

RESEARCH ARTICLE

Normalized real-time PCR for diagnosis of *H. pylori* infection

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ABSTRACT

Objectives: There is increasing interest in the use of quantitative PCR (q-PCR) for diagnosis of *H. pylori* infection. However, the assay remains largely unstandardized, making comparison between studies unreliable. The objective of this study was to assess accuracy of a normalized q-PCR assay for diagnosis of the infection.

Subjects and methods: Seventy-six fresh gastric biopsy specimens were collected from patients undergoing upper gastrointestinal tract endoscopy and examined by rapid urease test (RUT), culture, and a commercial TaqMan q-PCR assay targeting the *ureA* gene. Counts obtained from the latter assay were normalized to the human ACTB gene. A subject was considered to be infected if two or more assays were positive.

Results: The detection rates were 42.1%, 52.6%, and 78.9% by culture, RUT and q-PCR, respectively. Bacterial density ranged 0.005 to 4800 bacteria per 100 human cells. Because q-PCR showed low initial specificity (45.7%), the cutoff value for the assay was recalculated as 1 bacterium per 100 human cells, using ROC curve analysis. Accordingly, the sensitivities and specificities were 79.5% and 97.3%, respectively, for culture; 94.9% and 91.9%, respectively, for RUT; and 94.9% and 94.6%, respectively, for q-PCR. By gold standard, 39 of the dyspeptic patients (51.3%) were found to be infected.

Conclusions: With the identified cutoff value, the q-PCR assay diagnosed *H. pylori* infection with an accuracy slightly superior to that of RUT. However, the possibility that low counts detected only by q-PCR represent true infections warrants further investigation. Normalization of bacterial counts for standardization of q-PCR *H. pylori* assays is recommended.

Keywords: culture, *Helicobacter pylori*, normalization, rapid urease test, quantitative PCR

INTRODUCTION

Helicobacter pylori is a Gram-negative micro-aerophilic, curved bacterium. It is one of the most prevalent human pathogens infecting the stomachs of more than half of the world's population.¹ Its prevalence in developing countries is particularly high with an average of 80% compared to that of 30% in developed countries.² However, there is significant variation in its prevalence from one country to another in the same region.¹ In the Middle East for example, Saudi Arabia has the lowest prevalence (< 50%) while Egypt has the highest (80%).³ *H. pylori* has been subject to intense research since it was established as a risk factor for chronic active gastritis, peptic ulcer, gastric adenocarcinoma, and gastric lymphoma.^{4,5}

Various tests have been developed for the diagnosis of infection with *H. pylori* and/or confirmation of its eradication. Non-invasive tests include serology, urea breath test and antigen stool assay while invasive tests comprise histological detection, culture, rapid urease test (RUT) and polymerase chain reaction (PCR).⁶ The choice among these tests depends on the cost and availability, clinical setting, prevalence of *H. pylori* infection in the population and, most importantly, the performance of the test itself. So far, RUT seems to have the highest sensitivity and specificity as well as positive predictive value¹ and is increasingly being used as a reference.⁷ However, until today there is no consensus gold standard for the diagnosis of *H. pylori* infection.

The last decade witnessed an increasing interest in the use of quantitative PCR (q-PCR) in the detection of *H. pylori* in clinical samples.⁸ Studies have shown the technique to be particularly advantageous with certain types of samples, such as paraffin-embedded formalin-fixed specimens⁹ and biopsies obtained from patients during episodes of peptic ulcer bleeding¹⁰. It also has been found to be useful for assessment of bacterial density in the mucosa.⁹ In addition, q-PCR has been employed for simultaneous detection of both the bacterium as well as its resistance or virulence genes.^{11,12} However, there have been significant differences among the described assays in terms of design, detection limit and gold standard used for comparison.^{9,12-15} Furthermore, there has

Table 1. Demographic and clinical characteristics of patients included in the study (N = 76).

Gender M:F	39:37	
Age (mean ± SD)	35.5 ± 13.8	
Endoscopic finding*	No.	%
Gastritis	61	80.2
Duodenitis	8	10.5
Esophagitis	7	9.2
Gastric ulcer	5	6.6
Duodenal ulcer	10	13.1
Esophageal ulcer	1	1.3

* Some patients had more than one endoscopic finding

been no attempt to normalize bacterial counts to enable reliable comparison of results between studies. Q-PCR assays for the diagnosis of *H. pylori* infection remain, therefore, largely unstandardized.¹

The purpose of the current study was to describe a normalized q-PCR assay for comparable quantification of *H. pylori* in fresh biopsies from dyspeptic patients, and assess its accuracy in establishing diagnosis of the infection.

SUBJECTS AND METHODS

Study subjects and biopsy collection

Seventy-six subjects were recruited from among patients scheduled for upper gastrointestinal tract endoscopy at the University of Science and Technology Hospital, Sana'a, Yemen. Endoscopy and biopsy collection were performed under sedation by gastroenterologists using sterile forceps; three antral gastric mucosal biopsy specimens (approximately 2 cm) were obtained from each patient. The clinical features of the recruited patients is presented in Table 1. The study was approved by the Higher Education Council at Sana'a University; verbal consent was obtained from the patients to include their samples in the study.

Detection of *H. pylori* by rapid urease test (RUT)

One specimen from each subject was immediately assayed for the urease enzyme of *H. pylori* using the Pronto Dry test¹⁶ according to the manufacturer's instructions (Medical Instrument Corporation, Solothurn, Switzerland). The principle of the test is based on hydrolysis of urea in the supplied medium by bacterial urease into carbon dioxide and ammonia,

causing a rise in the pH that is detected colorimetrically.

Detection of *H. pylori* by culture

The second gastric biopsy specimen from each subject was immediately placed in 1 ml Tryptone Soy Broth containing 20% glycerol and stored at -80°C until cultured.¹⁷ Before culturing, the biopsy was thawed at room temperature, homogenized (Omni, USA), and 200 μl were inoculated on Columbia Blood Agar supplemented with Skirrow antibiotics supplement (5.0 mg vancomycin, 2.5 mg trimethoprim, and 1250 IU Polymyxin B), 5% human blood, and 5 ml horse serum.¹⁸ The inoculated plates were incubated in an anaerobic jar at 37°C for ten days under microaerophilic conditions created by using the CampyGen kit (Oxoid, England). Gram-negative bacteria forming tiny, translucent grey colonies that were urease-, catalase- and oxidase-positive, were identified as *H. pylori*.¹⁹

Detection of *H. pylori* by Q-PCR

The third biopsy specimen was placed in 1 ml sterile normal saline and stored at -25°C . Upon analysis, the specimen was thawed and a 25 mg, finely cut piece of it was used for DNA extraction using the PureLink Genomic DNA kit according to the manufacturer's instructions (Life Technologies, USA). DNA was eluted in 100 μl elution buffer.

A commercial TaqMan q-PCR assay (Primerdesign, UK) was used to quantify actin-beta (ACTB) gene copies in the extracts according to the manufacturer's instructions. This was done to validate the extraction and also to normalize bacterial counts as described below. Extracts showing inhibition in this assay or a threshold cycle (Ct) higher than 30 were not considered for further testing (low DNA yield) and extraction was redone.

For detection and quantification of *H. pylori* in the DNA extracts, an optimized, ready to use TaqMan q-PCR kit (Primerdesign, UK) was used. The kit included *ureA* gene-specific primers and FAM-labeled probes with propriety sequences, and a plasmid-based positive control for standard curve construction. Q-PCR reaction was carried out in total volume of 20 μl : 10 μl Precision MasterMix (Primerdesign, UK), 1 μl primer/probe mix, 4 μl water, and 5 μl DNA sample. The reactions were run on an ABI 7000 real-time PCR platform (Applied Biosystems, USA) using the program described by the manufacturer.

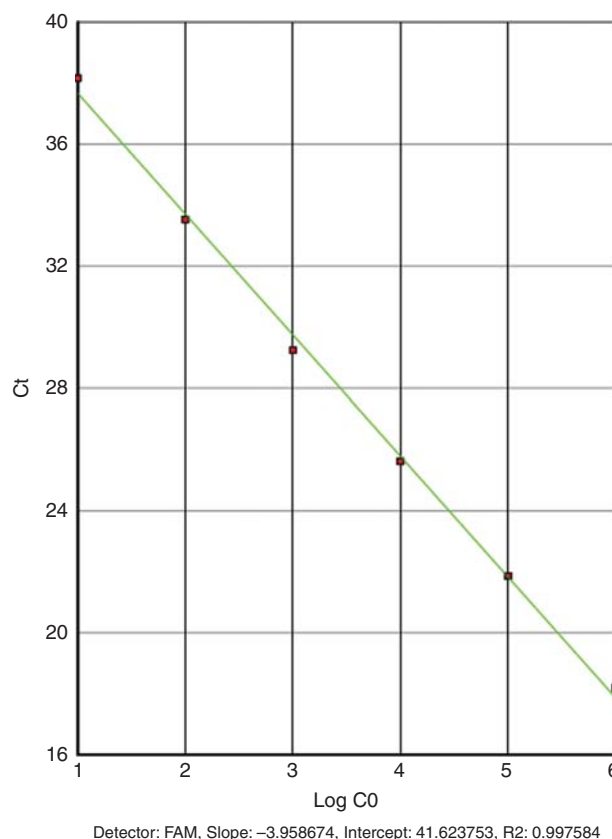


Figure 1. Standard curve of the *H. pylori* TaqMan q-PCR assay. Serial dilutions of plasmid-based positive control were prepared with final concentrations of 10^{-1} to 10^{-6} copies/reaction. Assay was run as described in the text. The curve was obtained by plotting log DNA copies count against threshold cycle (C_t) values.

Bacterial counts in copies/reaction were calculated using the obtained standard curve (Figure 1); a sample was considered positive if it resulted in an amplification plot with $C_t \leq 40$; i.e., 5 copies/reaction (equivalent to 4 bacteria per mg of the biopsy). Bacterial density were calculated by normalizing absolute counts to human ACTB gene copy number, and expressed as number of bacteria per 100 human cells, given there are two ACTB gene copies per human cell.

Statistical analysis

Data were summarized as means and standard deviations (SD) or percentages as appropriate. A patient was considered to be infected with *H. pylori* when two or more assays were positive (gold standard). Because q-PCR resulted in a high rate of false positives, a receiver operating characteristic (ROC) curve analysis was performed to determine

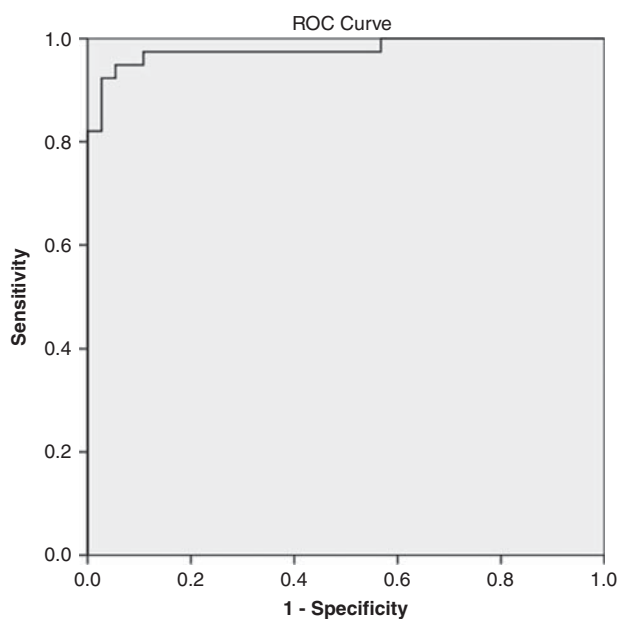


Figure 2. Receiver operating characteristic curve for the normalized q-PCR assay. The area under the curve is 0.98.

normalized q-PCR cut-off values that demonstrated the best sensitivity and specificity. Sensitivity, specificity, positive predictive-value (PPV) and negative predictive-value (NPV), as percentages and 95% confidence intervals, for each of the three assays against the defined gold standard were then calculated. Agreement among the three tests was assessed using Cohen's kappa statistic, while significance of differences in sensitivity and specificity were sought using McNemar's test. All statistical analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) software version 20.

RESULTS

Detection of *H. pylori* by the three assays

H. pylori was detected in 32 patients (42.1%) by culture, and in 40 (52.6%) by RUT. Using the initial cutoff value for q-PCR ($Ct \leq 40$), 60 patients (78.9%) tested positive. Bacterial density as determined by the later assay normalized to human DNA was in the range of 0.005 to 486 bacteria per 100 human cells (mean of 29). According to the gold standard definition (≥ 2 positive tests), 41 patients (53.9%) were initially considered as infected.

Recalculating the cutoff value for q-PCR

Compared to the gold standard, the q-PCR assay showed low specificity (45.7%) with 19 false-positive results. Because most of these false-positives

had low bacterial counts, the cutoff value was recalculated using ROC curve analysis of the normalized counts (bacterial density) (Figure 2). Based on the curve coordinates obtained, a cutoff value of 1 bacteria per 100 human cells was chosen to maintain the highest sensitivity and specificity (see below).

Ultimate performance of the three assays

The number of dyspeptic patients finally diagnosed with *H. pylori* infection according to the gold standard was 39; i.e., a prevalence of 51.3%. The performance of the three assays against this is presented in Table 2. Culture showed the highest specificity (97.3%) but the lowest sensitivity (79.5%). With the new cutoff value, 17 of the 19 initially false-positives by q-PCR became negative, dramatically increasing specificity of q-PCR to 94.9%; however, two of the positive cases by gold standard tested negative dropping initially 100% sensitivity to 94.6%. RUT showed an identical sensitivity to that of q-PCR but slightly lower specificity. Overall accuracy was 94.7%, 93.4% and 90.7% for q-PCR, RUT and culture, respectively. RUT and q-PCR showed the highest agreement ($\kappa = 0.736$), followed by q-PCR and culture ($\kappa = 0.712$), and finally RUT and culture ($\kappa = 0.635$). Differences in sensitivity and specificity were not found to be statistically significant.

DISCUSSION

A major aspect of strength in the current study was the use of normalized rather than absolute counts to measure bacterial load in the samples. Due to variations in the amount of biopsy used for DNA extraction as well as in the efficiency of extraction itself, comparison of bacterial loads between samples, even within the same study, using absolute counts is obviously not reliable. The importance of this has so far been highlighted only by Kobayashi et al., and Riberio et al., who normalized the counts of *H. pylori* to human DNA in picograms/nanograms.^{9,15} Another aspect of standardization in the current study was that extracts showing $Ct > 30$ in the ACTB gene assays were not considered for further testing and DNA extraction was redone. That ensured exclusion of extracts with low DNA yield or/and PCR inhibitors. Several real-time PCR assays for the detection of *H. pylori* have been described in the literature. Different genes have been used as targets in these assays including the *ureC*, *ureA*, 16S rRNA and 23S

Table 2. Sensitivity, specificity, PPV and NPV (as percentages and 95% confidence intervals) for each of the three assays used for detection of *H. pylori* in the biopsies.

Assay	Sensitivity*	Specificity*	PPV	NPV
Culture	79.5 (66.7-92.3)	97.3 (91.9-100)	96.9 (90.6-100)	81.8 (68.2-93.2)
RUT	94.9 (87.2-100)	91.9 (81.1-100)	92.5 (82.5-100)	94.4 (86.1-100)
Q-PCR	94.9 (87.2-100)	94.6 (86.5-100)	94.9 (87.2-100)	94.6 (86.5-100)

*Differences in sensitivity and specificity were not found to be statistically significant.

rRNA genes.^{9,12-14} The commercial kit used in the current study targets the *ureA* gene, which is present as a single copy per bacterial genome. However, what is probably most critical to the performance of PCR is the primers design rather than the target gene selected.²⁰ The detection limit of the assay was set at five copies per reaction, which is equivalent to four bacterial DNA per mg sample. This is consistent with the assay described by Schabereiter-Gurtner et al.,¹² in which a detection limit of 2.2 bacteria per PCR reaction and 5-8 bacteria per mg stool was reported. Lascols et al.,¹⁴ reported a higher detection limit at 30 bacteria per PCR reaction while He et al.,¹³ were only able to accurately quantify down to 1000 bacteria per reaction, although 10-100 bacterial DNA was qualitatively detectable. Obviously, without reporting detection limits as the number of bacteria per unit sample weight or volume, comparison between studies becomes difficult.

Regardless of the differences in assay design, the reported sensitivities and specificities for q-PCR for diagnosis of *H. pylori* have been high, being in the range of 97-100% for sensitivity, and 94.6-100% for specificity.^{9,12,14} The sensitivity in the current study was 100% initially and dropped to 94.9% after recalculating the diagnostic q-PCR cutoff value, which is still close to the reported range. The specificity, however, was initially very low (45.7%). The high number of false positive cases with q-PCR was probably because the bacterial load in these cases was too low to be detectable by RUT or culture. In the study by Lascols et al.,¹⁴ nine of the cases that were negative by both culture and histology tested positive by q-PCR. That was also attributed to a low bacterial count in the samples; however, the specificity of the assay was not significantly compromised because additional non-invasive tests were included and confirmed the diagnosis in four out of the nine cases. Similarly, He et al.,¹³ detected *H. pylori* DNA by q-PCR in 24 samples out of 27 that were culture negative.

Unlike the current study, however, the bacterial load in those samples was very high, which is difficult to explain.

The ability of q-PCR to detect bacteria at very low counts can compromise its specificity when other techniques such as culture, RUT or/and histology are used as gold standard. This becomes particularly evident when the proportion of study subjects harboring low counts is high, which was probably the case in the current study. To improve specificity of the assay, ROC curve analysis of normalized q-PCR counts was, therefore, performed. The identified cutoff value (1 bacteria per 100 human cells) dramatically improved the specificity, although sensitivity was slightly affected. A similar approach was used previously⁷ to calculate the delta cutoff value for urease breath test.

One possibility remains, however, that low bacterial counts detected only by q-PCR do represent true infections. This possibility is strongly supported by findings from the study of Ribeiro et al., in which *H. pylori* was detected by q-PCR in 16 out of 81 (19.7%) gastric biopsies that had previously tested negative by reference methods.¹⁵ Indeed, a significant proportion (20-50%) of dyspeptic patients are usually not diagnosed with *H. pylori* infection, which may be attributed to low sensitivity of the routinely used assays rather than actual absence of infection. As such, q-PCR may ultimately prove to be the most accurate method for diagnosis of *H. pylori* infection and replace currently used tests.

One limitation to the current study was that the specimens for culture were stored at -80°C until all of them were processed together. While this has been previously described in the literature,¹⁷ it may have probably reduced the sensitivity of the assay. Another limitation was that a proper history about recent antibiotic intake was not obtained with the assumption that patients admitted for diagnosis

rather than follow up, were unlikely to have received previous *H. pylori* eradication therapy. In other words, recent antibiotic use in some of the patients was not totally excluded. However, any such history may have only influenced the overall detection rate (prevalence) but not the comparisons among the assays.

CONCLUSION

In conclusion, with the identified cutoff value, the q-PCR assay diagnosed *H. pylori* infection with an accuracy slightly superior to that of RUT. However, if low counts detected only by q-PCR are considered as true infection, the assay will have much higher sensitivity than RUT, a scenario that warrants further

investigation. In any case, normalization of bacterial counts is probably an important aspect of standardization of q-PCR *H. pylori* assays since it enables reliable comparison of results among studies.

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