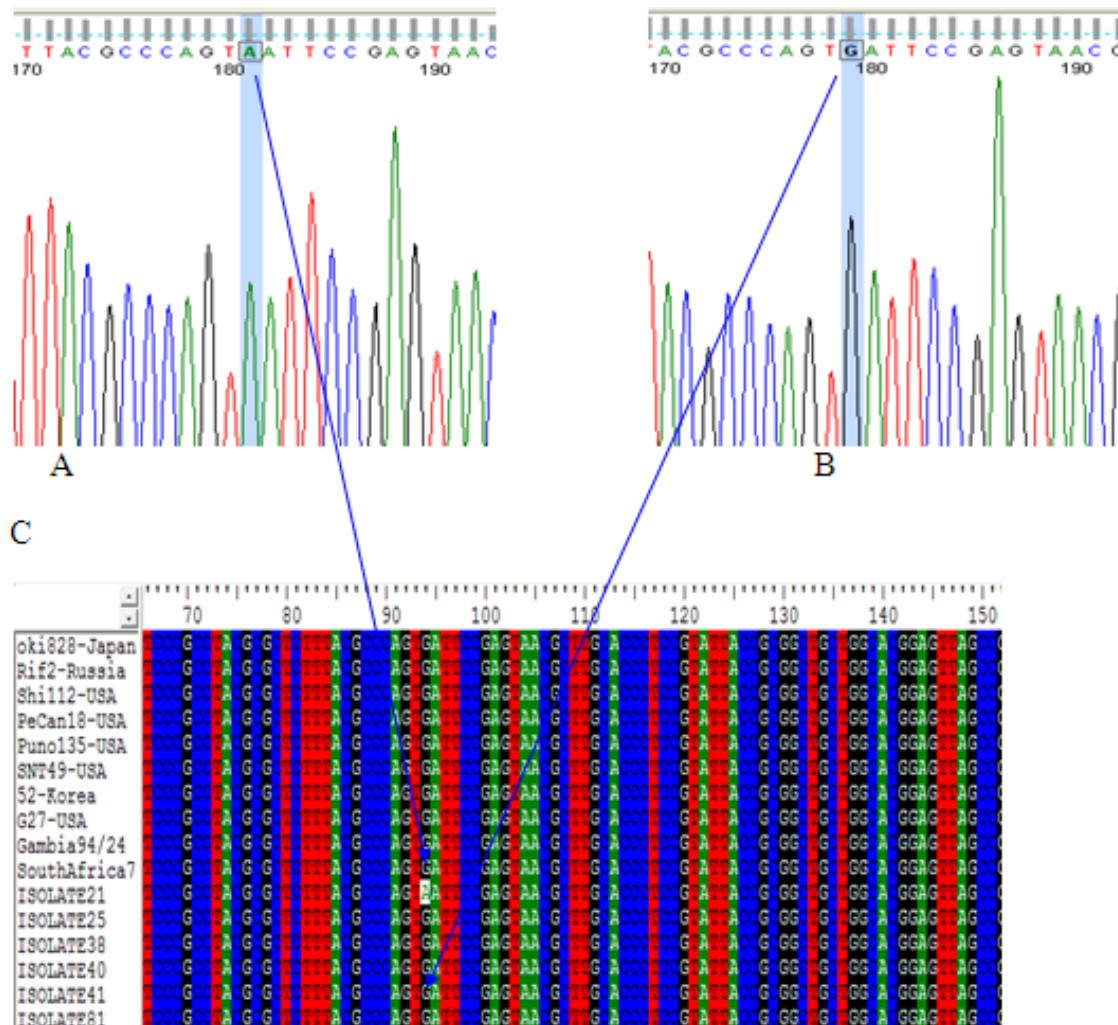


Figure 1. (A,B): Base pair substitution from G-A at isolate 21 which illustrated by arrows. Chromatograms edited using Finch TV software (C): Sequence alignment of 16S rRNA gene of six Sudanese *H. pylori* isolates compared with other selected strains obtained from GenBank databases using BioEdit software

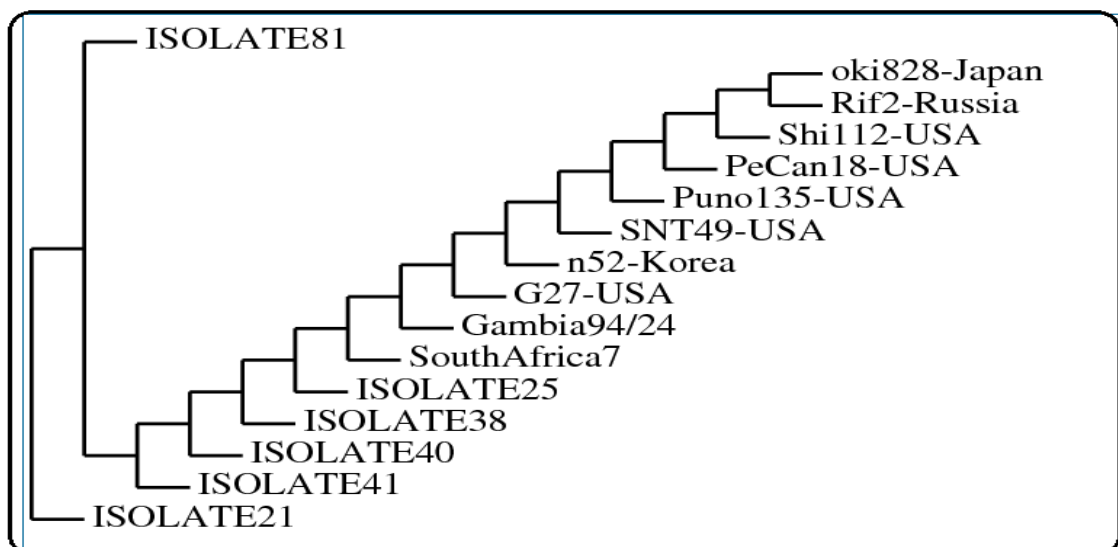


Figure 2. Phylogenetic relationship between 16s rRNA gene sequences of Sudanese *H.pylori* strains and selected reference strains

4. Discussion

In this study, we investigated a group of 81 patients using endoscopy and gastric biopsies for *H.pylori* infection. Our finding that *H.pylori* was more prevalent in male than female is agreed with many studies [30,31]. The majority of patients (86%) had gastritis, only (7%) of them were positive for PCR based on 16s rRNA, that detection rate was lower than findings from Nigeria when 52.38 % of gastritis samples were PCR positive [32]. DNA extraction method was important for detection of *H. pylori* 16s rRNA gene as confirmed by our study (60%) in comparison to (52.38%) by Smith et al and (14.3%) at Marais study [32,33]. While culturing of *H. pylori* considered as gold standard methods for detection in biopsies [34]. We found that PCR seems to be as sensitive as culture for *H. pylori* detection as confirmed by Vanzwet and his colleagues [35], and in contrast to the study from Gambia that showed (52.6%) for culture and (97%) for PCR [36]. Only 60% of positive samples were amplified 16s rRNA in comparison to other findings 48.3 % [37] and (91%) detected by Xu et al [38]. Percentages could be due to Sequence variations in 16s rRNA gene between different strains that affect at the PCR detection rate in agreement with Bruce et al [39]. Phylogenetic comparison of rRNA sequences has become a powerful method for the systematic classification of microbial organisms [40,41]. Analysis of 16S rRNA gene sequences has become the primary method for determining prokaryotic phylogeny, which is the current basis for prokaryotic systematics [31,42]. They amplified to confirm the presence of the isolated *H. pylori* strains [43,44]. Sequence analysis confirmed the identification of *H. pylori* isolates. 16S rRNA sequence analysis has demonstrated considerable genomic diversity between isolates, and numerous sequences. This diversity is characteristic to *H.pylori* as demonstrated by Taylor et al. [45]. A single nucleotide change (G-A) of the 16S rRNA of isolate 21 differentiated this strain from other isolates and selected ones as a kind of strain evolution. The phylogenetic tree showed that our isolates clustered on one branch with those from South Africa (CP002336.1), Gambia (CP002332.1), Korea (CP001680.1), Japan (CP006826.1), Russia (CP003906.1) and USA (CP002983.1, CP002982.1, CP003474.1, CP003475.1, and CP001173.1. Deviation of isolates 21 from the consensus sequence were from G-A and its occurrence at the root of the branch make it a common ancestor of that group and support the demonstration of Hushan and his colleagues that humans carried *H. pylori* out of Africa 60,000 years ago during their recent global expansions [46]. So *H.pylori* could be originated from Africa.

5. Conclusion

Additional studies are required on larger sample size to screen prevalent strains on Sudan and determine their characteristics and relatedness to reference strains and make data available to clinical application.

Competing Interests

The authors declare that they have no conflict of interests.

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