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RESEARCH ARTICLE

Large-Scale Evaluation of *ureC (glmM)* and *SSA* Conventional PCR for Rapid Direct Detection of *Helicobacter pylori* in Gastric Biopsies as Compared to *rpoB*-based Quantitative Real-Time PCR

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Abstract:

Background:

Conventional polymerase chain reaction (PCR)-based methods play a major role in the direct detection of *H. pylori* in clinical specimens, with time-saving as compared to culture-based methods. However, specificity and sensitivity vary among different varieties of these PCRs, which consequently could affect the accuracy of diagnosis of *H. pylori* infection. The study aimed to evaluate the utility of *ureC (glmM)* and *SSA* conventional PCR methods for rapid direct detection of *H. pylori* by comparing them with *rpoB*-based quantitative real-time PCR.

Methods:

A total of 402 non-repeated gastric biopsy specimens were subjected to DNA extraction followed by conventional *ureC (glmM)* and *SSA* PCR, and *rpoB*-based quantitative real-time PCR, which was used as the gold standard.

Results:

H. pylori was detected in 119 (29.6%), 126 (31.34%), and 187 (46.5%) of the tested specimens using *ureC* (*glmM*) PCR, *SSA* PCR, and real-time quantitative PCR, respectively. The specificity of the *SSA* PCR was higher than that of *ureC* (*glmM*) PCR (99.5% and 98.6%, respectively). The *SSA* PCR was more sensitive than the *ureC* (*glmM*), (66.8% and 62%, respectively). The diagnostic accuracy of *SSA* PCR (84.33%) was higher than that of *ureC* (*glmM*) PCR (81.59%).

Conclusion:

Overall, *SSA* PCR is more specific, sensitive, and diagnostically accurate than *ureC (glmM)* PCR, giving the *SSA* PCR assay superiority as a simple, rapid, and accurate diagnostic tool for direct detection of *H. pylori* in gastric tissue specimens.

Keywords: *Helicobacter pylori*, *ureC* (*glmM*) PCR, *SSA* PCR, Real-time PCR, Diagnostic accuracy, *rpoB*-based quantitative real-time, Gastric tissue.

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1. INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is one of the most widespread infections worldwide [1, 2]. It is considered a leading cause of chronic gastritis, peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma [3 - 6]. Early diagnosis of this infection is crucial to establishing an effective management plan and administering appropriate antibiotic therapy to eradicate this gastric pathogen and avoid its sequelae. *H. pylori* diagnostic approaches are

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invasive methods that are based on gastric biopsies and include urease tests, histopathology, culturing, polymerase chain reaction (PCR), or non-invasive methods that utilize the urea breath test, serological detection of antibodies, and antigen detection in stool [7 - 10].

PCR-based methods have great utility in the detection, genotyping, and antimicrobial resistance studies of *H. pylori* [11 - 13]. These methods include many conventional and real-time varieties that can be performed directly on gastric biopsy specimens. Overall, the main advantages of these molecular-based methods are higher sensitivity, specificity, avoidance of the time required for culture-based methods, and the ability to detect dead or inhibited bacteria resulting from the prior administration of antibiotics.

Both *ureC* and *SSA* PCRs are among the earlier conventional PCR assays used for *H. pylori* detection [14, 15]. The *ureC* PCR targets the urease C (*ureC*) gene, which was first wrongly believed to be associated with urease production [14], but later, it was proven to be responsible for the production of the phosphoglucosamine mutase, which is involved in the synthesis of bacterial cell walls, and renamed the *glmM* gene [16]. *SSA* PCR utilizes primers targeting the 26-kDa species-specific antigen (*SSA*) gene [15, 17].

An earlier study was carried out to compare conventional PCR methods used for the detection of *H. pylori* in gastric tissues, including these two PCRs, and demonstrated higher sensitivity and specificity for *ureC* PCR over *SSA* PCR [18]. However, this study used a limited number (50 cases) of gastric biopsy specimens and took the culture method as the gold standard for comparison, which can be biased by false negative cases resulting from dead or inhibited bacteria. The current study reevaluated and compared these two PCR methods in the presence of different test conditions either tested population or test methodology, to investigate if these differences could affect the sensitivity, specificity and diagnostic accuracy of these PCR methods. The study compared *ureC* (*glmM*) PCR and *SSA* PCR on a wide-scale using a large number of gastric biopsies (exceeding 400 specimens) obtained from dyspeptic Saudi patients and taking *rpoB* real-time PCR, which targets the RNA polymerase beta-subunit (*rpoB*) gene, as the gold standard to avoid false negative results of culture. The principal aim was to evaluate the diagnostic value and utility of these simple PCR approaches in the direct detection of *H. pylori* in gastric biopsies, aiming at a rapid and accurate diagnosis of *H. pylori* infection, which will serve to establish appropriate treatment plans for this common pathogen.

2. MATERIALS AND METHODS

2.1. Study Population and Clinical Specimens

The study included 404 Saudi patients attending gastroenterology clinics at general hospitals in the Jazan region of Saudi Arabia. All patients who participated in this study had dyspeptic symptoms. The study tested 402 non-repeated gastric biopsies obtained from 402 patients of the study population (one biopsy from each patient), while the remaining two biopsy specimen materials obtained from the remaining two patients were insufficient for further processing. Upper gastrointestinal endoscopy was performed to obtain the specimens.

2.2. DNA Extraction

The biopsy specimens were subjected to DNA extraction using a DNeasy blood and tissue kit (Qiagen) (Cat. No. 69504) according to the manufacturer's instructions.

2.3. Conventional PCR

Each specimen was examined by both *ureC* (*glmM*) PCR and *SSA* PCR. In *ureC (glmM*) PCR, the *H. pylori* DNA was amplified by targeting the *ureC* (*glmM*) gene, which is common for all *H. pylori* strains, using a forward primer (5′-AAG CTT TTA GGG GTG TTA GGG GTT T-3′) and a reverse primer (5′-AAG CTT ACT TTC TAA CAC TAA CGC-3′) (TIB MOLBIOL, Germany) [14]. In *SSA* PCR, the specimens were tested for the presence of species-specific DNA sequences coding for a protein antigen of 26 kDa molecular weight, which is present in all *H. pylori* strains, using the forward primer (5′-TGG CGT GTC TAT TGA CAG CGA GC-3′) and the reverse primer (5′-CCT GCT GGG CAT ACT TCA CCA TG-3′) (TIB MOLBIOL, Germany) [15]. In both PCR methods, reactions were carried out in volumes of 50 µl containing 2.5 U of FastStart *Taq* DNA polymerase (Roche, Germany), reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, deoxynucleoside triphosphate mix [0.2 mM each of dATP, dCTP, dGTP, and dTTP] (Roche, Germany), 0.4 µM of each primer, 7 µl of template DNA, completed to a 50 µl volume with sterile RNase/DNase-free water. Positive and negative controls were included in each PCR run. DNA extracted from a gastric biopsy specimen of a well-known *H. pylori*-positive patient was used as a positive control, while the negative control reaction contained RNase/DNase-free water instead of genomic DNA. PCR reactions were performed under the cycling conditions described previously [19, 20] with some modifications as follows: initial denaturation for 10 min at 94 ^{o}C, followed by 35 cycles of denaturation for 2 min at 94 ^{o}C, annealing for 2 min at 55 ^{o}C (for *ureC* (*glmM*) PCR), and 57 ^{o}C (for *SSA* PCR), and extension for 2 min at 72 ^{o}C, followed by a final extension for 10 min at 72 ^{o}C. The resulting products were separated in 2% agarose gels and 1x Tris-acetate-EDTA buffer, and images were captured after staining with ethidium bromide.

2.4. Real-Time PCR

Quantitative real-time PCR for the detection of *H. pylori* was used as the gold standard in this study. It was performed for all extracted DNA samples by targeting the RNA polymerase beta-subunit (*rpoB*) gene of *H. pylori* using the primer-probe based "genesig Quantification of *Helicobacter pylori* advanced kit" (PrimerDesign Ltd. Southampton, United Kingdom) as described previously [12].

2.5. Statistical Analyses

Sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy, and their 95% CI were determined for each of the tested conventional PCRs in reference to the quantitative real-time PCR, which was taken as the gold standard. Analyses were conducted using SPSS software.

3. RESULTS

A total of 402 gastric biopsy specimens were tested for the presence of *H. pylori* using *ureC* (*glmM*) PCR, *SSA* PCR, and *rpoB* quantitative real-time PCR, which was considered the gold standard test.

3.1. Quantitative Real-Time PCR

The quantitative real-time PCR assay detected *H. pylori* in 187 (46.5%) of the total tested specimens, while 215 (53.5%) cases were negative for *H. pylori* (Table **1**).

3.2. *ureC* (*glmM*) PCR

The *ureC* (*glmM*) PCR resulted in an amplified product of approximately 294 bp in size Fig. (**1**). Of 402 specimens, 119 (29.6%) were *H. pylori*-positive, while the remaining 283 (70.4%) were *H. pylori*-negative (Table **1**).

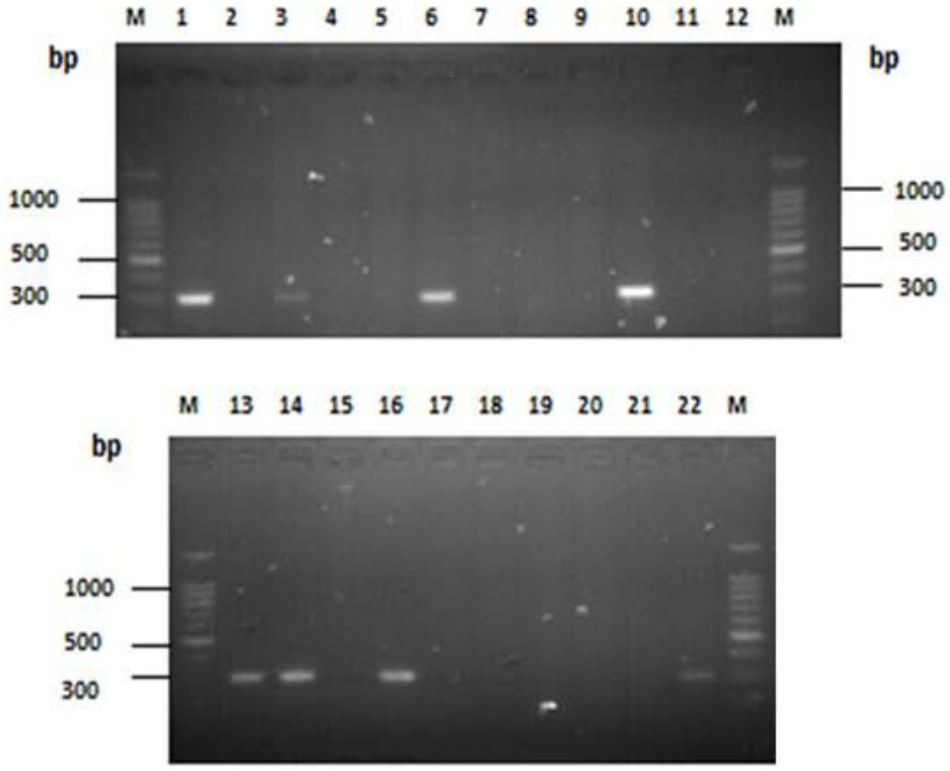

Fig. (1). Agarose gel electrophoresis of PCR products of the *ureC (glmM)* gene of DNA extracted from clinical gastric biopsy specimens obtained from dyspeptic patients. Lanes: M, molecular weight marker (Cleaver Scientific Ltd.); 1, 3, 6, 10, 13, 14, 16, and 22, clinical specimens positive for *H. pylori* (the size of the amplified product is approximately 294 bp); 2, 4, 5, 7–9, 11, 12, 15, and 17–21, clinical specimens negative for *H. pylori*.

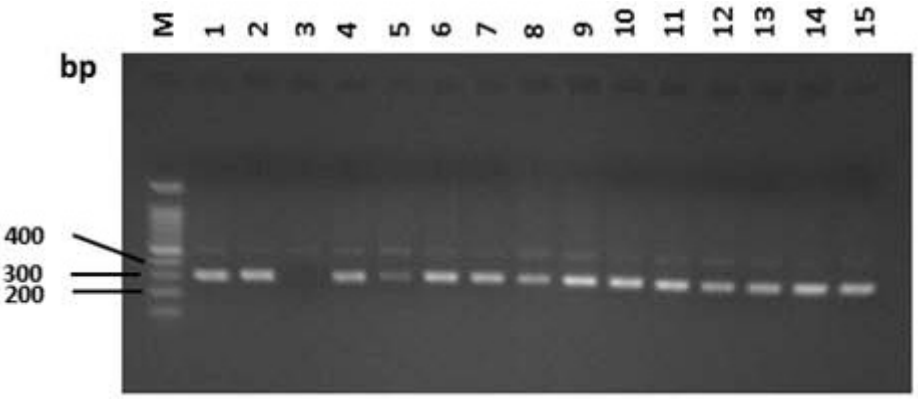

Fig. (2). Agarose gel electrophoresis of the PCR products of *SSA* gene (298 bp in size) of DNA extracted from clinical gastric biopsy specimens obtained from dyspeptic patients. Lanes; M; molecular weight marker (Cleaver Scientific Ltd.). All lanes present clinical specimens positive for *H. pylori* except lane 3 which presents a clinical specimen negative for *H. pylori*.

Table 1. Results of *ureC* (*glmM*) PCR and *SSA* PCR in reference to *rpoB*-based quantitative real-time PCR for detection of *H. pylori* in gastric biopsy specimens.

		***rpoB*-based quantitative real-time PCR**			**Sensitivity**	**Specificity**	**Positive Predictive Value**	**Negative Predictive Value**	**Diagnostic Accuracy**
		+ve (n)*	**-ve (n)**	**Total (n)**					
***ureC* (*glmM*) PCR**	**+ve**	116	3	119	62% (54.9-68.68)	98.6% (95.98-99.52)	97.5% (92.85-99.14)	74.9% (69.55-79.61)	81.59% (77.51-85.08)
	-ve	71	212	283					
Total		187	215	402					
***SSA* PCR**	**+ve**	125	1	126	66.8% (59.82-73.19)	99.5% (97.41-99.92)	99.2% (95.64-99.86)	77.5% (72.25-82.06)	84.33% (80.45-87.56)
	-ve	62	214	276					
Total		187	215	402					

95% CI for each diagnostic value is presented in the brackets.

3.3. *SSA* PCR

From the overall tested DNA samples, 126 (31.34%) were positive for the presence of *SSA* sequence, which gave the PCR amplified product of 298 bp in size (Table **1** and Fig. **2**).

A comparison of both conventional PCRs with quantitative real-time PCR is presented in (Table **1**). The *SSA* PCR was more sensitive than the *ureC* (*glmM*) (66.8% and 62%, respectively). Although both types of PCR showed high specificity, the specificity of the *SSA* PCR was higher than that of *ureC* (*glmM*) PCR (99.5% and 98.6%, respectively). Overall, the diagnostic accuracy of *SSA* PCR was higher than that of *ureC* (*glmM*) PCR (84.33% and 81.59%, respectively).

4. DISCUSSION

Routine conventional PCR methods play a major role in the direct detection of *H. pylori* in clinical specimens, with the advantage of saving time, compared to culture-based methods. However, the different varieties of these routine PCRs differ in specificity and sensitivity, which could affect the accuracy of the diagnosis of *H. pylori* infection.

This study compared two types of routine PCRs, namely *ureC* (*glmM*) PCR and *SSA* PCR, with the gold standard *rpoB* gene-based quantitative real-time PCR assay. The aim of these comparisons was to investigate the diagnostic accuracies of these conventional PCRs in the direct detection of *H. pylori* in gastric biopsies obtained from dyspeptic patients under test conditions different from previous studies, a namely large number of tested clinical specimens, patients from different geographic region, different DNA extraction methodology, and different gold standard.

The quantitative real-time PCR assay used in our study was taken as the gold standard because it is highly specific and sensitive for the detection of *H. pylori* [21]. It is highly specific because it targets the RNA polymerase beta-subunit (*rpoB*) gene of *H. pylori* using a primer-probe-based approach (both the primer and probe provided with the kit are highly specific for *H. pylori*). As stated by the manufacturer, this kit can detect all *H. pylori* strains as the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of kit design. Moreover, it is highly sensitive; by generating a standard curve using the positive control *H. pylori* DNA template provided with the kit, we found that the detection limit was two copies of the target gene of *H. pylori* per µl of the tested DNA (data not shown). Consequently, the use of such real-time PCR assay provided an advantage over the culture method by avoiding false negative results from dead or inhibited bacteria present in tested gastric biopsies.

Both tested conventional PCR methods exhibited high specificity (approximately 100%, with less than 1% difference, 98.6% and 99.5% for *ureC* (*glmM*) and *SSA* PCR, respectively). These findings are in complete concordance with Bickley *et al*. [14] and Lage *et al*. [19] who confirmed the specificity of *ureC* (*glmM*) PCR because the primers used did not amplify any DNA extracted from variable bacterial species other than *H. pylori*, including other members of the *Helicobacter* genus. This could be explained by the high specificity of the primers used and conservation of their target gene sequences in most *H. pylori* strains [14], which enables these primers to anneal specifically to their target genes.

Our results also concur with O'Toole *et al*. [17] who demonstrated, after sophisticated work for isolation and characterization of this specific antigen protein by determining the DNA sequence of its gene fragment, that the species-specific antigen (*SSA*) is unique for all *H. pylori* strains. These data were later used to design specific primers targeting this genomic sequence for utilization in PCR assays, which proved to be highly specific for *H. pylori* detection [15, 20]. Moreover, because of the universal presence of *SSA* in all *H. pylori* strains, it was used as a marker for the presence of *H. pylori*, acting as a positive control. Consequently, the *SSA* PCR assay was utilized in some studies to check the samples that were negative for *H. pylori* after other PCR methods used in genotyping studies [20, 22].

Despite the high specificity of both PCRs, very few false-positive results have been recorded, with three cases and one case, for *ureC* (*glmM*) and *SSA* PCR, respectively. This could be due to DNA cross-contamination during biopsy specimen processing, DNA extraction, or the PCR procedure itself [14].

Our results revealed 62% and 66.8% sensitivities for *ureC* (*glmM*) PCR and *SSA* PCR, respectively. Similarly, other studies have demonstrated variable sensitivities for both types of PCR. Lu *et al*. [18] found equal sensitivities for both *ureC* (*glmM*) and *SSA* PCR, with a detection limit equal to approximately 50 *H. pylori* bacterial organisms. By increasing cycle step durations to 2 min, Lage *et al*. [19] improved the

sensitivity of *ureC* (*glmM*) PCR to a detection limit of approximately two genomes of *H. pylori*. Compared to our quantitative real-time PCR gold standard, the false-negative results were clearly high, with 71 and 62 cases, for *ureC (glmM*) PCR and *SSA* PCR, respectively. Such false results can be explained by occasional sequence polymorphisms that may result from point mutations in target gene sequences [23, 24] leading to the inability of primers to anneal to their targets, or the presence of PCR inhibitors, mostly from the gastric tissue specimens [25], or low or bad quality fragmented or sheared DNA.

Overall, our results, using quantitative real-time PCR as the gold standard, revealed higher specificity, sensitivity, predictive values, and diagnostic accuracy of *SSA* PCR over *ureC* (*glmM*) PCR. This contrasts with the findings of Lu *et al*. [18], who found that *ureC* (*glmM*) PCR was equally sensitive to, but more specific than, *SSA* PCR, taking the culture method as the gold standard. They attributed the lower specificity of the *SSA* PCR method to its amplification of 10 culture-negative samples. Therefore, considering the culture method as the gold standard may be the cause behind the lower specificity of *SSA* PCR reported in this study, as these *SSA* PCR-positive 10 samples may be true positive, but the culture method could not detect them. Although the culture method is considered the most specific diagnostic tool, its sensitivity to *H. pylori* may be low in the presence of dead or inhibited bacteria in the biopsy specimens as a result of the use of antimicrobials or improper specimen transport. The differences observed between our findings and those of others could be attributed to differences in study conditions, including the number of specimens tested, demographic and social characters of the study population, specimen preparation and processing, DNA extraction methodology, the assigned gold standard, and PCR conditions (including differences in thermocycler ramp rates).

CONCLUSION

The study reevaluated the utility of *SSA PCR* and *ureC (glmM)* PCR in the diagnosis of *H. pylori* infection with modification in some study conditions, including enrollment of a large number of study population from a different geographic region, different gold standard and different DNA extraction methodology. Based on our large number of tested gastric biopsies (402 non-repeated specimens) and our highly specific and sensitive gold standard (*rpoB*-based quantitative real-time PCR), we conclude that *SSA* PCR is more specific, sensitive, and diagnostically accurate than the *ureC (glmM)* PCR, and that the *SSA* PCR assay is a simple, rapid, and accurate diagnostic tool for the direct detection of *H. pylori* in gastric tissue specimens.

LIST OF ABBREVIATIONS

PCR	=	Polymerase Chain Reaction
MALT	=	Mucosa-associated Lymphoid Tissue

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the research ethics committee at the faculty of Medicine - Jazan University with reference number (Ref: FMRERC-2012).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed written consent was obtained from each patient participating in the study.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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