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The validity evaluation of different 16srRNA gene primers for helicobacter detection urgently requesting to design new specific primers

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Molecular diagnosis of helicobacters by PCR is simpler, more accurate, and feasible compared to other diagnostic methods. Validity and accuracy are highly dependent on the PCR primer design, diffusion time, and mutation rate of helicobacters. This study aimed to design 16srRNA -specific primers for *Helicobacter spp.* and *H. pylori*. Application of comparative statistical analysis of the diagnostic utility of the most available 16srRNA genus-specific primers. The new primers were designed using bioinformatics tools (MAFFT MSA and Gblocks command line). A comparative study was applied on nine genus-specific 16srRNA primers in comparison to the ConsH using *in silico* and laboratory evaluation. The results demonstrated that the best specificity and sensitivity of the primers designed for this study compared to other primers. The comparative study revealed that the heminested outer/inner primers were the worst. Although H276, 16srRNA(a), HeliS/Heli-nest, and Hcom had acceptable diagnostic utility, false positive and false negative results were obtained. Specificity testing on clinical samples indicated a surprising result; that *H. pylori* was not the sole enemy that we were looking for, but the Non-*Helicobacter pylori* *Helicobacters* should be considered as a real risk prognostic for gastric diseases, consequently, a specific diagnosis and treatment should be developed. This study concluded that our designed primers were the most specific and sensitive in comparison with other primers. In addition, *in silico* evaluation is not accurate enough for primer assessment and that the laboratory evaluation is mandatory.

Genus *Helicobacter* is a microaerophilic, helical, Gram-negative bacterium responsible for gastrointestinal disorders¹. Thirty-four *Helicobacter spp.* have been identified so far according to LPSN (<http://www.bacterio.net/h/helicobacter.html>). It became evident that *Helicobacter spp.* can infect humans and various animal hosts and colonize different anatomical regions of the gastrointestinal tract².

Helicobacter pylori (*H. pylori*) was first recognized and considered a major cause of gastrointestinal disorders, including gastritis and peptic ulcers. Chronic infections are associated with gastric cancers and mucosal-associated lymphoid tissue lymphoma (type 1 gastric carcinogen by the International Agency for Cancer Research) in addition to several extra-gastric diseases^{1,3,4}.

Helicobacter spp. is assumed to be one of the most genetically diverse bacterial species studied to date⁵. Its genetic variability contributes to adaptation to changing environmental conditions via many approaches as antibiotic resistance development and the continuous surface antigen variation⁶. A wide range of genetic variability is attributed to substitution mutations⁷, insertion elements⁸, genetic rearrangement in the pathogenicity

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islands⁹, the presence of prophages that causes genetic variations¹⁰, and natural transformability demonstrated by many strains¹¹. *Helicobacter spp.* variability renders its diagnosis more challenging than many other genera.

Molecular diagnosis by polymerase chain reaction (PCR) is simpler, more accurate, and feasible compared to other invasive and non-invasive diagnostic procedures^{12–14}. PCR is used for the detection of *H. pylori* DNA in various samples including gastric mucosa, feces, saliva, dental plaque, and other environmental samples¹⁵. It has succeeded in demonstrating an actual correlation between *H. pylori* infection and extra gastric digestive carcinogenesis as hepatic carcinoma, bile duct cancer, pancreatic cancer, and colon cancer^{16,17}.

Molecular diagnostic methods using PCR especially nested PCR will be the gold standard in helicobacter diagnosis¹⁸. Although their use is still mainly restricted to research, it is gaining great popularity in the medical field¹⁹. It is noteworthy that the validity and accuracy of results are highly dependent on the PCR design and the time of its publication as new sequences are constantly submitted to databases^{16,20}.

Certain target genes have been extensively used for the PCR detection of *Helicobacter spp.* and *H. pylori*, including the 16S rRNA gene, the 26 K species-specific antigen gene, the *glmM* gene, the *ureA* gene, the *ureB* gene, the *cagA* gene, and the *vacA* gene^{21–33}. The most sensitive and widely used gene for the detection of *Helicobacter* infections is PCR that targets the genus-specific and conserved region of the housekeeping gene, 16S rRNA^{16,34}, which is present in all bacterial species. This small ribosomal subunit gene contains conserved regions that are used for the general amplification of bacterial DNA by utilizing universal primers. Comparison of DNA sequences from these PCR products is widely used in taxonomy, phylogenetic studies³⁵, and clinical microbiology³⁶. In addition to the conserved regions, 16S rRNA contains hypervariable regions that are highly specific for biological species or genera^{37,38}.

Despite the various advantages of PCR, high mutation rates of *Helicobacter spp.*, the short primers, the low melting temperature, polymorphism in binding site at 3' end in protein-coding genes, and high melting temperature difference between forward and reverse primers (more than the recommended 4 °C) affect amplification efficiency and may lead to false-negative results^{18,19,39,40}. False-positive results may be attributed to the usage of non-specific primers and the detection of cDNA from Non-*Helicobacter pylori* *Helicobacters* (NHPH), this is particularly important in environmental samples which may contain NHPH^{15,41}.

The present study aimed to design new sets of primers specific to *Helicobacter spp.* and *H. pylori* using bioinformatics tools. Evaluation of these designed primers was performed by *in silico* and experimental testing. Comparative Statistical analyses were applied for most 16srRNA primers for Genus *Helicobacter* detection.

Results

The newly designed 16srRNA specific primers for *Helicobacter spp.* and *H. pylori*. The conserved regions of the 16srRNA gene-specific to all helicobacters and all *H. pylori* strains are demonstrated in Table 1; whereas the conserved regions used in the design of highly specific primers are demonstrated in Table 2. The first conserved area of *Helicobacter spp.* was used for designing the genus-specific primer (ConsH) and the second conserved area of *H. pylori* was used for designing the *H. pylori* specific nested primer (PyloA/ PyloAN).

Evaluation of the newly designed primers. *In silico* evaluation by BLAST and *in silico* PCR amplification. ConsH primer corresponds to 383 *H. pylori* and 18 Non-*Helicobacter pylori* *Helicobacters* (NHPH) by primer-blast analysis. The nested primer (PyloA/PyloAN) corresponds to 281 *H. pylori* strains. *In silico* PCR amplification revealed that ConsH primer gave a positive band of 435 bp in 60 *Helicobacter* strains (96.8%). The nested primer (PyloA/PyloAN) revealed positive bands of 1274 bp (first nested, PyloA) and 160 bp (second nested, PyloAN) in 61 *Helicobacter* strains (98.4%).

Laboratory evaluation.

- **Specificity testing:** The ConsH obtained positive bands of 435 bp with all helicobacters and negative results with all non-helicobacter strains. The *H. pylori*-specific nested primer (PyloA/PyloAN) produced positive bands of 1274 bp in the first pair and 160 bp in the second pair with all five *H. pylori* strains and negative results with all Non-*Helicobacter pylori* *Helicobacter* (NHPH) and non-helicobacter strains. The results of PCR application in 243 clinical biopsy samples revealed 99 positive samples for *Helicobacter spp.* by ConsH primer (40.7%), from these positive samples, 66% were *H. pylori* and 33% were Non-*Helicobacter pylori* *Helicobacter* (NHPH). The positive cases were confirmed by sequencing
- **Sensitivity testing:** The detection limit of ConsH primer was 250 and 25 pg in stool and biopsy clinical samples, respectively, while the detection limit of PyloA was 0.25 fg and 0.25 pg in stool and biopsy clinical samples respectively.

Comparative analysis between the newly designed primer ConsH and different 16srRNA primers for G: *Helicobacter* detection. *In silico* comparative analysis. Primer Blast revealed that H276 primer correlates with 277 *H. pylori* and 56 Non-*Helicobacter pylori* *Helicobacters* (NHPH), whereas 16srRNA(b) matches 401 *H. pylori*, outer/inner primers are compatible with 283 *H. pylori* and 48 NHPH, HeliS-Helinet match 329 *H. pylori*, and 115 NHPH. 16srRNA(a) doesn't match any *Helicobacter*. Hcom corresponds to 282 *H. pylori* and 13 NHPH. BFHpyl matches 312 *H. pylori* and 3 NHPH. Heid correlates with 297 *H. pylori* and 2 NHPH. The positive percent of *in silico* PCR amplification is explained as follows: H276 and outer/inner primers gave positive in 60 *Helicobacters* (96.8%), Hcom1 gave positive with 61 *Helicobacters* (98.4%). 16srRNA (a) and

Genus/spp.	Conserved area
Genus Helicobacter	GTGGATTAGTGGCGCACGGGTGAGTAACGCATAGGTTATGTGCCTCTTAGTTTGGGATAGCCATTGGAA ACGGTGATTAATACCAGATATCCCTACGGGGGAAAGATTTATCGTAAAGATAGACCCATATGCCCTAT CAGCTTGTGGTAAGGTAATGGCTTACCAAGGCTATGACGGGTATCCGGTCTGAGAGGGTGAACGGACA CACTGGAACTGAGACACGGTCCAGACTCTACGGGAGGCAGGAGTGGGAATATTGCTCAATGGGGGAA ACCTGAAGCAGCAACGCCGCTGGAGGATGAAGGTTTTAGGATTTGAAACTCCTTTTGTACAGAGAAGA TAATGACGGTATCTGACGAATAAGCACGGGCTAACTCCGTGCCAGCAGCCGGTAATACGGAGGGTGC AAGCGTTACTCGGAATCACTGGGCGTAAAGAGCGCGTAGGCGGGATAGTCAGTCAGGTGTGAAATCCTA TGGCTTAACCATAGAAGTGCATTTGAAACTGCTATTCTAGAGTGTGGGAGAGGCAGGTGGAATTTCTGG TGTAGGGGTAATAATCCGTAGAGATCAAGAGGAATACTCATTCGCAAGGCGACCTGCTAGAACATGACTG ACGCTGATTCGCGGAAAGCGTGGGGAGCAACAGGATTAGATACCTTGGTAGTCCACGCCCTAAACGAT GGATGCTAGTTGTTGGAGGGCTTAGTCTCTCCAGTAATGCAGCTAACGCCTTAAGCATCCCGCCTGGGG AGTACGGTTCGCAAGATTAATACTCAAAGGAATAGACGGGGACCCGCAAGCGGTTGGAGCATGTGGTTT AATTCGAAGATACACGAAGAACTTACCTAGGCTTGACATTGAGAGAATCCCGTAGAAATACGGCGGTTG TCTGGCTTGCAGACCTTGAACACAGGTGCTGCACGGCTGTGCTCAGCTCGTGTGAGATGTTGGGT TAAGTCCCGCAACGAGCGCAACCCCTTTCTTAGTTGCTAACAGGTCATGCTGAGAAGTCTAAGGAGAC TGCCTCCGTAAGGAGGAGGAAGTTGGGGACGACGCTCAAGTATCATGGCCCTTACGCCCTAGGGTACAC ACGTGCTACAATGGGGTGCACAAAGAGAAGCGATACTGCGAAGTGGAGCCAACTTCAAAAACATCTCTC AGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCTGGAATCGTAGTAATCGCAAATCAGCCATGT TGGCGTGAATACGTTCCCGGGTCTT
Helicobacter pylori	GAGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGCTGCCTAATACATGCAAGTCAACGATGAAGCTT TCTAGCTTGTAGAAAGGCTGATTAGTGGCGCACGGGTGAGTAACGCATAGGTTATGTGCCCTCTTAGTTT GGGATAGCCATTGGAAACGATGATTAATACCAGATACTCCCTACGGGGGAAAGATTTATCGTAAAGAGA TCAGCCTATGCTCTATCAGCTTGTGGTAAGGTAATGGCTTACCAAGGCTATGACGGGTATCCGGCCTG AGAGGGTGAACGGACACACTGGAACCTGAGACACGGTCCAGACTCCTACGGGAGGCAGGAGTGGGAATA TTGCTCAATGGGGGAAACCCCTGAAGCAGCAACGCCGCTGGAGGATGAAGTTTATAGGATTGAAACTC CTTTGTTAGAGAAGATAATGACGGTATCTAACGAATAAGCACGGCTAACCTCCGTGCCAGCAGCCGCG GTAATACGGAGGGTGAACGGTTACTCGGAATCACTGGGCGTAAAGAGCGCGTAGGCGGGATAGTCAGT CAGGTGTGAAATCCTATGGCTTAACCATAGAAGTGCATTTGAAACTACTATTCTAGAGTGTGGGAGAGG TAGGTGGAATCTTGGTGTAGGGGTAAATCCGTAGAGATCAAGAGGAATACTCATTCGCAAGGCGACC TGCTGGAACATTACTGACGCTGATTGCGCGAAAGCGTGGGGAGCAACAGGATTAGATACCCCTGGTAGT CCACGCCCTAAACGATGGATGCTAGTTGTTGGAGGGCTTAGTCTCTCCAGTAATGCAGTAAACGCATTA AGCATCCCGCCTGGGGAGTACGGTTCGCAAGATTAATACTCAAAGGAATAGACGGGGACCCGCAAGCC GTGGAGCATGTGGTTAATTGCAAGATACAGGAAGAACCTTACCTAGGCTTGACATTGAGAGAATCCGC TAGAAATAGTGGAGTGTCTGGCTTGGCAGACTTGAACACAGGCTGACAGGCTGCTGCTGAGTCTGCTG TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTTCTTAGTTGCTAACAGGTCATGCTG AGAAGTCTAAGGATAGTGCCTCCGTAAGGAGGAGGAAGTTGGGGAGCAGCTCAAGTATCATGGCCCTT ACGCCTAGGGCTACACACGCTGCTACAATGGGGTGCACAAAGAGAAGCAATACTGCGAAGTGGAGCCAA CTTCAAAACACCTCTCAGTTCCGATTGTAGGCTGCAACTCGCCTGCATGAAGCTGGAATCGTAGTAAT CGCAAATCAGCCATGTTGGCGTGAATACGTTCCCGGGTCTGTACTCACCGCCCGTCAACACAGCGACT GGGTGAAGTTCGTAACAAGGTAACCGTA

Table 1. The conserved sequence of 16srRNA gene in *G. Helicobacter* and *H. pylori*.

Primer name	Sequence	GC content	TM	Product size bp	
ConsH	Forward primer	TCG CTA AGA GAT CAG CCT ATG TCC T	48	65.8	435
	Reverse Primer	ATT CCA CCT ACC TCT CCC ACA CT	52	64.7	
PyloA	Forward primer	TTG ATC CTG GCT CAG AGT GAA CG	52	64.7	1274
	Reverse primer	TGC AGC CTA CAA TCC GAA CTG AG	52	64.7	
PyloAN	Forward primer	GGT GGA ATT CTT GGT GTA GGG GT	52	64.7	160
	Reverse primer	TAG CAT CCA TCG TTT AGG GCG TG	52	64.7	

Table 2. Sequence of the newly designed primers in the study.

BFHpyl gave positive with only one Helicobacter (*H. pylori* 26695 and *H. pylori* G27, respectively). 16srRNA (b) and Heid didn't produce any bands with all Helicobacters.

Laboratory comparative study. H276, outer/inner, Hcom1, Heli-nest/HeliS, and 16srRNA (a) yielded positive results with all eight Helicobacters, 16srRNA (b) relented positive results with six Helicobacters, BFHpyl revealed a positive band with only one *H. pylori* while Heid primer gave negative with all Helicobacter spp. Non-specific results were obtained with other Non-helicobacters as follows: H276 (*S. mutans*), Heli-nest/HeliS (*Salmonella* spp.), Hcom1 and BFHpyl (*K. pneumoniae*), 16srRNA (a) (*E. fecalis*), Heid (*E. coli*) while outer/inner primer gave positive results with all non-helicobacters.

Statistical evaluation of the diagnostic utility of different 16srRNA primers for Helicobacter spp. detection. ConsH matched all criteria considered for the gold standard selection. The statistical evaluation parameters according to in silico PCR amplification are described in Table 3. The outer/inner primers had 100% specificity and sensitivity with a P-value of 0.0001. Hcom1, H276, and HeliS/Heli-nest had 50% SP. The utility is measured also by the receiver operating characteristics (ROC) analysis expressed by the area under the curve (AUC), the ROC analysis of the in silico evaluation is demonstrated in Fig. 1. The laboratory evaluation showed 0.00% SP with heminested outer/inner primers with P-value=1. The H276, 16srRNA(a), HeliS/Heli-nest, and Hcom had

Gold standard primer	Screening primers	SEN (%)	SPC (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)	Acc (%)	BA (%)	LR +	LR-	DOR	X ²	P value
CONSH	Hcom1	100	50	98.4	100	50	0.0	98.4	75	2.0	0.0	-	7.12	0.01
	H276	98.3	50	98.3	50.0	50.0	1.7	96.8	74.2	1.97	0.033	59	3.140	0.076
	Outer + Inner	100	100	100	100	0.0	0.0	100	100	NA	0.0	-	34.10	0.0001
	Heli-nestS + Heli	8.3	50	83.3	1.8	50	91.7	9.7	29.2	0.17	1.83	0.09	0.555	0.456

Table 3. Screening primers test results of In silico PCR for detection of 62 *Helicobacter* strains. SEN: sensitivity; SPC: specificity; PPV: Positive predictive value; NPN: Negative predictive value; FPR: False positive rate; FNR: False-negative rate; ACC: Accuracy; BA: Balanced accuracy; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; DOR: Diagnostic odds ratio. 16S rRNA, hspG, and Heid primers tested negative in the detection of all 62 *Helicobacter* species. 16S rRNA and BFHpyl primers tested negative in the detection of 61 *Helicobacter* species.

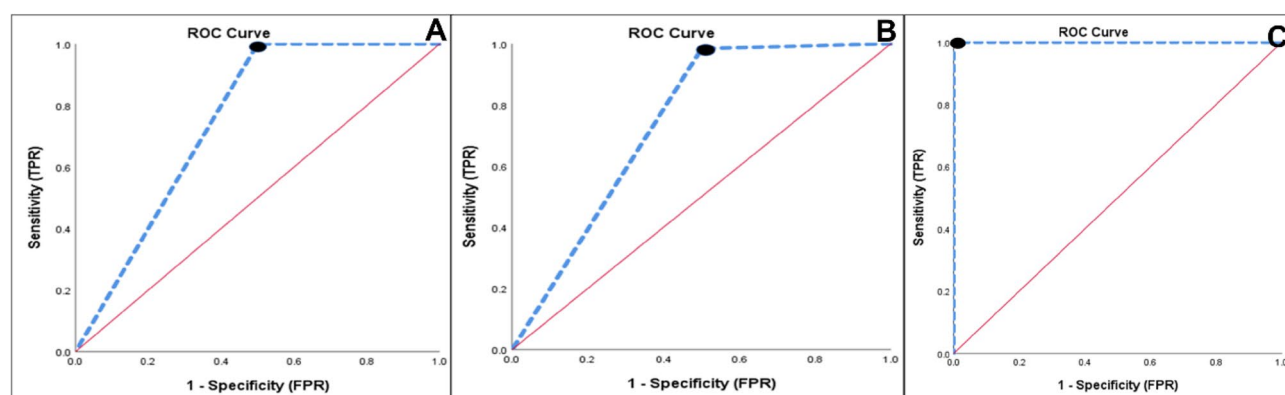


Figure 1. ROC curve of In silico comparison by PCR amplification. Null hypothesis: true area = 0.5, AUC. (A), ROC curve of ConsH and Hcom1 primers for detection of 62 *Helicobacter* strains, AUC represents the accuracy of the screening test (0.750). (B), ROC curve of ConsH and H276 primers, AUC represents the accuracy of the screening test (0.742). (C), ROC curve of ConsH and Outer + Inner primers, AUC represents the accuracy of the screening test (1.00).

90% specificity with P -value = 0.001. Although 16srRNA(b) had 100% specificity, it had 75% sensitivity with a P -value = 0.004. The ROC analysis of laboratory comparison is demonstrated in Fig. 2 and Table 4.

Discussion

The present study demonstrated the whole process of designing novel sets of highly specific primers targeting 16srRNA conserved region by bioinformatics tools, ConsH for detecting *G. Helicobacter*, and nested primers for *H. pylori* detection (PyloA/PyloAN). The design process was followed by evaluation of the diagnostic utility of the primers in comparison to a group of widely used 16srRNA primers in literature, and statistical monitoring of the diagnostic utility of the comparable primers used in *Helicobacter spp.* diagnosis.

Efficient PCR performance is highly dependent on PCR primer design, consequently one must spend a notable effort on the primer design. Well-designed PCR primers not only augment specificity and sensitivity but also reduce the effort spent on the experimental optimization⁴².

PCR diagnosis especially nested PCR may be regarded as the gold standard for *Helicobacter* diagnosis through the construction of specific primers. Nested PCR provides higher sensitivity by excluding false-negative results due to low bacterial counts and PCR inhibitors¹⁸. PCR yielded a higher detection rate (40.8%) compared to histopathology (36.7%) and can be suitable for patients unfit for endoscopic examination⁴³.

The choice of 16srRNA gene in PCR diagnosis of *Helicobacter* diseases was supported by a systematic review and meta-analysis conducted on various sources, including MEDLINE, Web of Sciences, and the Cochrane Library from April 1, 1999, to May 1, 2016. The most diagnostic candidate genes according to statistical parameters were 23S rRNA, 16S rRNA, and *glmM*⁴⁴. The urgent demand for rapid accurate *Helicobacter* detection puts an obligation for the construction of novel specific primers.

Both the ConsH and PyloA/PyloAN primers were synthesized according to the bioinformatic tools with primer designs criteria which ensures efficient PCR performance. The primer lengths fall within the recommended 18–30 nucleotides, shorter primers can produce non-specific results, and longer primers can form secondary structures and reduce PCR efficiency. Primers GC contents are within 48–52%, and the difference between the melting temperatures of the forward and reverse primers was within 4 °C versus other compared

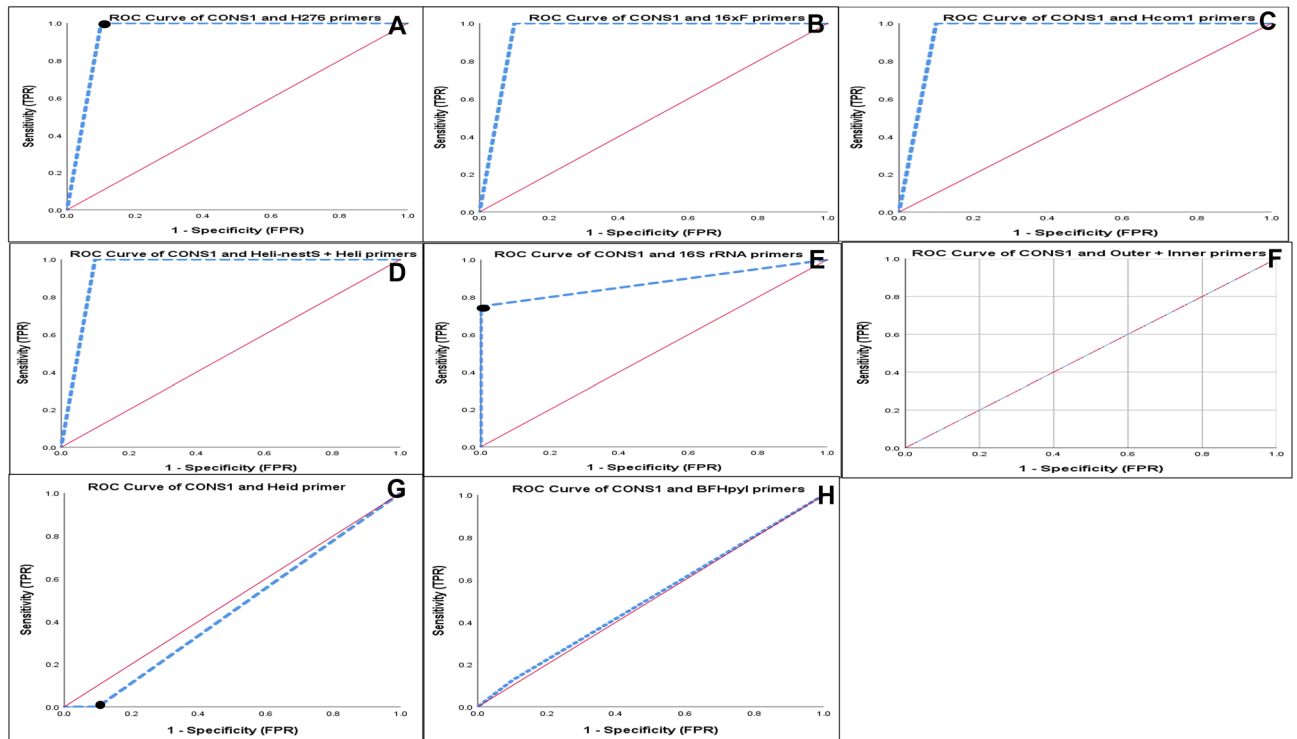


Figure 2. ROC curve of laboratory comparison of all primers using *Helicobacter* spp. (n = 8) and non-*Helicobacter* spp. (n = 10). Null hypothesis: true area = 0.5, AUC: Area under the curve. (A), ROC curve of ConsH and H276 primers, AUC represents the accuracy of the screening test (0.950). (B), ROC curve of ConsH and 16srRNA (a) primers, AUC represents the accuracy of the screening test (0.950). (C), ROC curve of ConsH and Hcom1 primers, AUC represents the accuracy of the screening test (0.950). (D), ROC curve of ConsH and Heli-nestS + Heli primers, AUC represents the accuracy of the screening test (0.950). (E), ROC curve of ConsH and 16S rRNA primers, AUC represents the accuracy of the screening test (0.875). (F), ROC curve of ConsH and Outer + Inner primers, AUC represents the accuracy of the screening test (0.500). (G), ROC curve of ConsH and Heid primers, AUC represents the accuracy of the screening test (0.450). (H), ROC curve of ConsH and BFHpyl primers, AUC represents the accuracy of the screening test (0.513).

Gold standard Primer	Screening Primer	SEN (%)	SPC (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)	Acc (%)	BA (%)	LR+	LR-	DOR	X ²	P value
CONSH	H276	100	90.0	88.9	100.0	10.0	0.0	94.4	95	10.0	0.0	-	11.025	0.001
	16 XF	100	90.0	88.9	100.0	10.0	0.0	94.4	95	10.0	0.0	-	11.025	0.001
	Heli-nestS + Heli	100	90.0	88.9	100.0	10.0	0.0	94.4	95	10.0	0.0	-	11.025	0.001
	Hcom1	100	90.0	88.9	100.0	10.0	0.0	94.4	95	10.0	0.0	-	11.025	0.001
	16S rRNA	75.0	100	100	83.3	0.0	25	88.9	87.5	-	0.25	-	8.128	0.004
	Outer + Inner	100	0.0	44.4	-	100	0.0	44.4	50	1.0	-	-	-	-
	BFHpyl	12.5	90	50	56.3	10	87.5	55.6	51.3	1.25	0.97	1.29	0.00	1
	Heid	0.0	90	0.0	52.9	10	100	50.0	45	0.0	1.11	0.0	0.00	1

Table 4. Laboratory evaluation of the primers validity for the detection of *Helicobacter* spp. (n = 8) and Non-*Helicobacter* spp. (n = 10). SEN: sensitivity; SPC: specificity; PPV: Positive predictive value; NPN: Negative predictive value; FPR: False positive rate; FNR: False-negative rate; ACC: Accuracy; BA: Balanced accuracy; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; DOR: Diagnostic odds ratio.

primers as 16srRNA (a), Hcom, and Heli-nest primers which have larger than 4 °C difference between the primers. This may cause false negatives^{45,46}.

The evaluation of the newly designed primers was performed by in silico tools and laboratory test (PCR). The results of the in silico evaluation were very promising as ConsH matched with *Helicobacter*s only and no mismatching with other bacteria, also the nested primers for *H. pylori* (PyloA/PyloAN) matches only with *H. pylori*. In silico PCR amplification showed highly encouraging results. The advantages of in silico evaluation are low cost and timesaving for evaluation as it gives a preliminary decision about the newly designed primer sets.

Nonetheless, the in silico evaluation is not a confirmatory method for evaluation due to continuously submitting uncurated sequences into GenBank, therefore, laboratory evaluation is mandatory⁴⁷.

In the laboratory evaluation, the newly designed primers showed sufficient results. The specificity testing revealed significant results as ConsH detected all Helicobacters and the nested primer (PyloA/PyloAN) identified all five *H. pylori* strains without mismatching with Non-Helicobacter pylori Helicobacters (NHPH). They did not select any non-Helicobacter bacteria. The clinical precision testing by PCR was applied on gastric biopsies from dyspeptic patients introduced to the endoscope unit in the mentioned hospitals, revealing that all PCR results are consistent with the invasive methods applied (RUT and Histopathology). The positive PCR by sequencing confirmed the presence of Helicobacter DNA in the samples^{48–50}. The ConsH detected 40.7% positive samples, from the positive Helicobacter *spp.* PCR, the nested PCR detected 61.3% *H. pylori* and 39.7% Non-Helicobacter pylori Helicobacters (NHPH). These findings are concerning because there is an unexpected hidden unexpected enemy in the form of Non-Helicobacter pylori Helicobacters (NHPH), hence, diagnosis and treatment guidelines should be changed to consider the Non-Helicobacter pylori Helicobacter (NHPH) beside *H. pylori*.

In the present study, the designed primers showed considerable sensitivity to low concentrations of specific DNA in clinical samples, allowing sensitive detection in different types of contaminated samples⁵¹.

Here comes the answer to an important question about the need for a newly designed set of specific primers instead of the primers used in the literature. In silico and laboratory comparative analysis should be established to determine the diagnostic utility of 16srRNA primers for genus-level identification of Helicobacter *spp.* In silico comparison showed reasonable results for H276, 16srRNA(a), heminested outer/inner, and nested HeliS-Helinet by primer-blast but did not achieve substantial findings with 16srRNA(b). In silico PCR amplification gave promising results with H276 (96.8%), Hcom (98.4%), nonetheless, the results were poor for 16srRNA(b), BFHpyl, 16srRNA(a), and Heid primers. From the mentioned results, the in silico evaluation is not an accurate method for a real evaluation of any primer but is only a preliminary step, so laboratory evaluation is the confirmatory method for accurate evaluation. The specificity test revealed that ConsH primer is the best one in this marathon, as it has all considerations to be the gold standard primer for statistical evaluation of the comparable primers. The results of the laboratory concluded that the comparable primers offered false-positive results by non-specific binding to non-Helicobacter bacteria and some of them gave false-negative results as BFHpyl, Heid, and 16srRNA(b).

The comparative study was analyzed statistically to assess the diagnostic utility. The diagnostic parameters and ROC analysis of in silico and laboratory evaluation are demonstrated in Tables 3, 4, and Figs. 1, 2, which concluded that the in silico evaluation is not accurate enough to assess the diagnostic utility of any primer. That was evident in the case of heminested outer/inner primer which showed excellent diagnostic significance ($P < 0.0001$), 100% in most diagnostic parameters, and the best score of AUC (1.00) in ROC analysis. These results differ in the laboratory evaluation. This heminested primer produced false-positive results because it achieved positive PCR with all non-Helicobacter bacteria with 0.00% specificity (Table 4) and AUC (0.5) (Fig. 2), which demonstrated the unreliability of heminested outer/inner primer for G: Helicobacter diagnosis. The aforementioned findings disagree with Qin et al. who confirmed its reliability for G: Helicobacter identification and that it is a powerful diagnostic tool⁵². Hcom1, H276, and nested primer (HeliS/Heli-nest) indicated non-significant results for their use in G: Helicobacter diagnosis by in silico PCR amplification (Table 3) and considered insignificant tool for diagnosis, as they had AUC less than 0.8 in ROC analysis tool. The laboratory evaluation of these primers demonstrated a major difference with in silico evaluation as the statistical analysis revealed that they had significant diagnostic utility ($P < 0.001$) and were considered reliable for Helicobacter *spp.* diagnosis with AUC (0.950) in ROC analysis^{53–55}. However, the results of the current study are incompatible with Flahou et al. that Hcom1 is suitable for the identification of Helicobacter *spp.*⁵⁶, as it may give false-positive results with *K. pneumoniae*. H276 may give positive results with *S. mutans* found in the buccal cavity⁵⁷. The present study disagrees with Riley et al. who developed this primer and concluded that it was sensitive and specific to detect several numbers of Helicobacters and it was suitable for routine diagnosis⁵⁴. The nested primers (HeliS/Heli-nest) gave positive results with Salmonella *spp.* which is alarming due to misdiagnosis of gastrointestinal disturbance of Helicobacteriosis and Salmonellosis⁵⁵.

16srRNA (a, b) were unreliable based on in silico evaluation, but have a significance for diagnosis of Helicobacters ($P < 0.001$ and 0.004, respectively) and an acceptable AUC (0.875, 0.950, respectively) in ROC analysis. However, false-positive results may be detected in 16srRNA (b) with *E. fecalis*. Low sensitivity obtained with 16srRNA(a) (Se 75%). Consequently, these results disagree with Idowu et al. and tiwari et al. who concluded their specificity and sensitivity in Helicobacter *spp.* diagnosis^{55,58}. Finally, the worst primers that appeared in our study were clearly demonstrated in BFHpyl and Heid which have the lowest significance in in silico and laboratory evaluation ($P < 1.00$) and low AUC (0.513, 0.450, respectively) by ROC analysis (Table 4) (Figs. 1, 2). These results disagree with Flahou et al. and Farshad^{56,59}.

Our study explained a suitable comparative analysis of different primers for Helicobacters diagnosis by using ROC analysis and different statistical diagnostic parameters. Use of bioinformatics tools and command line to extract conserved regions of 16srRNA gene in Helicobacter *spp.* and *H. pylori* are used in the design of primers. Considerable detailed evaluation of the newly designed primers by specificity using sequenced Helicobacters and non-Helicobacters carefully selected from GIT microbes. Application of the designed primers in clinical samples (gastric biopsies) using sufficient representative sample size from dyspeptic patients introduced to three hospitals. The results were compared with the routine diagnosis in the hospitals and sequencing of the PCR products. Sensitivity testing was performed to find the detectable DNA concentration in highly contaminated samples (Stool). However, more non-Helicobacters and sequenced local Helicobacter strains should be used, and that will be considered in our future studies.

Conclusion

Our designed primers ConsH and PyloA/PyloAN are highly sensitive and specific and could be used for accurate diagnosis of *Helicobacter* diseases from different clinical samples. Moreover, PyloA/PyloAN indicated the abundance of Non-*Helicobacter pylori* *Helicobacter* (NHPH) and their unrecognized role in *Helicobacteriosis*. It can also be concluded that *in silico* evaluation is not adequately accurate to assess the diagnostic utility of the primers and must be accompanied by the laboratory evaluation. Also, we recommend more research on Non-*Helicobacter pylori* *Helicobacter* (NHPH) and correlation with the clinical aspects of the disease in comparison with *H. pylori*.

Material and methods

Ethical statement. This study was approved by the Research Ethics Committee at Educational Hospitals and institutions committee (GOTHI) (Approval number; HAM00116). This committee confirms that all experiment was performed in accordance with relevant guidelines and regulations. Informed consent was assigned by all participants in the study (provided in supplementary files).

Design new 16srRNA specific primers for G: *Helicobacter* and *Helicobacter pylori*. Selected strains for multiple sequence alignment (MSA) and determination of the G: *Helicobacter* and *H. pylori* conserved regions; 62 reference *Helicobacter* strains were used as mentioned below. *Helicobacter pylori* (2017, 2018, 26695, 26695, 35A, 51, 52, 83, 908, 17, Aklavik86, B38, B8, BM0(12A, 12S), Cuz20, ELS37, F(16, 30, 32, 57), G27, *Gambia*94/24, HPAG1, HUP-B14, India7, Lithuania75 OK113 DNA, OK310, P12, PeCan (18, 4), Puno(120, 135), Rif(1, 2), SJM180, SNT49, Sat464, Shi(112, 169, 417, 470), South Africa(20, 7), UM(032, 037, 066, 298, 299), XZ274, v225d, strain J99. Non-*Helicobacter pylori* *Helicobacter* (NHPH) as *Helicobacter acinonychis* str. Sheeba, *Helicobacter bizzozeronii* CIII-1, *Helicobacter cetorum* (MIT 00-7128 and MIT 99-5656), *Helicobacter cinaedi* (ATCC BAA-847 DNA and PAGU611). *Helicobacter felis* ATCC 49179, *Helicobacter hepaticus* ATCC 51449, *Helicobacter mustelae* 12198.

Multiple sequence alignment (MSA). All 16srRNA gene sequences of all selected *Helicobacter* strains were downloaded and saved in FASTA format to be used in MSA. MAFFT online version <https://mafft.cbrc.jp/alignment/server/> was used for MSA of all 16srRNA gene sequences of the selected strains with a distance matrix by counting the number of shared 6mers between every sequence pair. A guide tree was built, and the sequences were aligned progressively according to the branching order, then the tree was re-constructed, and finally, a second progressive alignment was carried out. MAFFT was applied twice to obtain the outputs of Pearson/FASTA and Clustal.

Gblocks tool command line for the determination of conserved regions. To detect the conserved regions of the alignment, the Gblocks tool was used to yield an htm file that can be viewed using any browser. The MSA of all *Helicobacter spp.* 16srRNA gene sequences were collected in a file named All_strains_16s.aln and MSA of all *H. pylori* strains 16srRNA gene sequences were collected in a file named All_H_pylori.aln. both files were used in The Gblocks command line in Terminal.

```
$ Gblocks All_Strains_16s.aln -t=d -p=y.
```

```
$ Gblocks All_H_pylori.aln -t=d -p=y.
```

The design of new specific PCR primers for *Helicobacter spp.* (ConsH) and nested primers for *H. pylori* (PyloA/PyloAN). The conserved regions of all *Helicobacter spp.* and *H. pylori* were used in ThermoFisher oligonucleotides design online version https://www.thermofisher.com/eg/en/home/life-science/oligonucleotides-prime-rs-probes-genes/custom-dna-oligos.html?s_kwcid=AL13652!3!506722412345!p!g!!thermo%20primer&ef_id=CjwKCAjw_o-HBhAsEiwANqYhp_8UC-ah9JbbfjH6_l3wIt2XPuxGgjhQjVGVJBB5ny9X8hWdECQ3hxoCa7oQAvD_BwE:G:s&s_kwcid=AL13652!3!506722412345!p!g!!thermo%20primer&cid=bid_mol_pch_r01_co_cp1358_pjt0000_bid00000_0se_gaw_bt_pur_con&gclid=CjwKCAjw_o-HBhAsEiwANqYhp_8UC-ah9JbbfjH6_l3wIt2XPuxGgjhQjVGVJBB5ny9X8hWdECQ3hxoCa7oQAvD_BwE, Which was used to design primers for PCR. Many probabilities were obtained for forward and reverse primers. Every pair was tested to select the most suitable pair.

Evaluation of the newly designed primers. *In silico* evaluation by primer-Blast and *in silico* PCR amplification. ConsH and PyloA/PyloAN primers were examined by primer-Blast online software <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>. The primers were also subjected to *in silico* PCR amplification using online software [http://In silico.ehu.es/](http://In%20silico.ehu.es/) against 62 *Helicobacter* strains including 53 *Helicobacter pylori* strains and 9 Non-*Helicobacter pylori* *Helicobacter* (NHPH) strains.

Laboratory evaluation. DNA extraction: The whole genome was extracted from three different samples, local isolate, gastric biopsies, and stool samples. DNA extraction using different extraction kits, Thermo scientific GeneJET Genomic DNA Purification kit#K0722, QIAamp DNA stool Mini kit#51604, and GF-1 Tissue DNA Extraction kit Vivantis cat.no: GF-TD-100.

PCR: The PCR mixture included 1x master mix (amaR OnePCR™, Cat.No. SM213-0250, GeneDireX, Inc.), 0.25 μmol forward and reverse primers, 10 ng DNA, and up to 20 μl nuclease-free water.

The amplification cycle for ConsH, pyloA/PyloAN was as follow: initial denaturation at 94 °C/3 minutes, 30 cycles of the following temperatures: 94 °C/30 sec, annealing at 60 °C/1 minutes, and cyclic extension at 72 °C/2

Primer	Sequence	Length	GC%	T _m °C	PCR product	References
H276 F	5'-CTA TGA CCG GTA TCC GGC-3'	18	61.1	55.4	376 bp	57
H676 R	5'-ATT CCA CCT ACC TCT CCC A-3'	19	52.6	54.8		
16S rRNA(b)-F	5'-CTG GAG AGA CTA AGC CCT CC-3'	20	60	57.4	112 bp	59
16S rRNA(b)-R	5'-ATT ACT GAG GCT GAT TGT GC-3'	20	45	52.8		
16srRNA(a)-F	5'-TAA GAG ATC AGC CTA TAT GTC C-3'	22	40.9	50.1	534 bp	58
16srRNA(a)-R	5'-TCC CAG GCT TTA AGC GCA AT-3'	20	50	57.8		
Hcom1	5'-GTA AAG GCT CAC CAA GGC TAT-3'	21	47.5	54.5	390 bp	56
Hcom2	5'-CCA CCT ACC TCT CCC ACA CTC-3'	21	61.9	59.8		
BHpyl_F	5'-AAA GAG CGT GGT TTT CAT GGC G-3'	22	50	58.9	217 bp	59
BHpyl_R	5'-GGG TTT TAC CGC CAC CGA ATT TAA-3'	24	45.8	57.9		
Heid F	5'-ATG GCT TAC AAC CCT AAA ATT TTA CAA-3'	27	34.4	57.4	1278 bp	62
Heid R	5'-TCA CAT GTT TTC AAT CAT CAC GC-3'	23	39.1	53.6		
Outer forward	5'-CTG GCG GCG TGC CTA ATA C-3'	19	63.2	59.7	1054 bp	55
Outer reverse	5'-CTC ACG ACA CGA GCT GAC-3'	18	61.1	56.2		
Inner forward	5'-CTG GCG GCG TGC CTA ATA C-3'	19	63.2	59.7	256 bp	55
Inner reverse	5'-ACC CTC TCA GGC CGG ATA CC-3'	20	65	62.1		
Heli-nestS	5'-ATT AGT GGC GCA CCG GTG AGT AA-3'	23	52.2	61.1	1315 bp	58
Heli-nestR	5'-TTT AGC ATC CCG ACT TAA GGC-3'	21	47.6	54.7		
Heli-S	5'-GAA CCT TAC CTA GGC TTG ACA TTG-3'	24	45.8	55.4	426 bp	58
Heli-R	5'-GGT GAG TAC AAG ACC CGG GAA-3'	21	57.1	59.3		

Table 5. The sequence of different primers for detection of G: Helicobacter.

minutes, final extension at 72 °C/5 minutes. Half a microliter of PCR product for the first nested PCR (PyloA) was amplified in the second PCR (PyloAN) for 20 cycles. Gel electrophoresis of the PCR products was performed in 1.5% agarose (Vivantis, cat.no: pco701) against a 100 bp DNA ladder (Vivantis, cat. no: NL1405).

Specificity testing. PCR application of newly designed primers using eight sequenced Helicobacter strains (5 *H. pylori* and 3 Non-Helicobacter pylori Helicobacters (NHPH), *H. hepaticus*, *H. cinidiae*, and *H. felis*) and 10 non-Helicobacter bacteria present in GIT included *E. coli*, *Klebsiella pneumoniae*, *Salmonella spp.*, *Proteus mirabilis*, *Enterococcus faecalis*, *Enterobacter spp.*, *Staphylococcus aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *Candida albicans*. All bacterial strains were sequenced reference. The newly designed primers were applied clinically as mentioned before on 243 gastric biopsies taken from dyspeptic patients admitted to Ahmed Maher Hospital (180 samples), El-Maadi Military Hospital (38 samples), and Military production Hospital in Helwan (25 samples). The patients were tested in the hospital by different invasive methods like endoscopic pictures, histopathology, and rapid urea test (RUT). The PCR positive samples were confirmed by the different invasive methods and sequencing of the PCR products.

Sensitivity testing. Serially diluted DNA of the whole genome of *H. pylori* (Local isolate) was introduced into a negative stool and biopsy samples in the following concentration (5 ng, 0.5 ng, 50 pg, 5 pg, 0.5 pg/μl). The final concentration in total volume PCR mixture (250 pg, 25 pg, 2.5 pg, 0.25 pg, 25 fg)⁶⁰.

Comparative analysis between the newly designed primer ConsH and different 16srRNA primers for Helicobacter spp. detection. The 16srRNA specific primers used in the comparative study. The comparative study was performed on 9 primers for the detection of G: Helicobacter compared to the newly designed primer in this study (ConsH) (Table 5).

In silico comparative study. These primers were examined by primer-blast and subjected to in silico PCR amplification.

Laboratory comparative study (specificity testing). PCR application of the comparable primers was performed on the sequenced Helicobacter strains and non-helicobacter strains.

Statistical evaluation of the diagnostic utility of different 16srRNA primers for G: Helicobacter detection in the literature. **Gold standard primer selection.** The selected gold standard primer for evaluation was the most specific one which gave negative results with all non-Helicobacter strains and positive results with all sequenced Helicobacter strains.

Statistical methodology. The sensitivity (true positive rate, TPR), specificity (true negative rate, TNR), positive predictive value (PPV) and negative predictive value (NPV), false-positive rate (FPR), false-negative rate (FNR),

positive likelihood ratio (LR+), negative likelihood ratio (LR-), accuracy (ACC), balanced accuracy (BA) and diagnostic odds ratio (DOR) were calculated for in silico and laboratory comparison of the nine comparable primers. Statistical analyses of screening tests for *Helicobacter spp.* using different primers were performed using Chi-square. Receiver Operating Characteristics (ROC) analysis for each screening test, the true positive rate (TPR) against false positive rate (FPR) can be measured compared to the gold standard. Values of $p \leq 0.05$ were considered statistically significant. Analyses were performed via SPSS version 26 software (SPSS Inc, Chicago, IL, USA) and R version 4.1.1 (R Foundation for Statistical Computing), with the 'epiR' package^{61,62}.

Data availability

The sequenced data were submitted to NCBI Genebank at these accession numbers, OL631161, OL631152, OL634838, OL634782, OL631585, OL631225, OL631133, OL630959, OL631249.

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References

- Parsonnet, J., Shmueli, H. & Haggerty, T. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *J. Am. Med. Assoc.* **282**, 2240–2245 (1999).
- Recordati, C. *et al.* Spatial distribution of *Helicobacter spp.* in the gastrointestinal tract of dogs. *Helicobacter* **14**, 180–191 (2009).
- Malferteiner, P. *et al.* Management of *Helicobacter pylori* infection—the Maastricht V/Florence consensus report. *Gut* **66**, 6–30 (2017).
- Yamaoka, Y. & Ansari, S. Current understanding and management of *Helicobacter pylori* infection: An updated appraisal. *F1000Research* **7**, 1–10 (2018).
- Falush, D. *et al.* Traces of human migrations in *Helicobacter pylori* populations. *Science* **299**, 1582–1585 (2003).
- Wang, G., Taylor, D. E. & Humayun, M. Z. Mutation as an origin of genetic variability in *Helicobacter pylori*. *Trends Microbiol.* **7**, 488–493 (1999).
- Suerbaum, S. & Josenhans, C. *Helicobacter pylori* evolution and phenotypic diversification in a changing host. *Nat. Rev. Microbiol.* **5**, 441–452 (2007).
- Kalia, A. *et al.* Evolutionary dynamics of insertion sequences in *Helicobacter pylori*. *J. Bacteriol.* **186**, 7508–7520 (2004).
- Khatoun, J., Prasad, K. N., Prakash Rai, R., Ghoshal, U. C. & Krishnani, N. Association of heterogeneity of *Helicobacter pylori* cag pathogenicity island with peptic ulcer diseases and gastric cancer. *Br. J. Biomed. Sci.* **74**, 121–126 (2017).
- Hanafiah, A. & Lopes, B. S. Genetic diversity and virulence characteristics of *Helicobacter pylori* isolates in different human ethnic groups. *Infect. Genet. Evol.* <https://doi.org/10.1016/j.meegid.2019.104135> (2020).
- Suerbaum, S. & Blaser, M. J. *Helicobacter pylori*. *Encycl. Microbiol.* <https://doi.org/10.1016/B978-012373944-5.00188-7> (2009).
- Engstrand, L., Nguyen, A. M., Graham, D. Y. & El-Zaatari, F. A. Reverse transcription and polymerase chain reaction amplification of rRNA for detection of *Helicobacter* species. *J. Clin. Microbiol.* **30**, 2295–2301 (1992).
- Myung, S. J. *et al.* Detection of *Helicobacter pylori* DNA in human biliary tree and its association with hepatolithiasis. *Dig. Dis. Sci.* **45**, 1405–1412 (2000).
- Monstein, H. J. & Ellnebo-Svedlund, K. Molecular typing of *Helicobacter pylori* by virulence-gene based multiplex PCR and RT-PCR analysis. *Helicobacter* **7**, 287–296 (2002).
- Sugimoto, M. *et al.* Unreliability of results of PCR detection of *Helicobacter pylori* in clinical or environmental samples. *J. Clin. Microbiol.* **47**, 738–742 (2009).
- Bulajic, M. *et al.* PCR in *Helicobacter spp.* diagnostic in extragastric malignancies of digestive system. *Eur. J. Gastroenterol. Hepatol.* **24**, 117–125 (2012).
- Fox, J. G. *et al.* Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* **114**(4), 755–763. [https://doi.org/10.1016/S0016-5085\(98\)70589-x](https://doi.org/10.1016/S0016-5085(98)70589-x) (1998) (PMID: 9516396).
- Patel, S. K., Pratap, C. B., Jain, A. K., Gulati, A. K. & Nath, G. Diagnosis of *Helicobacter pylori*: What should be the gold standard?. *World J. Gastroenterol.* **20**, 12847–12859 (2014).
- Calvet, X. Diagnosis of *Helicobacter pylori* infection in the proton pump inhibitor era. *Gastroenterol. Clin. North Am.* **44**, 507–518 (2015).
- Poynter, S., Phipps, J. D., Naranjo-Pino, A. & Sanchez-Morgado, J. M. Difficulties in the molecular diagnosis of *Helicobacter* rodent infections. *Vet. Microbiol.* **134**, 272–278 (2009).
- Goosen, C. *et al.* Evaluation of a novel heminested PCR assay based on the phosphoglucosamine mutase gene for detection of *Helicobacter pylori* in saliva and dental plaque. *J. Clin. Microbiol.* **40**, 205–209 (2002).
- Li, C. *et al.* High prevalence of *Helicobacter pylori* in saliva demonstrated by a novel PCR assay. *J. Clin. Pathol.* **48**, 662–666 (1995).
- Chisholm, S. A., Owen, R. J., Louise Teare, E. & Saverymuttu, S. PCR-based diagnosis of *Helicobacter pylori* infection and real-time determination of clarithromycin resistance directly from human gastric biopsy samples. *J. Clin. Microbiol.* **39**, 1217–1220 (2001).
- Koehler, C. I. *et al.* *Helicobacter pylori* genotyping in gastric adenocarcinoma and MALT lymphoma by multiplex PCR analyses of paraffin wax embedded tissues. *Mol. Pathol. MP* **56**, 36–42 (2003).
- Hulten, K. *et al.* *Helicobacter pylori* in the drinking water in Peru. *Gastroenterology* **110**, 1031–1035 (2001).
- Lage, A. P. *et al.* Diagnosis of *Helicobacter pylori* infection by PCR: Comparison with other invasive techniques and detection of cagA gene in gastric biopsy specimens. *J. Clin. Microbiol.* **33**, 2752–2756 (1995).
- Mapstone, N. P. *et al.* Identification of *Helicobacter pylori* DNA in the mouths and stomachs of patients with gastritis using PCR. *J. Clin. Pathol.* **46**, 540–543 (1993).
- Miyabayashi, H., Furihata, K., Shimizu, T., Ueno, I. & Akamatsu, T. Influence of oral *Helicobacter pylori* on the success of eradication therapy against gastric *Helicobacter pylori*. *Helicobacter* **5**, 30–37 (2000).
- Park, C. Y., Kwak, M., Gutierrez, O., Graham, D. Y. & Yamaoka, Y. Comparison of genotyping *Helicobacter pylori* directly from biopsy specimens and genotyping from bacterial cultures. *J. Clin. Microbiol.* **41**, 3336–3338 (2003).
- Song, Q., Haller, B., Schmid, R. M., Adler, G. & Bode, G. *Helicobacter pylori* in dental plaque: A comparison of different PCR primer sets. *Dig. Dis. Sci.* **44**, 479–484 (1999).
- Peek, R. M. *et al.* Detection of *Helicobacter pylori* gene expression in human gastric mucosa. *J. Clin. Microbiol.* **33**, 28–32 (1995).
- Smith, S. I. *et al.* Comparison of three PCR methods for detection of *Helicobacter pylori* DNA and detection of cagA gene in gastric biopsy specimens. *World J. Gastroenterol.* **10**, 1958–1960 (2004).
- Wang, J. *et al.* Comparison of cytotoxin genotypes of *Helicobacter pylori* in stomach and saliva. *Dig. Dis. Sci.* **47**, 1850–1856 (2002).
- Battles, J. K. *et al.* Diagnostic assay for *Helicobacter hepaticus* based on nucleotide sequence of its 16S rRNA gene. *J. Clin. Microbiol.* **33**, 1344–1347 (1995).
- Rosselló-móra, R. Minireview towards a taxonomy of bacteria and archaea based. **14**, 318–334 (2012).
- Sontakke, S. *et al.* Use of broad range 16S rDNA PCR in clinical microbiology. *J. Microbiol. Methods* **76**, 217–225 (2009).

37. Chakravorty, S., Helb, D., Burday, M. & Connell, N. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J. Microbiol. Methods* **69**, 330–339 (2007).
38. Schriefer, A. E. *et al.* A multi-amplicon 16S rRNA sequencing and analysis method for improved taxonomic profiling of bacterial communities. *J. Microbiol. Methods* **154**, 6–13 (2018).
39. Miifahussurur, M. & Yamaoka, Y. Diagnostic methods of helicobacter pylori infection for epidemiological studies: Critical importance of indirect test validation. *BioMed Res. Int.* <https://doi.org/10.1155/2016/4819423> (2016).
40. Mounsey, A. & Leonard, E. A. Noninvasive diagnostic tests for helicobacter pylori infection. *Am. Fam. Phys.* **100**, 16–17 (2019).
41. Abadi, A. B. T. Diagnosis of Helicobacter pylori using invasive and noninvasive approaches. *J. Pathog.* **2018**, 1–13 (2018).
42. Guo, J., Starr, D. & Guo, H. Classification and review of free PCR primer design software. *Bioinformatics* <https://doi.org/10.1093/bioinformatics/btaa910> (2020).
43. Ismail, H., Morgan, C., Griffiths, P., Williams, J. & Jenkins, G. A newly developed nested PCR assay for the detection of helicobacter pylori in the oral cavity. *J. Clin. Gastroenterol.* <https://doi.org/10.1097/MCG.0000000000000310> (2016).
44. Khadangi, F., Yassi, M. & Kerachian, M. A. Review: Diagnostic accuracy of PCR-based detection tests for Helicobacter Pylori in stool samples. *Helicobacter* <https://doi.org/10.1111/hel.12444> (2017).
45. Bustin, S. A., Mueller, R. & Nolan, T. Parameters for Successful PCR Primer Design. *Methods in molecular biology* (ed Clifton, N. J.) 5–22 (2020).
46. Borah, P. Primer designing for PCR (2011).
47. Hinchliff, C. E. & Smith, S. A. Some limitations of public sequence data for phylogenetic inference (in Plants). *PLoS ONE* <https://doi.org/10.1371/journal.pone.0098986> (2014).
48. Deng, L., He, X. Y., Tang, B., Xiang, Y. & Yue, J. J. An improved quantitative real-time polymerase chain reaction technology for Helicobacter pylori detection in stomach tissue and its application value in clinical precision testing. *BMC Biotechnol.* **20**, 1–9 (2020).
49. Park, C. G., Kim, S., Jeon, H. S. & Han, S. Validation of loop-mediated isothermal amplification to detect Helicobacter pylori and 23S rRNA mutations: A prospective, observational clinical cohort study. *J. Clin. Lab. Anal.* **35**, 1–8 (2021).
50. Sohrabi, A. *et al.* Efficacy of loop-mediated isothermal amplification for h. Pylori detection as point-of-care testing by noninvasive sampling. *Diagnostics* **11**, 1538 (2021).
51. Qiu, E., Li, Z. & Han, S. Methods for detection of Helicobacter pylori from stool sample: current options and developments. *Braz. J. Microbiol.* <https://doi.org/10.1007/s42770-021-00589-x> (2021).
52. Qin, H. *et al.* Diagnosis of Genus Helicobacter through a hemi-nested PCR assay of 16S rRNA. *Saudi Pharm. J.* **24**, 265–272 (2016).
53. Choi, Y. K., Han, J. H. & Joo, H. S. Identification of novel Helicobacter species in pig stomachs by PCR and partial sequencing. *J. Clin. Microbiol.* **39**, 3311–3315 (2001).
54. Riley, L. K., Franklin, C. L., Hook, R. R. & Besch-Williford, C. Identification of murine helicobacters by PCR and restriction enzyme analyses. *J. Clin. Microbiol.* **34**, 942–946 (1996).
55. Tiwari, S. K., Khan, A. A., Ibrahim, M., Habeeb, M. A. & Habibullah, C. M. Helicobacter pylori and other Helicobacter species DNA in human bile samples from patients with various hepato-biliary diseases. *World J. Gastroenterol.* **12**, 2181–2186 (2006).
56. Flahou, B. *et al.* Diversity of zoonotic enterohepatic Helicobacter species and detection of a putative novel gastric Helicobacter species in wild and wild-born captive chimpanzees and western lowland gorillas. *Vet. Microbiol.* **174**, 186–194 (2014).
57. Forssten, S. D., Björklund, M. & Ouw, A. C. Streptococcus mutans, caries and simulation models. *Nutrients* **2**, 290–298 (2010).
58. Idowu, A. *et al.* Detection of Helicobacter pylori and its virulence genes (cagA, dupA, and vacA) among patients with gastro-duodenal diseases in Chris Hani Baragwanath Academic Hospital, South Africa. *BMC Gastroenterol.* **19**, 1–10 (2019).
59. Farshad, S., Rasouli, M. & Alborzi, A. Simultaneous detection of Helicobacter genus and Helicobacter pylori species using a multiplex PCR method. *Iran. Biomed. J.* **8**, 205–209 (2004).
60. Lu, J. J. *et al.* Comparison of five PCR methods for detection of Helicobacter pylori DNA in gastric tissues. *J. Clin. Microbiol.* **37**, 772–774 (1999).
61. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria. Available: <https://www.R-project.org/>. Accessed 29 Aug 2021.
62. Stevenson, M. & Sergeant, E. with contributions from Nunes, T., Heuer, C., Marshall, J., Sanchez, J., Thornton, R., Reiczigel, J., Robison-Cox, J., Sebastiani, P., Solymos, P., Yoshida, K., Jones, G., *et al.* epiR: Tools for the analysis of epidemiological data. Available: <https://CRAN.R-project.org/package=epiR>. Accessed 29 Aug 2021.

Author contributions

Sh.A.; Conception, Study Design, Methodology, Data interpretation, Acquisition, Drafting, Revision, Submission. K.Sh.; Methodology, Drafting. W.H.; Funding, Conception, Study Design. M.A.; Clinical sample collection. A.A. Clinical sample collection. S.A. Clinical sample collection. M.A. Clinical sample collection. M.M. Clinical sample collection. M.A. Statistical analysis and visualization.

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Competing interests

The authors declare no competing interests.

Additional information

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