Infection and Drug Resistance

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ORIGINAL RESEARCH

Development and Validation of Multiplex Quantitative PCR Assay for Detection of *Helicobacter pylori* and Mutations Conferring Resistance to Clarithromycin and Levofloxacin in Gastric Biopsy

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Aims and Objectives: More than half of the world's population is infected with *Helicobacter pylori*, which can cause chronic gastritis. WHO has regarded clarithromycin-resistant *H. pylori* as a high priority pathogen. Hence, accurate diagnosis and detection of clarithromycin- and levofloxacin-resistant *H. pylori* strains is essential for proper management of infection. The objective of this study was to develop and optimize multiplex quantitative PCR assay for detection of mutations associated with clarithromycin and levofloxacin resistance in *H. pylori* directly from the gastric biopsies.

Materials and Methods: Specific primers and probes were designed to amplify *ureA* and mutations in 23S rRNA and *gyrA* genes. Singleplex and triplex qPCR assays were optimized and the assay's sensitivities and specificities were determined. The optimized multiplex qPCR assay was performed on 571 gastric biopsies.

Results: In this study, 14.7% (84/571) of the gastric biopsies were positive for *H. pylori* by conventional methods and 23.8% (136/571) were positive by the *ureA*-qPCR with 96.4% sensitivity and 88.5% specificity, while the +LR and −LR were 8.72 and 0.04, respectively. The *ureA*-positive samples (n=136) were subjected to multiplex qPCR which detected A2142G and A2143G mutations in the 23S rRNA gene (20.6%, 28/136) conferring clarithromycin resistance and *gyrA* mutations N87K, N87I, D91N, and D91Y (11.8%, 16/136) leading to levofloxacin resistance. The sensitivity and specificity of qPCR of 23S rRNA gene were 100% and 98.7%, respectively, while 100% and 99.8% for qPCR of *gyrA*, respectively.

Conclusion: The effectiveness of this qPCR is that it is sensitive in detecting low bacterial load and will help in timely detection of clarithromycin- and levofloxacin-resistant strains, especially in case of mixed infections. Since it is culture independent, it can inform clinicians about antibiotics to be included in the first-line therapy, thereby improving the management of *H. pylori* infection at a much greater pace.

Keywords: *Helicobacter pylori*, multiplex qPCR, resistance, mutation, clarithromycin, levofloxacin

Introduction

Helicobacter pylori is a common pathogen that infects nearly 50% of the global population.[1] Infection with this pathogen causes chronic gastritis, which can cause chronic gastroduodenal diseases such as gastritis, gastric ulcer, duodenal ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) B-cell lymphoma.[2] Although *H. pylori* is the strongest known risk factor that account

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for 80% or more of gastric cancers, it is noteworthy that only 3% of infected patients subsequently develop gastric cancer.[3]

Antibiotic resistance in *H. pylori* is an important factor affecting the effectiveness of current therapeutic regimens. In the general population, the prevalence of bacterial resistance has been associated with antibiotic consumption and it varies in different geographic areas.[4] Antimicrobial resistance is the main factor leading to therapeutic failure, especially in the case of clarithromycin, which can induce a 70% loss of effectiveness in eradication of *H. pylori* as a part of proton pump inhibitor (PPI)/amoxicillin-based triple therapy.[5,6] Levofloxacin has been recommended as salvage therapy and is shown to be more effective than quadruple therapy in second- and third-line treatments of *H. pylori* infection.[7] However, resistance to clarithromycin or levofloxacin is the major reason for eradication failure after clarithromycin- or levofloxacin-based triple therapy, respectively.[8]

Clarithromycin and levofloxacin resistance are exclusively caused by specific mutations in a small region of the responsible genes, therefore molecular methods offer an alternative to conventional techniques for detection of these mutations.[9] Clarithromycin resistance in *H. pylori* is caused by mutations in the peptidyltransferase region encoded in domain V of the *H. pylori* 23S ribosomal RNA (rRNA) gene in the 50S ribosomal subunit. Clarithromycin stimulates the release of peptidyl-tRNA from the A site (aminoacyl accepter site), blocking the elongation of the nascent peptide chain during bacterial protein synthesis.[10] Mutations in the V domain of the 23S rRNA gene are the main cause of clarithromycin resistance in *H. pylori*. The most frequent mutations are A2143G, A2142G, and A2142C, which account for more than 80% of clarithromycin resistance in *H. pylori*. Other mutations such as A2115G, G2141A, C2147G, T2190C, C2195T, A2223G, and C2694A have also been reported, but their role in clarithromycin resistance is unclear.[11] The quinolone resistance determining region (QRDR) is a short conserved region within the *gyrA* (codon 74–113) and *gyrB* (codon 500–538) genes that has been shown to be associated with levofloxacin resistance.[12] Resistance to levofloxacin in *H. pylori* is mediated by the mutations at amino acid position 87 (Asn87) and 91 (Asp91) in the QRDR of the *gyrA* gene which has a more critical impact than *gyrB*.[13,14] Mutations in the *gyrB* are also reported in levofloxacin resistant strains, but often occur along with *gyrA* mutations.[15] The knowledge of resistance mechanisms may contribute to elaborate more rational antibiotic combinations with the aim of improving treatment success. There are various methods used to detect the presence of *H. pylori* infection and it can be categorized into two types, ie, invasive and non-invasive methods. These methods have their own advantages, and limitations.[16] Culture of *H. pylori* from biopsies is the gold standard for antibiotic susceptibility testing and is the most specific method for *H. pylori* detection. However, sensitivity of culture may be compromised, by strain-fastidiousness, the effect of therapy causing low cell density or altered morphologic features and loss of viability and/or overgrowth of contaminating microorganisms.[16]

Development of a rapid and reliable high-throughput method for detection of *H. pylori* resistance is expected to be highly useful for assisting in *H. pylori* management. This is particularly relevant in recognizing that culture and susceptibility testing of *H. pylori* should be mandatory after first treatment failure. Molecular detection of *H. pylori* using a PCR-based method is able to detect low amounts of bacterial DNA and can aid in detection of *H. pylori* at a much higher rate. Development of molecular methods for the detection of mutations involved in clarithromycin and/or levofloxacin resistance *H. pylori* in stool samples has been reported in several studies.[17–21] Quantitative PCR (qPCR) represents a highly sensitive and powerful technology for the quantification of *Helicobacter* DNA in gastrointestinal samples. As compared to other available methods for clinical and research purposes, qPCR is highly reliable, rapid, and sensitive for diagnosis of *H. pylori* infection.[22] To date, qPCR has been used extensively for the evaluation of antibiotic sensitivity of *H. pylori* in biopsy samples, specifically for the detection of clarithromycin resistance.[21,23–25] Conversely, there are only a few available qPCR assays for detection of *H. pylori* and mutations conferring resistance to both clarithromycin and levofloxacin antibiotics.[26,27] Therefore, the objective of this study was to develop and evaluate a multiplex qPCR assay for detection of *H. pylori* and gene mutations conferring clarithromycin and levofloxacin resistance in *H. pylori* in gastric biopsy specimens. This assay will provide us with a new tool along with rapid detection method and standard protocols for management and monitoring, and in improving outcomes of *H. pylori* eradication therapy.

Materials and Methods

Patients and Gastric Biopsy Specimens

Two hundred and eighty-eight patients with chronic dyspepsia undergoing upper oesophagogastroduodenoscopy (OGDS) at the Endoscopy Unit, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Kuala Lumpur,

Malaysia from April 2014 to August 2015 were included in this study. Patients on antibiotics or nonsteroidal anti-inflammatory drugs within 4 weeks prior to OGDS and those with gastric malignancy or immunosuppression were excluded. Three biopsies were obtained from antrum and/or corpus of the stomach from each patient. The biopsies were placed individually in different tubes for determination of *H. pylori* by conventional methods including culture, rapid urease test, and histological examination, as highlighted in the previous study.[28] A total of 571 gastric biopsies were obtained and *H. pylori* infection was determined in these biopsies by culture (n=571), rapid urease test (n=571), and histological examination (n=569; two biopsies were not suitable).

Clinical *H. pylori* Isolates

Culture was deemed positive when *H. pylori* growth was identified as Gram-negative curved/spiral rods by Gram staining and urease, catalase, and oxidase positive.[28] A total of 59 *H. pylori* were isolated by culture. Antibiotic susceptibility testing of clarithromycin and levofloxacin was performed as described in our previous study[28] using E-test and mutations were identified by gene sequencing.

Among the 59 *H. pylori* isolates; *H. pylori* strains A3, A26, C81, C55, A142, A129, A150, A177, and C192 were used as positive controls. All are curved/spiral shaped rods, urease-, catalase-, and oxidase-positive and positive for *glmM*,[29] as determined by conventional PCR. *H. pylori* strain C55 was used as a positive control for *H. pylori* detection by *ureA*-qPCR. Sequencing confirmed the genetic mutations of the 23S rRNA gene and *gyrA* of *H. pylori* in strains A3, A26, C81, A142, A129, A150, A177, and C192, which were used as positive controls for detection of clarithromycin or levofloxacin resistance. *H. pylori* strains A177 and A142 harboured the A2142G and A2143G mutations, respectively. *H. pylori* strains A129, A150, and C192 were wild-type strain, strain harbouring the N87K and N87I mutations, respectively. While *H. pylori* strains A3, A26, and C81 were wild-type strain, strain harbouring the D91N and D91Y mutations, respectively.

DNA Extraction

DNA from 59 *H. pylori* isolates and 571 gastric biopsies (used for rapid urease test) were extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corporation, Taiwan) according to the manufacturer's instructions. The extracted DNA was stored at −20°C until use. Concentration and purity of DNA sample were measured by determining the absorbance of optical density at the wavelength of 260 nm and 280 nm using an ultraviolet (UV) spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA). Samples with low (<1.5) or high (>2.0) OD_{260}/OD_{280} ratio were not used for qPCR, and the extraction was repeated.

Primers and Probes Design

GenBank was searched for sequences of *H. pylori ureA* gene. The published sequences of *H. pylori* 23S rRNA gene (Accession no. KC556778), *gyrA* (Accession no. L29481), the wild type *H. pylori* ATCC 26695, and sequences from clinical *H. pylori* strains were aligned using CLUSTAL W Multiple Alignment[30] in Bioedit Version 7.2.5, and primers and probes were designed by using the online PrimerQuest Tool on the IDT website (https://sg.idtdna.com/PrimerQuest/Home/Index). Primers and probes for *ureA* were selected from a region with 100% homology between all published *H. pylori ureA* encoding sequences. The BLAST search revealed 100% homology to the *H. pylori*-specific *ureA* gene only. Primers and probes for the 23S rRNA gene were designed to target the location of the two adjacent adenines where mutations A2142 and A2143 occur conferring resistance to clarithromycin in the 23S rRNA gene. Primers and probes for the *gyrA* gene were designed targetting mutations in codon positions 87 and 91 of the *H. pylori gyrA* (N87K and N87I, and D91N and D91Y). A BLAST search was performed to check the specificity of the DNA sequences of the primers and probes (http://www.ncbi.nlm.nih.gov/BLAST/). The properties of primers and probes were assessed for the optimized melting temperatures, secondary structure, base composition, and amplicon lengths. Potential self-annealing and hairpin formation were checked using free oligo self-complementarity check software (http://biotools.nubic.northwestern.edu/OligoCalc.html). The primers and probes were synthesized by Integrated DNA Technologies (Singapore Science Park II, Singapore) and Macrogen (Synapase, Singapore) and are listed in Table 1.

Detection of *H. pylori* in Biopsy Samples by qPCR

For the *ureA* detection, the reaction mixture was prepared in a final volume of 20 µL at the final concentration

Table 1 Oligonucleotides Sequence Used in This Study

Primers/Probes	Sequences (5′→3′)	Targets	Product (bp)
UreA-F[a]	AGTTCCTGGTGAGTTGTTCTT	*ureA*	120
UreA-R[b]	TGGAAGTGTGAGCCGATTT		
Cla-F[a]	CTGCGCATGATATTCCCGTT	23S rDNA	157
Cla-R[b]	CGACCTGCATGATGGCGTA		
Lev-F[a]	CGCCATCAATAGAGCCAAAG	*gyrA*	136
Lev-R[b]	GTGGGTGATGTGATTGGTAAAT		
UreA-P[c]	FAM-AAATGTTGGCGACAGACGGTTC-TAMRA	*ureA*	
Cla-PWT[c]	Cy5-AAGGTCCACGGGGTCTTTCCGTC-BHQ2	Wild type	
Cla-P2142G[c]	HEX-AGGTCCACGGGGTCTTCCCGTCT-BHQ2	A2142G	
Cla-P2143G[c]	FAM-AGGTCCACGGGGTCTCTCCGTCTT-BHQ2	A2143G	
Lev-N87WT[c]	HEX-CCG+C+G+TTA+T+CG-IowaBlack®FQ	Wild type	
Lev-N87K[c]	6-FAM-CCG+C+T+TT+A+T+CGC-IowaBlack®FQ	N87K	
Lev-N87I[c]	Cy5-CCG+C+A+AT+A+T+CGC-IowaBlack®RQ-Sp	N87I	
Lev-D91WT[c]	HEX-TA+T+G+AT+GCG+CT-IowaBlack®FQ	Wild type	
Lev-D91N[c]	Cy5-TTA+T+A+ATG+C+G+CTAGT-IowaBlack®RQ-Sp	D91N	
Lev-D91Y[c]	6-FAM-TTA+T+T+ATG+C+G+CTAGT-IowaBlack®FQ	D91Y	

Notes: [a]Forward primer, [b]Reverse primer, [c]TaqMan probe.

containing 10 µL of 1×Probe PCR master mix reaction buffer (contains Tris-Cl, KCl, NH_4Cl, $MgCl_2$, dNTP, and QuantiNova DNA Polymerase) (QuantiNova™ Probe PCR kit, Qiagen, Corbett Robotics, Australia), 0.5 µM primer UreA-F, 0.5 µM primer UreA-R, 0.2 µM of UreA-probe, 2.5 µL template DNA, and RNase free water. The amplification reaction was performed using Rotor-Gene Q (Qiagen, Corbett Robotics, Australia), with preliminary denaturation for 2 minutes at 95°C, followed by 40 amplification cycles of denaturation at 95°C for 5 seconds and combined annealing/extension step for 5 seconds at 60°C. Samples were run in duplicate and were considered positive if both reactions were positive. DNAs of two *H. pylori* reference strains (ATCC 43526 and J99) and one clinical strain C55 were used as positive with negative controls containing DNA template replaced with sterile distilled water. A standard curve was generated using 10-fold serially diluted samples of DNA extracted from *H. pylori* strain C55, varying from 100 ng/µL to 1 fg/µL (10^0 to 10^{-7}) of 1.22×10^8 CFU/mL which were used as a template for qPCR. The fluorescence reading for each sample was taken at the annealing step green channel [6-carboxyfluorescein (FAM)]. To verify the amplified *ureA*, all qPCR products were electrophoresed on 1.5% agarose gel and visualized under UV light with GelStain staining.

In order to exclude the effect of human DNA on the *H. pylori* DNA quantification, the human albumin gene (human genome consists of only one copy of the albumin gene) was amplified to quantify the number of human cells in the biopsy using the following primers: Forward (5′-CTGCATTGCCGAAGTGGAA-3′) and Reverse (5′-CAAACATCCTTACTTTCAACAAAATCA-3′), and the probe (5′-FAM-TGCCTGCTGACTTGCCTTCATT AGCTG-3′-TAMRA).[31] DNA extracted from human whole blood was used to determine the DNA concentration and purity, and used as a template for standard curve for human cells. Conversion of DNA template (ng) to copy number (C, copies/µL) in each target was carried out using the formula as described previously.[32] *H. pylori* DNA load in gastric biopsy was expressed as the quotient between the copy number of microorganisms and copy number of human cells in each gastric sample[31] multiplied by 100 to provide the number of copies of *H. pylori* DNA per 100 human cells.[33]

The limit of detection of the *ureA* qPCR assay was determined using 10 serially diluted DNA samples of *H. pylori* strain C55. DNA concentration ranging from 100 ng/µL to 0.01 fg/µL were used as a template for qPCR. The specificity of the primers and probes were investigated by applying the DNA of various bacterial species, including urease producing bacterial strains (clinical isolates) such as *Staphylococcus aureus, Acinetobacter* spp., *Vibrio parahaemolyticus, Proteus mirabilis, Serratia* spp., *Staphylococcus epidermidis, Enterobacter* spp., *Klebsiella pneumoniae*, and *Bacillus subtilis*. *H. pylori* ATCC 43526 and *H. pylori* J99 were used as positive control.

Detection of Point Mutation in the 23S rRNA Gene of *H. pylori* in Biopsy Samples by Multiplex qPCR

The *ureA*-positive samples were subjected to multiplex qPCR for the detection of mutation in 23S rRNA gene. The reaction mixture was prepared in a final volume of 20 µL containing five µL of 1× Multiplex PCR master mix (contains QuantiNova DNA Polymerase, QuantiNova multiplex PCR buffer and dNTP mix) (QuantiNova™ Multiplex PCR kit, Qiagen, Corbett Robotics, Australia), 0.4 µM primer Cla-Fw, 0.4 µM primer Cla-Rw, 0.25 µM of ClaPWT, Cla-P2142G and Cla-P2143G TaqMan probe, 2.5 µL of template DNA and RNase free water. The amplification was performed using Rotor-Gene Q (Qiagen, Corbett Robotics, Australia) with conditions of preliminary denaturation for 2 minutes at 95°C, followed by 40 amplification cycles of denaturation at 95°C for 5 seconds and combined annealing/extension step for 30 seconds at 60°C. Samples were run in duplicate and were considered positive if both reactions were positive. *H. pylori* J99 strain and clarithromycin resistant A177 (mutation A2142G) and A142 (mutation A2143G) were used as positive control with sterile distilled water used in negative control. The standard curves were generated individually using 10-fold dilutions of DNA ranging from 72 ng to 7.2 fg of 1.15×10^8 CFU/mL for wild-type strain (*H. pylori* J99), 150 ng to 15 fg of 1.1×10^8 CFU/mL for *H. pylori* A177, and 100 ng to 10 fg of 1.38×10^8 CFU/mL for A142. The seven 10-fold serial dilutions of each DNA were used as a template for qPCR. The fluorescence reading for each sample was taken at the annealing step on red channel (Cy5), yellow channel (HEX), and green channel [6-carboxyfluorescein (FAM)]. To verify the amplified 23S rRNA gene sequence, all qPCR products were electrophoresed on 1.5% agarose gel and visualized under UV light with GelStain staining.

Singleplex PCR was performed before conducting multiplex reactions in order to validate the performance of qPCR assay. The reaction mixture was prepared in a final volume of 20 µL containing 5 µL of 1×Multiplex PCR master mix (QuantiNova™ Multiplex PCR kit, Qiagen, Corbett Robotics, Australia), 0.4 µM primer Cla-Fw, 0.4 µM primer Cla-Rw, 0.25 µM of ClaPWT or Cla-P2142G or Cla-P2143G TaqMan probe, and 2.5 µL of template DNA and RNase free water. For multiplex reaction, the reaction mixture was prepared in a final volume of 20 µL containing 5 µL of 1×Multiplex PCR master mix (QuantiNova™ Multiplex PCR kit, Qiagen, Corbett Robotics, Australia), 0.4 µM primer Cla-Fw, 0.4 µM primer Cla-Rw, 0.25 µM of ClaPWT, Cla-P2142G, and Cla-P2143G TaqMan probe and 2.5 µL of template DNA and RNase free water.

The above reactions of singleplex and multiplex were proceeded independently and the C_q values of the assay were compared. The values obtained for a given target in the singleplex and multiplex assays should not differ significantly.

In order to evaluate the sensitivity of the multiplex qPCR assay, 10-fold dilutions of DNA ranging from 72 ng to 0.0072 fg of 1.15×10^8 CFU/mL for wild-type strain (*H. pylori* J99), 150 ng to 0.0015 fg of 1.1×10^8 CFU/mL for *H. pylori* A177 and 100 ng to 0.001 fg of 1.38×10^8 CFU/mL for *H. pylori* A142 were prepared independently. The bacterial DNA was used as a template for qPCR.

The specificity of the 23S rRNA gene qPCR assay was determined by testing other bacterial strains including *Acinetobacter* spp., *B. subtilis, S. flexneri, P. mirabilis, Serratia* spp, *S. typhimurium, E. coli, S. epidermidis, P. aeruginosa, Enterobacter* spp., *K. pneumoniae, V. parahaemolyticus, V. cholera, S. aureus*, and *Flavobacterium* spp. *H. pylori* ATCC 43526 and *H. pylori* J99 were used as positive controls.

Detection of Point Mutation in *gyrA* from Biopsy Samples by Multiplex qPCR

The *ureA*-positive samples were subjected to multiplex qPCR for detection of the two different sites of mutation in *gyrA* (amino acids positions 87 and 91). For *gyrA* amplification at amino acid position 87 (136 bp), the reaction mixture was prepared in a final volume of 20 µL containing 5 µL of 1×Multiplex PCR master mix (QuantiNova™ Multiplex PCR kit, Qiagen, Corbett Robotics, Australia), 0.4 µM primer Levo-Fw, 0.4 µM primer Levo-Rw, 0.25 µM of Levo-N87WT, Levo-N87K and Levo-N87I TaqMan probe, 2.5 µL of template DNA and RNase free water. *H. pylori* A129 (the wild-type), *H. pylori* A150 (levofloxacin-resistant harbouring mutation N87K) and *H. pylori* C192 (levofloxacin-resistant harbouring mutation N87I) were used as positive controls with sterile distilled water in the negative control. The standard curves were generated individually using 10-fold dilutions of DNA ranging from 100 ng to 10 fg of 1.28×10^8 CFU/mL for wild-type strain (*H. pylori* A129), 160 ng to 16 fg of 1.45×10^8 CFU/mL for *H. pylori* A150, and 145 ng to 14.5 fg of 1.31×10^8

CFU/mL for *H. pylori* A192. The 10-fold serial dilutions of each DNA were used as a template for qPCR.

For *gyrA* amplification at amino acid position 91 (136 bp), the reaction mixture was prepared in a final volume of 20 µL at a final concentration containing 5 µL of 1×Multiplex PCR master mix (QuantiNova™ Multiplex PCR kit, Qiagen, Corbett Robotics, Australia), 0.4 µM primer Levo-Fw, 0.4 µM primer Levo-Rw, 0.25 µM of Levo-D91WT, Levo-D91N and Levo-D91Y TaqMan probe, 2.5 µL of template DNA, and RNase free water. *H. pylori* strains A3 (wild-type), A26 (levofloxacin-resistant harbouring mutation D91N), and C81 (levofloxacin-resistant harbouring D91Y) were used as positive controls with sterile distilled water added in the negative control. The standard curves were generated individually using 10-fold dilutions of DNA ranging from 120 ng to 12 fg of 1.59×10^8 CFU/mL for *H. pylori* strain A3, 110 ng to 11 fg of 1.25×10^8 CFU/mL for *H. pylori* A26, and 180 ng to 18 fg of 1.98×10^8 CFU/mL for *H. pylori* C81. The 10-fold serial dilutions of each DNA were used as a template for qPCR.

The amplification reaction was performed using Rotor-Gene Q (Qiagen, Corbett Robotics, Australia) with conditions of preliminary denaturation for 2 minutes at 95°C, followed by 40 amplification cycles of denaturation at 95°C for 5 seconds and combined annealing/extension step for 30 seconds at 60°C. Samples were run in duplicate and were considered positive if both reactions were positive. The fluorescence reading for each sample was taken at the annealing step on red channel (Cy5), yellow channel (HEX), and green channel [6-carboxyfluorescein (FAM)]. To verify the detected *gryA* sequence, all qPCR products were electrophoresed on 1.5% agarose gel and visualized under UV light with GelStain staining.

In order to validate the *gyrA*-N87 and *gryA*-D91 multiplex qPCR assay, the primer optimization, the performance of singleplex and multiplex reactions, the sensitivity and specificity of the multiplex qPCR assay, and the performance of multiplex qPCR to detect the multiple strains in the same biopsy sample were performed following the protocol as described above for detection of mutations in the 23S rRNA gene.

The discrepancy of results between qPCR and E-test were analyzed by sequencing using BigDye terminator v3.1 sequencing kit on an ABI 3730×L sequencer (MyTACG Bioscience Enterprise, Selangor, Malaysia). The primers used were Cla primers for detection of clarithromycin and Lev primers for detection of levofloxacin resistance (Table 1). After sequencing, a BLAST search was performed to check the sequence similarity/identity of the DNA sequences.

qPCR Parameters and Acceptance Criteria

The qPCR measures the fluorescence at each annealing step. Rotor-Gene Q series software was used to analyze the data. An amplification plot was generated showing the increase in the reporter dye fluorescence (Rn) with each cycle of PCR. Quantification of input target amount was analyzed by quantification cycle (Cq) value that is the point at which the sample of PCR amplification plot crosses the threshold. An acceptable standard curve was considered if a difference of −3.1±0.5 cycles was demonstrated between each of the 10-fold dilutions, and highly efficient and reproducible reactions if the correlation coefficient (R^2) from all independent experiments were in the range of 0.90–1.0 (R^2=0.90–1).

Statistical Analysis

Statistical analysis was performed using SPSS software version 23 (SPSS Inc, Chicago, IL, USA). Differences between groups of the categorical data were statistically evaluated using Pearson Chi-square (χ^2) test. The independent samples *t*-test is used to determine the positivity results between singleplex and multiplex qPCR. Differences of qPCR-negative and qPCR-positive were statistically evaluated using one sample Chi-square (χ^2) test. Kappa and McNemar tests were used to compare consistency between different detection methods. Differences were considered significant when the *P*-value was <0.05. The accuracy, sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio (+LR), and negative likelihood ratio (-LR) of the qPCR results were calculated.

Results

Detection of *H. pylori* in Biopsy Samples by qPCR

The specificity of the species-specific primers and qPCR protocol was optimized for the reference strains of *H. pylori* (*H. pylori* ATCC 43526 and *H. pylori* J99) and nine urease-producing strains. The *H. pylori* reference strains yielded specific qPCR amplification at $C_q\leq37$ cycles with expected amplicon of 120 bp. The species of different genera show amplification at $C_q\geq37$ cycles (Supplement 1) suggesting that other bacterial DNA of

different species did not cross-react with the primers, hence our primers were specific for *H. pylori*. The standard curve for quantification was generated using the optimized qPCR amplification assay conditions based on DNA concentration ranging from 100 ng to 0.01 fg. The standard curves showed correlation coefficients (R^2) of 0.990, and the quantification detection limits were 1 fg per reaction (Supplement 1). If one bacterium corresponds to 1.8 fg of DNA (1,667,867 bp) per genome,[34] so 1 fg equals 0.56 bacterial cell. Agarose gel electrophoresis of the qPCR products (120 bp) confirmed the desired quality of amplification. The qPCR products were sequenced to verify the amplified *ureA* with 100% homology with *H. pylori ureA* in GenBank.

The optimized qPCR was performed to amplify *ureA* fragments of *H. pylori* in all 571 gastric biopsies. The cut-off values that distinguish the positive and negative detection were calculated using Receiver Operating Characteristic (ROC) curve analysis for the normalized counts (bacterial density) (Supplement 2). The area under the ROC curve (AUC) was obtained (Table 2). The optimal cut-off points were calculated by definition of a positive result as the number of microorganisms that maximized the weighted combination of sensitivity and specificity (ie, that maximized the Youden index (*J*)=sensitivity+specificity−1).[39] Based on the curve coordinates obtained, a sample was considered positive if the value off $\geq$ 0.5 copies number per 100 human cells was chosen to maintain the highest Youden index of 98.5% (Table 2). The area under the ROC curve was 0.999 (95% confidence interval: 0.998–0.999) (Table 2), which indicates a good predictive of qPCR using this cut-off value.

The distribution of C_q values among qPCR-positive and -negative biopsy samples were analyzed to determine the C_q cut-off (Supplement 3). The distribution of C_q values allowed us to define an optimal cut-off at ≤30 cycles. The cut-off point at $\geq$ 0.5 copies number is confirmed by the fact that C_q values obtained from all positive biopsy samples (n=136) were lower than and equal to 30 cycles. No biopsy samples that were positive for qPCR of *ureA* exhibit at C_q values higher than 30 cycles (Supplement 3). Therefore, the qPCR result was considered positive if it resulted in an amplification plot with $C_q \leq 30$, ie, $\geq$ 0.5 copies number per 100 human cells.

For the detection of *H. pylori* by conventional methods for comparison, a biopsy sample was regarded as positive when it was positive by either culture, rapid urease test, or histology. The positive detection rate for qPCR assay was significantly higher than conventional methods [23.8%, (136/571) vs 14.7% (84/571), $P<0.0001$]. A total of 136 *H. pylori* positive biopsies by *ureA*-qPCR were detected in 92 out of 288 patients. These biopsies were from antrum and/or corpus stomach of the patients. The qPCR using biopsies from different sites of the stomach showed consistent results. The performances of qPCR in comparison to the results of conventional methods as the gold standard were analyzed. Based on the optimal cut-off value for qPCR, the sensitivity, specificity, and accuracy of the qPCR were 96.4% (95% confidence interval [CI]=89.1–99.1%), 88.5% (95% CI=85.3–91.1%) and 89.7%, respectively (Table 2). The qPCR results in the present study show acceptable sensitivity, specificity, and accuracy for detection of *H. pylori* in biopsy samples. The positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR), negative likelihood ratio (−LR) and were 58.8% (95% CI=50.1–67.1%), 99.3% (95% CI=97.8–99.8%), 8.72, and 0.04, respectively (Table 2). The Kappa value indicates

Table 2 Performance of the qPCR of *ureA* for Detection of *H. pylori* in 571 Biopsy Samples[a]

AUC[b] (95% CI)	Cut-Off	*J* (%)	No. of Biopsies with the Following Result:				Sp (%) (95% CI)	Se (%) (95% CI)	PPV (95% CI)	NPV (95% CI)	+LR	–LR	Accuracy (%)
			TP	TN	FP	FN							
0.999 (0.998–0.999)	0.5	98.5	80	432	56	3	88.5 (85.3–91.1)	96.4 (89.1–99.1)	58.8 (50.1–67.1)	99.3 (97.8–99.8)	8.72	0.04	89.7

Notes: [a]True-positive (TP) biopsies were defined as biopsies positive by both the qPCR and conventional methods. True-negative (TN) biopsies were defined as biopsies negative by both the qPCR and conventional methods. False-positive (FP) biopsies were defined as biopsies negative by conventional and positive by the qPCR technique. False-negative (FN) biopsies were defined as biopsies positive by conventional and negative by qPCR. [b]AUC=Area under the curve ROC, Cut-off denotes the cut-off points considering as positive any number of microorganisms (qPCR≥0.5 copy number/100 human cells) that is maximized the weighted combination of sensitivity and specificity (ie, that maximized the Youden index). *J*, Youden index=sensitivity+specificity-1. Kappa=0.67, *P* (McNemar)<0.0001.

Abbreviations: Sp, specificity; Se, sensitivity; PPV, positive predictive value; NPV, negative predictive value; +LR, positive likelihood ratio; –LR, negative likelihood ratio.

a substantial degree of agreement (Kappa=0.67, $P<0.0001$) of the qPCR assay and conventional methods for detection of *H. pylori* in gastric biopsy samples.

The qPCR assay enabled detection and quantification of *H. pylori* in biopsy samples. A total of the 571 biopsies were tested, 23.8% (136/571) were positive by qPCR based on the optimal cut-off value ($\geq$ 0.5 copies number per 100 human cells at the $C_q \leq 30$ value), while 76.2% (435/571) were negative for *H. pylori* (<0.5 copy number in the sample at the $C_q>30$ value). The *H. pylori*-positive biopsies by qPCR yielded amplification products with a predicted size of 120 bp. The sequences alignment showed 100% homology with published *H. pylori ureA* in GenBank. There was a significant difference among the *H. pylori*-positive and *H. pylori*-negative samples detection by qPCR ($p<0.0001$).

Optimization of multiplex qPCR of 23S rRNA gene, multiplex qPCR of *gyrA*87, and multiplex qPCR of *gyrA*91

The parameters used for validation of multiplex qPCR of 23S rRNA gene, qPCR of *gyrA*87, and qPCR of *gyrA*91 were primer concentration, singleplex qPCR reaction, triplex qPCR reaction, and singleplex versus triplex qPCR reactions. The optimum of triplex qPCR reaction determined the limit of triplex qPCR detection, specificity of triplex qPCR, and detection of multiple strains in the same biopsy samples. The key factors examined were the performance of singleplex versus triplex qPCR reaction. The results showed that multiplex reaction of 23S rRNA gene (Supplement 4), qPCR of *gyrA*87 (Supplement 5) and *gyrA*91 (Supplement 6) gave the same efficiency as the singleplex reaction. The tested *H. pylori* strains displayed only one C_q value for one genotype which showed no cross-reactivity with other non-targeted strains. There was no statistical significance (independent *t*-test *P*-value>0.05) of C_q value obtained for a given target in the singleplex and multiplex assays (Supplement 7–9) which proved that the probe for detection of 23S rRNA gene and *gyrA* are specific for each mutation. No fluorescence signal from other bacterial strains tested was obtained in all fluorescence colors (Supplement 10–12) and no PCR band was amplified.

Detection of Point Mutation in the 23S rRNA Gene of *H. pylori* in Biopsy Samples by Multiplex qPCR

The optimized multiplex qPCR of 23S rRNA gene was performed to amplify 23S rDNA fragments of *H. pylori* in 136 positive samples. The detection of point mutations in 23S rRNA gene by multiplex qPCR is shown in Table 3. Overall, wild-type strains were detected in 79.4% (108/136) of the biopsies and 20.6% (28/136) of the biopsies were infected with *H. pylori*-resistant strains. Among the 28 biopsies, 22 biopsies were infected with single genotype (A2142G genotype in three biopsies and A2143G genotype in 19 biopsies) while six biopsies were infected with mix genotype (wild-type and mutation strains). A mixture of resistant and susceptible was found in six biopsies, in which 66.7% (4/6) of the biopsies had wild-type and A2142G strains, while 33.3% (2/6) biopsies harboured wild type and A2143G strains. The performance of the multiplex qPCR of 23S rRNA gene is shown in Table 4. The result was obtained when multiplex qPCR of the 23S rRNA gene was compared with E-test as the gold standard. The sensitivity, specificity, and accuracy of qPCR were

Table 3 Detection of Clarithromycin Susceptibility Testing by E-Test, Sequencing and the Multiplex qPCR of 23S rDNA

No. of Samples	Culture	E-Test[b]	Sequencing[b] (No. of Samples)	qPCR of *ureA*	Multiplex qPCR of 23S rDNA[c] (No. of Samples)	Median of Bacterial Load (Bacteria)
38	Positive	Sensitive	T2182 (34) WT[a] (4)	Positive	WT[a] (38)	10^3
21	Positive	Resistance	A2142G (2) A2143G (19)	Positive	A2142G (2) A2143G (19)	10^2 10^3
70	Negative	Negative	Negative	Positive	WT[a] (70)	10^3
7	Negative	Negative	Negative	Positive	A2142G (1) A2142G + WT[a] (4) A2143G + WT[a] (2)	10^2 10^2 (A2142), 10^3 (WT) 10^2 (A2143), 10^3 (WT)

Notes: [a]No mutation at nucleotide position 2142 and 2143. [b]E-test and sequencing were performed on *H. pylori*-positive isolates by culture (59 clinical isolates). [c]Multiplex qPCR of 23S rDNA was performed on *H. pylori*-positive samples by qPCR of *ureA* (136 biopsy samples).

Table 4 Performance of the Multiplex qPCR of 23S rDNA and Multiplex qPCR of *gyrA* Assays for Detection of Clarithromycin and Levofloxacin Resistance in Biopsy Samples[a]

	No. of Biopsies with the Following Result:				Sp (%) (95% CI)	Se (%) (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (%)	Kappa	*P* (McNemar)
	TP	TN	FP	FN							
Clarithromycin resistance	21	542	7	0	98.7 (97.3–99.4)	100 (80.8–100)	75 (54.8–88.6)	100 (99.1–100)	98.8	0.851	0.016
Levofloxacin resistance	15	554	1	0	99.8 (98.9–99.9)	100 (74.7–100)	93.8 (67.7–99.7)	100 (99.1–100)	99.8	0.967	1.000

Notes: [a]True-positive (TP) biopsies were defined as biopsies resistant by both the qPCR and E-test. True-negative (TN) biopsies were defined as biopsies susceptible by both the qPCR and E-test. False-positive (FP) biopsies were defined as biopsies susceptible by E-test and resistant by the qPCR. False-negative (FN) biopsies were defined as biopsies susceptible by qPCR and resistant by E-test.
Abbreviations: Sp, specificity; Se, sensitivity; PPV, positive predictive value; NPV, negative predictive value.

100%, 98.7%, and 98.8%, respectively. Positive and negative predictive values were 75% and 100%, respectively.

Detection of Point Mutation in the *gyrA* Gene in Biopsy Samples by Multiplex qPCR-*gyrA*

The detection of point mutation in the *gyrA* gene in 136 positive samples by multiplex qPCR of *gyrA* is shown in Table 5. Overall, wild-type strains detected in biopsies were 88.2% (120/136) compared to 11.8% (16/136) with levofloxacin resistant *H. pylori*. Among the 16 biopsies, one biopsy was infected with a single genotype while 15 biopsies had a mixed genotype. The performance of the multiplex qPCR of *gyrA* assay is shown in Table 4. The sensitivity, specificity, and accuracy were 100%, 99.8%, and 99.8%. Positive and negative predictive values were 93.8% and 100%, respectively.

Comparison of Clarithromycin and Levofloxacin Susceptibility Testing by E-Test and qPCR

Clarithromycin and levofloxacin susceptibility testing by E-test and sequencing methods were performed on 59 *H. pylori* isolates, while qPCR was carried out on 136 *H. pylori*-positive biopsies. Comparison of the methods (Table 4) showed that Kappa values were 0.85 (P=0.016) and 0.97 (P=1.00), indicating the multiplex qPCR of 23S rRNA gene and qPCR of *gyrA* methods had a substantial degree of agreement with the gold standard of E-test for

Table 5 Detection of Levofloxacin Susceptibility Testing by E-Test and Sequencing to the Multiplex qPCR of *gyrA*

No. of Samples	Culture	E-Test[d]	Sequencing[d] (No. of samples)	qPCR of *ureA*	Multiplex qPCR *gyrA*[e] (No. of Samples)	Median of Bacterial Load (Bacteria)
44	Positive	Sensitive	WT[a] (44)	Positive	N87WT[b]+D91WT[c] (44)	10^5 (N87WT), 10^6 (D91WT)
15	Positive	Resistance	N87I (2) N87K (8) D91N (2) D91Y (3)	Positive	N87K (1) N87WT[b]+D91N (2) N87WT[b]+D91Y (2) N87K+D91Y (1) N87K+D91WT[c] (5) N87I+D91WT[c] (2) N87WT[b]+N87K+D91WT[c] (2)	10^1 10^5 (N87WT), 10^5 (D91N) 10^1 (N87WT), 10^6 (D91Y) 10^1 (N87K), 10^6 (D91Y) 10^4 (N87K), 10^7 (D91WT) 10^4 (N87I), 10^6 (D91WT) 10^4 (N87WT), 10^3 (N87K), 10^6 (D91WT)
76 1	Negative Negative	Negative Negative	Negative Negative	Positive Positive	N87WT[b]+D91WT[c] (76) N87K +D91WT[c] (1)	10^5 (N87WT), 10^6 (D91WT) 10^1 (N87K), 10^5 (D91WT)

Notes: [a]No mutation at amino acid position 87 and 91; [b]No mutation at amino acid position 87; [c]No mutation at amino acid position 91. [d]E-test and sequencing were performed on *H. pylori*-positive isolates by culture (59 clinical isolates). [e]Multiplex qPCR of *gyrA* was performed on *H. pylori*-positive samples by qPCR of *ureA* (136 biopsy samples).

detection of clarithromycin and levofloxacin resistance. For detection of clarithromycin-resistance, 59 biopsy samples were positive by culture and multiplex qPCR of 23S rRNA gene. From 571 biopsy samples, 77 were negative by culture but had amplification of *H. pylori* DNA and 23S rRNA gene by qPCR. Among the 77 multiplex qPCR of 23S rRNA gene-positive samples, 90.9% (n=70) had clarithromycin-susceptible strains, 1.3% (n=1) were clarithromycin-resistant (A2142G mutation) and 7.8% (n=6) contained mixed genotypes (four had wild-type+A2142G mutation and two had wild-type+A2143G mutation) (Table 3).

The 59 culture-positive *H. pylori* samples had 38 wild-type and 21 mutant genotypes detected by sequencing were also detected by multiplex qPCR for the 23S rRNA gene. Among the seven samples infected with resistant genotype as detected by multiplex qPCR of 23S rRNA gene, the median of bacterial load of the A2142G strains in the biopsies was 10^2, while biopsies with mixed genotype had 10^3 and 10^2 bacterial load of the wild type and the mutant genotypes, respectively (Table 3).

Detection of levofloxacin resistance by qPCR was concordant with E-test method where 44 out of 136 positive biopsies showed the presence of wild-type strains (Table 5). Fifteen samples had a single mutant genotype as determined by sequencing, however multiplex qPCR of *gyrA* showed a mixed genotype in 14 samples. Seventy-seven culture-negative biopsies had mixed infection by multiplex qPCR of *gyrA* assay. In 76 multiplex qPCR *gyrA*-positive samples, the median bacterial load in biopsies with mixed infection of wild type N87 and D91 were 10^5 and 10^6, respectively. On culture-negative biopsy, the median of bacterial load of mixed genotype strains of wild type D91 and N87K strains were 10^5 and 10^1, respectively.

Detection of Mixed Genotypes

Table 6 shows the detection of different resistance genotypes in biopsies from different anatomical sites. A mixed resistance genotype was identified in eight patients, of which five patients had different genotypes of clarithromycin, while three had different levofloxacin resistance genotypes. For the clarithromycin resistance genotype, there was a predominant infection at corpus site of the stomach with *H. pylori* strains than the antrum site. For levofloxacin, the antrum site showed mixed infection with wild type and mutant resistance genotype, while the corpus site was infected with either single wild type or mutant genotype.

Discussion

Diagnostic methods for detection of *H. pylori* infection are available, diverse, and the choice of one method or another depends on their advantages and disadvantages in each technique. The limitations associated with conventional techniques such as low concentration of *H. pylori* in biopsy sample is one of the main causes of failure to detect this bacterium by culture and other conventional methods. The molecular approaches based on DNA amplification have been used for *H. pylori* detection in clinical and environmental samples. The *ureA* gene has been reported as highly specific in *Helicobacter* spp. detection.[35] Therefore, in the present study, the *ureA* gene was selected for detection by qPCR method because of the highly conserved region and no cross-homologies to other closely related bacteria or subspecies of *Helicobacter*.

The successful quantification, limit of detection, and specificity of qPCR assay were investigated. In the present study, the lowest *H. pylori* DNA concentration detected was one femtogram. The correlation coefficient obtained

Table 6 Detection of Mixed Resistance Genotype from Antrum and Corpus Biopsies

Patient	Biopsy No.	Resistance Genotype	
		Antrum	Corpus
1	ETP34	Cla-wild type	A2142G mutation
2	ETP40	Cla-wild type	A2142G mutation
3	ETP177	A2142G mutation	Cla-wild type
4	ETP245	Cla-wild type	A2142G mutation
5	ETP250	Cla-wild type	A2142G mutation
6	ETP111	N87K mutation, D91-wild type	Lev-wild type
7	ETP274	N87-wild type, N87K mutation, D91-wild type	N87K mutation
8	ETP275	N87-wild type, N87K mutation, D91-wild type	N87K mutation, D91Y mutation

Abbreviations: Cla, clarithromycin; Lev, levofloxacin.

by linear regression analysis from this experiment was R^2=0.99, indicating highly efficient and reproducible reactions. The limit of qPCR in this study (one fg) was lower compared to the previous report by Ribeiro et al,[36] who reported 10 fg. The *ureA* qPCR assay is also specific for *H. pylori* detection because it positively detected *H. pylori* strains at C_q values of ≤30 with no fluorescence signal from other urease-producing bacterial strains detected at these C_q values. Experimental testing of the primer pair of qPCR assay resulted in high specificity for detection of *H. pylori* strains. The result showed that the qPCR positive result was detected in each tested *H. pylori* strains and negative for other tested bacterial species. This result suggested that the proposed primer sets gave a specific detection of *H. pylori* clinical strains and might be useful for diagnosis of *H. pylori* infection directly from gastric biopsies.

The cut-off point was determined and used to distinguish positive from negative results. The optimal cut-off point was defined as positive by the presence of any number of microorganisms (real-time PCR>0 microorganism/human cell) proposed by Saez et al,[31] while Al-Moayad et al[33] suggested a cutoff value of one bacterial per 100 human cells to distinguish cases from controls. Due to differences in laboratory practices, it is not feasible to directly compare the suggested cut-off point. Therefore, in this study the cut-off value was calculated using ROC curve analysis to distinguish positive from negative qPCR assays. Based on the obtained ROC curve coordinates, the value of ≥ 0.5 copies per 100 human cells was chosen to be a cut-off point that has the highest sensitivity and specificity (highest Youden index of 98.5%). Moreover, the distribution of C_q values clearly shows the C_q values of all positive biopsy samples were lower than and equal to 30 cycles (Supplement 3). Thus, biopsy samples with ≥ 0.5 copies which were reproducible at C_q values ≤30 were considered positive by qPCR.

With the new optimal cut-off point (≥ 0.5 copies number) obtained from this study, qPCR method used for detecting *H. pylori* in biopsy samples is a reliable test because of its acceptable sensitivity (96.4%) and specificity (88.5%) with an AUC of 0.999.

The prevalence of *H. pylori* infection in Malaysia varied from 14.1–30.4%.[37–39] Positive and negative predictive values are directly related to the prevalence of the disease/infection in the population.[40] In the present study, we observed a lower prevalence rate of *H. pylori* infection (16.7%) by conventional methods compared to previous studies. In 2010, 30.8% prevalence rate was obtained by conventional methods, while 19.2% (positivity was determined by culture) was reported in 2013.[41,42] Thus, the low prevalence rate of *H. pylori* infection in the present study might reflect the PPV and NPV of the test. Likelihood ratio is more informative to measure the clinically useful of qPCR in diagnosis of *H. pylori* infection because this parameter is independent of prevalence. In the present study, +LR of 8.72 indicates a 9-fold increase in the odds of *H. pylori*-infected patient has a positive qPCR result. The value of +LR greater than one and –LR is smaller than one obtained by qPCR compared to conventional methods indicate qPCR developed this study is very useful for detection of *H. pylori* directly from gastric biopsies.

The qPCR assay targeting *ureA* gene used to detect *H. pylori* in biofilm and drinking water has been conducted previously.[35,43] Saez et al[31] showed the sensitivity of qPCR targeting *ureA* gene used to detect *H. pylori* in the gastric antrum in bleeding and non-bleeding patients was 97.9% and 94.7%, respectively, while it was 68.4% in non-bleeding and 91.5% in bleeding patients for the gastric corpus. The specificity in both antrum (53.1% versus 73%) and corpus (56.3% versus 78.4%) was less in bleeding than in non-bleeding patients. It was found that the sensitivity and/or specificity obtained using *ureA*-qPCR in this study was higher than in previous reports.[21,31,33,44] A higher +LR (8.72 versus 4.3) and good –LR (0.04 versuss 0.63) was obtained in this study compared with that of Ramirez-Lazaro et al.[45]

The qPCR method shows a statistically significant higher percentage of *H. pylori* positivity compared to conventional methods (23.8% versus 14.7%, p<0.0001). Agreement between the tests according to Kappa analysis was at substantial degree (0.67) for qPCR and conventional methods. Our study showed that the false-negative and false-positive cases were determined as a low density of *H. pylori* by conventional methods, so the accuracy of qPCR may be decreased. The lower detection rate by conventional methods due to low concentration of *H. pylori* in biopsy samples and the microaerobic growth characteristic of *H. pylori* strains[46] are causes of failure to detect by culturing and other conventional techniques. The sensitivity of the qPCR method in this study was higher than that of the standard conventional methods, supporting the findings of other studies.[21,33,44] The lower specificity refers to more false positive results which was found in the test, however

good specificity value is obtained (>80%). The high number of false positive cases found by qPCR was probably due to detection of *H. pylori* DNA in the samples with low bacterial count, while the microorganism was not detected using conventional methods.[33] Therefore, by using a hypothetical ideal gold standard, the specificity of qPCR would be greater.[47] Similarly, He et al[48] reported that *H. pylori* DNA was detected by qPCR in 24 samples out of 27 that were negative by culture. Lascols et al[49] detected *H. pylori* DNA by qPCR in nine of the cases that were negative by both culture and histology. The sensitivity and specificity of *ureA* qPCR method depend on the optimum cut-off point. The cut-off value will improve the specificity of the assay, although sensitivity was slightly affected.[33] The ability to detect bacteria at very low counts by qPCR method can compromise its specificity when other techniques are used as gold standard.[33] This becomes particularly evident when the proportion of study subjects harbouring low counts is high, which was probably the case in the present study. An increase in the number of biopsy specimens taken might increase the bacterial load in the samples which the cut-off value needs to be recalculated and this improved the specificity of the assay.[31] In addition, a possible reason for the differences of detection with various methods is the topographic distribution of *H. pylori* colonization in the stomach causing the bias in sampling. This might interfere with the detection rate of *H. pylori* in each method[45] and sampling errors that might generate differences in colonization density.[36] Several studies have shown that the eradication of *H. pylori* in the treatment for some gastrointestinal diseases can be affected by bacterial density or localization.[50,51]

The primer pairs and probes were designed for multiplex qPCR assay to detect the two targets of point mutations in 23S rRNA gene and one target of wild-type sequence in the biopsy samples. In this study, multiplex assays require validation and optimization to confirm the results obtained from multiplex reaction was the same with the results if the reactions were performed separately. The quantitative sensitivity of multiplex qPCR of 23S rRNA gene assay was <10 bacteria per reaction tube, ie, <20 copies of the 23S rRNA gene (two gene copies of 23S rRNA gene present in genome of *H. pylori*) could be detected. These results are similar to previously published reports.[48,52] The results confirm that qPCR can accurately quantify the *H. pylori* density in gastric mucosa, while other conventional methods are unable for quantification. Multiplex qPCR of 23S rRNA gene assay is specific for *H. pylori* detection because there was no fluorescence signal from other bacterial species tested obtained in all three fluorescence colours and no PCR band was amplified. Multiplex qPCR of the 23S rRNA gene able to detect the two strains in a mixture with the lowest proportion of the mutant DNA was 1% (99/1). This result is better than those published for real-time PCR assays using allele-specific scorpion (5%, 95/5)[53] and using LightCycler (10%).[54–56]

In this study, a total of 35.6% (21/59) of strains were resistant to clarithromycin by E-test. The multiplex qPCR of 23S rRNA gene on the biopsy samples enabling detection of clarithromycin resistance in 20.6% (28/136) of the samples. Among these samples, 75% (21/28) were *H. pylori* positive detected by both culture and qPCR. In seven *H. pylori*-negative biopsies by culture, the qPCR was able to detect the presence of both single mutated genotype and mixed genotype. The median of bacterial load of *H. pylori* mixed genotype (strains harbouring both A2142G and A2143G mutations) is high, with a bacterial density in the range of 10^2 bacterial cells. This result indicates that the qPCR assay could be used to detect *H. pylori* clarithromycin resistance and possible to determine the number of microorganisms present in the biopsy samples. Other techniques are also useful for bacterial quantification; however, each technique has weaknesses. Culture is only semiquantitative and it is time-consuming.[49] Histology is also semiquantitative, but its accuracy is relatively weak.[47] Therefore, qPCR technique allows diagnosis of *H. pylori* and antibiotic susceptibility testing in the same day, which is much more rapid than culture which requires at least 5–10 days. The discrepant result between culture and qPCR among the *H. pylori*-negative biopsies were analyzed using sequencing method. Although sequencing was unable to detect mixed genotypes on the same alleles, it shows only mutated genotypes either A2142G or A2143G in the biopsy samples indicates that this mutant strain might be an infective strain. A reasonable explanation could be that strains of *H. pylori* harbour two copies of the 23S rRNA gene in the chromosomal DNA with a mutation in one gene copy is shown to confer resistance to clarithromycin.[57] Seven biopsies with single mutated (n=1) and mixed genotype (n=6) of *H. pylori* strains were considered as false negative by culture. This might be due to the low concentration of bacterial density, probable dead cells or decreased viability

of the microorganisms present in the specimens that could not be detected by culture.[58] *H. pylori* exist in viable non cultivable (coccoid) form cannot be cultured or detected by rapid urease test or histology, however the nonviable bacterial DNA were available for PCR amplification.[36,48,59] This result shows that the genotypic method is more sensitive in detecting bacterial populations in the same samples than the phenotypic method.[58]

The high performances of multiplex qPCR of 23S rRNA gene (sensitivity, 100%; specificity 98.7%) obtained in this study are comparable with previous reports which showed the range of the sensitivity and specificity were 90–100% and 92.5–99%, respectively.[22,49,56,60,61] The potential factors that could explain low specificity, including negative culture might be due to low density of bacterial cells and presence of dead bacterial cells. Compared with phenotypic clarithromycin resistance tested by E-test, mutations detected by qPCR revealed almost perfect concordance with E-test, since all of the resistance detected by E-test was able to be detected by qPCR (Kappa=0.85). The only discrepancy concerned was the mixture of susceptible and resistant genotypes, which were detected by qPCR than E-test. This approach allows us to recommend its use for detection of clarithromycin resistance directly on biopsy samples.

Development of molecular method to detect *gyrA* mutations is expected to be highly useful for management of the *H. pylori* eradication therapy with fluoroquinolones-containing regimens. In this study, the quantitative sensitivity of *gyrA* qPCR assay was <10 bacteria per reaction indicates that qPCR can accurately quantify the *H. pylori* density in gastric mucosa. The multiplex qPCR-*gyrA* able to detect the two strains in a mixture with the lowest proportion of the mutant DNA (1%; 99/1 ratio). The available commercial genotyping tests for levofloxacin resistance in *H. pylori* were not designed to detect the N87 and D91 mutations using triplex *gyrA* qPCR.[62] As such, because the N87 and D91 mutation were common in strains resistant to levofloxacin tested in this study, therefore the inability to detect these resistance strains would likely result in treatment failure.[62] Therefore, new primers were designed to detect both mutations (N87 and D91) in *gyrA* gene. Validation study of the multiplex qPCR-*gyrA* suggested that the proposed primer sets resulted in a specific detection of *gyrA* gene mutations in *H. pylori* clinical strains.

In this study, resistance to levofloxacin was observed in 25.4% (15/59) of the samples as determined by E-test. However, 11.7% (16/136) was resistance to levofloxacin as determined by multiplex qPCR of *gyrA*. Among these samples, 93.8% (15/16) were *H. pylori* positive detected by both culture and qPCR. In one *H. pylori*-negative biopsy by culture, the qPCR was able to detect the presence of mixed genotype (D91 wild-type with N87K mutation). Interestingly, it found that the mixtures of susceptible (D91 wild-type) and resistant (N87K) strain had high bacterial load (D91 wild-type, 10^5; N87K, 10^1 bacterial cells). This has been confirmed by sequencing which shows the presence of both genotypes (D91 wild-type and N87K mutation). The culturing of *H. pylori* from gastric biopsies is often difficult and does not always recover all the bacterial cells present in the sample. This finding shows that the genotypic method is more sensitive than the phenotypic method.[58]

This study reports very high performances of multiplex qPCR of *gyrA* (with sensitivity, 100%; specificity, 99.8%) for detection of levofloxacin resistance. Agreement between the tests according to Kappa analysis was almost perfect degree (0.97) for qPCR and E-test, indicating a qPCR result revealed a good concordance with E-test. There is only a discrepancy concerning whether the qPCR was better for detection of the mixtures between susceptible and resistant genotypes than by E-test. These results indicate that multiplex qPCR of *gyrA* exhibited high performance for detection of *H. pylori* levofloxacin resistance in biopsy samples.

The present study reveals that the presence of more than one genotype in a single biopsy specimen might be due to either multiple strains which coexist in the same site in the gastric mucosa or the presence of mutated and wild-type alleles of the same strain.[62–64] Kargar et al[24] revealed that acquisition of antibiotic resistance may be caused by the horizontal transferring from resistant cells to susceptible cells of the same strain which results in increasing the population of resistant strains. Hetero-resistance will also be misdetected unless a specific method to detect sequence diversity in resistance-related genes is employed.[65] In this study, the qPCR was able to detect the presence of mixed genotypes in *H. pylori*-negative biopsies by culture and mixed genotypes are still giving the bacterial load (10^1 bacterial cells for N87K and 10^5 bacterial cells for D91WT). A reasonable explanation could be that these mixed sequences are due to mixed infection[27] because there is a single-copy of *gyrA* gene presence in the *H. pylori* genome.[34] The presence of a mixed *H. pylori*

population in the same sample detected by qPCR is an advantage when compared with culture.

Analysis of the resistance genotype in different anatomical sites of gastric biopsies (antrum and corpus) shows the presence of mixed genotypes of resistance *H. pylori* in patients. We identified that clarithromycin resistance strains predominantly infected the corpus site, while levofloxacin resistance strains infected both the antrum and corpus. In addition, the mixed genotype of levofloxacin resistance (wild type and mutant strains) were detected in the antrum site. Although the number of samples with mixed infection were small, it supports the hypothesis that the existence of heterogenous *H. pylori* strains in different anatomical sites of the stomach occurs.[66] This shows multiplex qPCR assay in this present study is able to detect different resistance genotypes of *H. pylori* strains which cannot be detected by culture method. There is a possibility that the mixed genotypes of antibiotic resistance might not be found in a single individual biopsy and lead to undetected antibiotic resistance if a single biopsy is analyzed for antimicrobial susceptibility testing. The heterogeneity of *H. pylori* has important clinical significance and suggests that a single biopsy site might be unable to be considered representative of an antimicrobial susceptibility profile.[65] Several authors have indicated that multiple gastric biopsies from both the antrum and corpus should be taken for the assessment of antibiotic resistant *H. pylori* strains.[66–69]

In conclusion, we have developed a multiplex qPCR assay that permits reliable, accurate, fast, cost-effective detection and could easily be performed and quantify *H. pylori* infection directly on gastric biopsies as well as providing determination of the mutation resistance to clarithromycin and levofloxacin in a rapid manner. The qPCR test for *ureA* for *H. pylori* detection and two evaluated multiplex qPCR tests for detection of mutations in 23S rRNA gene and *gyrA* can overcome the weaknesses of culture and other conventional methods. Therefore, the results of the present study might be important for clinical management of the infection. Detection of a mixed population in the same biopsy sample by qPCR is an advantage to overcome the misinterpretation of the overall antibiotic susceptibility profile of the strain. The good performance of the developed multiplex qPCR assay of the present study is useful for diagnosis of *H. pylori* infection and detection of mutations in clarithromycin- and levofloxacin-resistance strains. These will help in improving the management of *H. pylori* infection by reducing the development of secondary resistance which has a negative impact on eradication of *H. pylori* infection.

Data Sharing Statement

Data will be shared and available from the corresponding author on reasonable request.

Ethical Approval and Informed Consent

The study was approved by the Medical Research Ethic Committee of the University (UKM 1.5.3.5/244/ETP-2013-042) and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors declare no conflicts of interest in this work.

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