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Pitfalls of UBT, SAT, and Nested PCR Reliability for Diagnosing *Helicobacter pylori*

Janka Klingová †, Bianka Prokopová †, Barbora Šipková, Vanesa Bujková and Pavol Sulo *

Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Ilkovicova 6, 842 15 Bratislava, Slovakia; janka.klingova@gmail.com (J.K.); prokopova32@uniba.sk (B.P.); barbora.sel@gmail.com (B.Š.); vaneska.bujkova@gmail.com (V.B.)

* Correspondence: pavol.sulo@uniba.sk

† These authors contributed equally to this work.

Abstract

Helicobacter pylori is the leading cause of chronic gastrointestinal tract diseases, with a worldwide prevalence of around 50%. For identification in medical practice, non-invasive methods such as the immunochromatographic test for antigen in stool (SAT) and the urease breath test (UBT) are widely used. Recently, we developed a highly sensitive and specific nested PCR (NPCR) that involves two amplification reactions and uses primers designed to target the variable regions of the 16S rRNA gene to amplify a short 148 bp amplicon. The aim of this study was to compare two classical methods, SAT and UBT, with the 148 bp amplicon NPCR. We examined samples from 137 volunteers, and found 46 positives with NPCR using stool samples, 34 with UBT, and only 24 with SAT. *H. pylori* origin of the 148 bp amplicons was confirmed by sequencing. NPCR had the highest detection rate in this cohort, suggesting that a portion of the population may be misdiagnosed, particularly by SAT. Due to the cost and simple performance in practice, SAT is a method of choice for initial screening. However, in cases of negative results and persistent digestive problems, we recommend the more sensitive UBT. NPCR may be a useful complementary method, especially in discordant or clinically suspicious cases.

Keywords: *H. pylori*; diagnostic reliability; nested PCR; detection limit

1. Introduction

Chronic diseases are defined as long-term health problems with slow progression and are among the most common causes of death worldwide [1,2]. The pathogenesis of most of them is unknown. However, it is believed that the leading cause of their development is the persistence of various pathogens in the human body [1–4]. We focused our study on the bacterium *Helicobacter pylori*, which is the leading cause of chronic gastrointestinal tract diseases, including gastritis, peptic ulcer disease, and stomach cancer. It is also prevalent in approximately half of the human population; however, its occurrence varies significantly across geographic regions [1,5–7]. It is a microaerophilic, Gram-negative bacterium with a typical S-shaped spiral. It is highly motile, propelled by a unipolar bundle of 2-6 flagella, and its movement resembles a corkscrew. As a result, it colonizes the mucus layer of the gastric epithelium [6,8]. The bacterium is highly successful due to its various adaptations that facilitate colonization. These include its aforementioned flagella, shape, movement, and ability to adhere, as well as its secretion of many toxins. It moves along pH gradients and, to protect itself from unfavorable pH conditions,

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it also possesses urease activity, which enables it to increase the pH. Otherwise, it penetrates epithelial cells, causing inflammation [6,8]. Recent studies have shown that *H. pylori* can also influence the occurrence of various extragastric diseases. In many cases, this connection is based solely on epidemiological studies, and the causal relationship has not been established. The diseases with sufficiently well-established causal relationships are immune thrombocytopenic purpura, iron deficiency anemia, and vitamin B12 deficiency [9–11]. Reliable diagnostics are essential for treatment. We categorize the tests into two groups based on the sample used: invasive and non-invasive. In invasive diagnostics, a sample is obtained via endoscopy and then tested using a rapid urease test (RUT), histological examination, culture, or molecular methods [10–16].

Non-invasive diagnostics are based on detecting bacterial enzymes in the urea breath test (UBT), antigens in the stool antigen test (SAT), antibodies in serology, or DNA sequences from stool and saliva [10–16]. Ideally, the test correctly identifies all patients with and without the disease. However, current tests also show false-positive and false-negative results. Individual methods should therefore be sensitive and specific. The gold standard of invasive diagnostics is considered to be endoscopy, followed by subsequent histology. Among non-invasive methods, UBT is preferred; however, SAT is also recommended. DNA amplification methods (PCR) have not yet been established for various reasons [10–18]. Most of them had serious limitations and mistakes in primer design [18]. In addition, there are concerns about their sensitivity and specificity. Several PCR modifications are available; however, the preferred option for better sensitivity and specificity is nested PCR (NPCR) [16,18,19]. This PCR variant includes two rounds of PCR reactions. The first reaction amplifies a larger DNA region that serves as a template in the second reaction, which then amplifies a narrower subregion [16,18,19]. Due to this feature, NPCR is more specific, allowing DNA to be amplified from samples with fewer target molecules than in simple PCR [16,18,19]. At the beginning, we developed a nested PCR for the unambiguous identification of *H. pylori* in biopsy, stool, and saliva samples using primers targeting variable regions of the 16S ribosomal RNA (rRNA) gene, amplifying a 454 bp DNA segment [18]. This assay was sensitive for *H. pylori* DNA isolated from stomach biopsies but not sensitive enough to detect it in stool samples [18]. Therefore, we developed an NPCR assay to amplify a shorter 148 bp region of the 16S rRNA gene and compared it with SAT using many stool samples. In this case, due to DNA degradation in stool, short NPCR detected many more positive samples than SAT [19]. The main goal of this study was to demonstrate the power of NPCR using stool samples and compare its sensitivity with other non-invasive methods (SAT and UBT) used in medical practice.

2. Materials and Methods

2.1. Patients and Samples

Stool and breath samples, necessary for performing UBT and SAT, were collected from volunteers at the Faculty of Natural Science of Comenius University (PRIFUK) and the Faculty of Physical Education and Sport (FTVŠ UK) between 2022 and 2024. A total of 137 stool samples were analyzed (52 men and 85 women; mean age, 39.3 years; range, 11–83 years). The conditions for inclusion in the study were a minimum of 4 weeks without antibiotic use and at least 2 weeks without proton pump inhibitor (PPI) use. All research procedures were conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. The PRIFUK ethics committee approved the study. All participants provided written informed consent, as outlined in the Declaration of Helsinki. In the event of a positive finding, the volunteers received a consultation and, if necessary, were treated according to standard procedures by a gastroenterologist or general practitioner. All samples were stored in a freezer at −20 °C. A culture of *H. pylori* J493, with a partial

16S rRNA sequence identical to that of clinical isolate B12823, which is used as a standard, was provided by Silvia Vašková, Laboratories, Piešťany, s.r.o. Slovakia [18].

2.2. Urease Breath Test (UBT)

The ^{13}C urease breath test was performed using the Helicobacter Test INFAI® (INFAI GmbH, Cologne, Germany) according to the manufacturer's instructions. The $^{13}C/^{12}C$ ratio was determined using a laser-assisted ratio infrared spectroscopy analyzer at Endocentrum—SanitStom, Banská Bystrica, Slovakia. An increase (Δδ-value) of more than 4‰ in the exhaled carbon dioxide after 30 min was considered indicative of infection [18].

2.3. Stool Antigen Test (SAT)

The presence of *H. pylori* in stool was determined using the "euroSCREEN HP" immunochromatographic test (Eurolab Lambda a.s., Trnava, Slovakia), which is based on monoclonal antibodies, according to the manufacturer's instructions [19].

2.4. DNA Isolation

DNA from stool samples (180–220 mg) was obtained using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. In the final step, the DNA was eluted into 40 µL of an AE solution. The extracted DNA was stored at −20 °C for future analysis [18,19]. After several cycles of thawing and freezing, most DNA preparations yielded the same results. If in doubt, we isolated DNA again from frozen stool samples.

2.5. Nested PCR

The primers for specific NPCR amplicons and the amplification conditions are listed in Table 1.

Table 1. Primers and PCR conditions.

Name	PCR Product Size	Primers 5′ → 3′	Amplification Cycling
148-amplicon	192 bp	Heli Strim 5′-AAGAACCTTACCTAGGCTTGACATTG-3′ Sat out 5′-AGTATCCTTAGAGTTCTCAGCATGACCT-3′	94 °C—3 min, 35x (94 °C—45 s, 55 °C— 1 min, 72 °C—1 min), 72 °C—5 min, 14 °C
148-amplicon	148 bp	Hp up 5′-TGAGAGAATCCGCTAGAAATAGTG-3′ Sat in 5′-CATGACCTGTTAGCAACTAAGAAAGG-3′	94 °C—5 min, 25x (94 °C—45 s, 55 °C— 1 min, 72 °C—1 min), 72 °C—5 min, 14 °C
1vial	206 bp	1viout 5′AGATACACGAAGAACCTTACCTAGGCTTGACATTG-3′ 2viout 5′-GAGGCAGTATCCTTAGAGTTCTCAGCATGACCT-3′ 1viin 5′-AGAGAATCCGCTAGAAATAGTGG-3′ 2viin 5′-ATGACCTGTTAGCAACTAAGAAAG-3′	94 °C—3 min, 25x (94 °C—45 s, 65 °C— 1 min, 72 °C—1 min), 35x (94 °C—45 s, 55 °C— 1 min, 72 °C—1 min), 72 °C—3 min, 14 °C
panA	204 bp	Heli Strim 5′-AAGAACCTTACCTAGGCTTGACATTG-3′ psatout 5′-CCTTACGAAGGCAGTCTCCYTAGAG-3′	94 °C—3 min, 35x (94 °C—45 s, 55 °C— 1 min, 72 °C—1 min), 72 °C—5 min, 14 °C
panA	148 bp	phpup 5′-TGAAAGAATCTGCTAGARATAGYGG-3′ psatin 5′-CGAACTGYTAGCAACTAAGGACGA-3′	94 °C—5 min, 25x (94 °C—45 s, 50 °C— 1 min, 72 °C—1 min), 72 °C—5 min, 14°

DNA was amplified using FIREPol® DNA Polymerase (BioDyne Tartu, Estonia) in a 25 µL solution that contained 1X buffer B; 0.2 mM deoxynucleotides (dNTPs); 2.5 mM $MgCl_2$; 1X Super PCR Enhancer (S-Solution) (BioDyne); 1 pmol/µL of each primer; 0.5 µL of DNA; and 0.2 U/µL enzyme. For inconclusive cases, the DNA volume was increased up

to 5 µL. The second PCR reaction contained the same components, except for the second primer pair and 1 µL of DNA from the first reaction. Amplification was performed in a thermal cycler (Eppendorf Mastercycler Nexus GSX1 Thermal Cycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany). To avoid contamination, two negative controls were included after each sample in triplicate experiments [18,19].

2.6. Analysis of PCR Products and Their DNA Sequences

The size of the PCR products was determined by electrophoresis in a 1% agarose gel and 1X Tris-borate-ethylenediaminetetraacetic acid (TBE) solution. All PCR products were sequenced after EPPiC Fast (A&A Biotechnology, Gdansk, Poland) treatment using the ABI–100 Avant and BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were edited in CHROMAS, trimmed to the same length, and compared with each other, with sequences in the GenBank database, and with the sequences for all accepted *Helicobacter* species [20] using the BlastN program from the National Center for Biotechnology Information (NCBI). Sequences were aligned using the Clustal W program [18,19].

2.7. Statistical Analysis

Sensitivity and specificity were calculated according to [21], McNemar's test results were calculated according to [22], and Cohen's Kappa values were calculated according to [23].

2.8. Other Procedures

Other procedures were performed as described in [15,18,19].

No generative artificial intelligence (GenAI) was used in this paper.

3. Results

First, we searched for suitable patients in the gastroenterological outpatient clinic; however, the UBT limited our options. It was challenging to find patients who had not taken PPIs for at least 2 weeks, so we began testing volunteers from our faculty and the Faculty of Physical Education and Sports. This volunteer-based cohort limits direct extrapolation to symptomatic clinical populations, even though 40% had various digestive problems and 30% were treated by a gastroenterologist after testing. In total, we examined 137 volunteers, determined the presence of *H. pylori* using the breath UBT, and compared it with SAT using stool samples and NPCR using DNA isolated from stool samples. The results are summarized in Table 2.

Table 2. *H. pylori* positivity in different diagnostic tests.

Number	Results		
	UBT	SAT	NPCR
20	+	+	+
9	+	–	+
5	+	–	–
2	–	+	+
2	–	+	–
15	–	–	+

Table 2. *Cont.*

Number	Results		
	UBT	**SAT**	**NPCR**
83	–	–	–
1	–	–	i
Total	34/137	24/137	46/137

i = inhibition of the amplification in the first PCR reaction.

NPCR proved to be the most sensitive of all three diagnostic methods, detecting the highest number of positive cases, specifically 33.6%. With UBT, the rate was only 24.8%, and with SAT, it was even lower at 17.5% (Table 2). UBT is often used as the gold standard for sensitivity and specificity calculations. Therefore, we first calculated these values with respect to the UBT results (Table 3). Since NPCR was more sensitive, we also recalculated the sensitivity and specificity with respect to the NPCR results (Table 4).

Table 3. Sensitivity and specificity of diagnostic tests compared to UBT.

	SAT	**95% CI**	**NPCR**	**95% CI**
Sensitivity (%)	58.8	±16.5	85.3	±11.3
Specificity (%)	96.1	±6.5	83.3	±12.6
Positive Predictive Value	83.3		63.0	
Negative Predictive Value	87.6		94.4	

Table 4. Sensitivity and specificity of diagnostic tests compared to NPCR.

	UBT	**95% CI**	**SAT**	**95% CI**
Sensitivity (%)	63.0	±13.9	52	±14.4
Specificity (%)	94.4	±6.8	97.8	±4.4
Positive Predictive Value	85.3		91.7	
Negative Predictive Value	83.3		78.6	

The odds ratio for NPCR and UBT, which was used as the control, calculated using McNemar's test was 3.4, with a 95% confidence interval ranging from 1.2 to 11.8. The Cohen's Kappa result was 0.586 at a 0.05 significance level, indicating moderate agreement. For the SAT/UBT pair, the odds ratio from McNemar's test was 0.29, with a 95% confidence interval extending from 0.07 to 0.9. The Cohen's Kappa result was 0.608 at a 0.05 significance level. However, a key limitation of these interpretations is the choice of reference method. SAT and UBT are widely used in medical practice. A comparison of the results of these tests with those from NPCR indicates that some individuals may have been misdiagnosed.

We would like to emphasize the NPCR's specificity, as the origin of any amplified DNA segment can be identified by sequencing, and comparisons with GenBank and with 16S rRNA from 55 accepted *Helicobacter* [20] species confirmed the *H. pylori* origin. The 46 sequences produced 11 different variants (Figure 1).

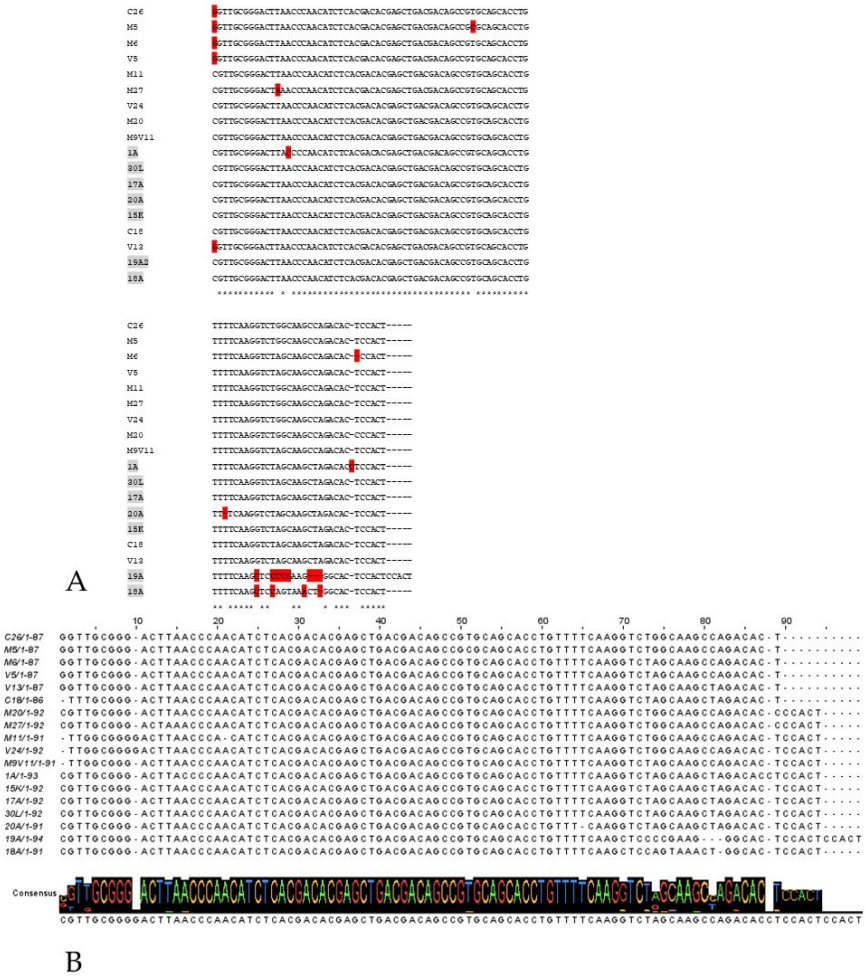


Figure 1. Different variants of the 92 nt long segment of *H. pylori* 16S rRNA amplified from stool. (**A**) ClustalX alignment. Divergent positions are marked in red. SAT-positive but NPCR-negative samples are marked in gray. C26, M5, M6, etc., are codes for particular samples. (**B**) Degree of conservation at divergent positions.

Only 103 samples produced the same result with all three tests (Table 2). Five samples were positive according to UBT but negative according to SAT and NPCR. Two samples were positive according to SAT but negative according to NPCR and UBT. Fifteen samples were positive only according to NPCR.

4. Discussion

Why are there samples that cannot be identified correctly by all methods, no matter whether they are non-invasive or invasive? UBT is described as a gold standard non-invasive method for *H. pylori* diagnosis [9,12,14]. We did not conduct UBT in the parallel or repeated experiments. However, a cohort of 19 out of the 137 participants were positive according to SAT and NPCR but negative according to UBT (Table 2). The most plausible explanation is the metabolic or physiological state of *H. pylori*, where, for some reason, urease was not induced [9,12]. Perhaps it was due to the use of orange juice instead of citric acid, as recommended in recent guidelines [12,24]. It could be associated with delta-over-baseline (DOB) values that depend on factors such as gender, age, Body Mass Index (BMI), socioeconomic status, and smoking habits [12]. However, we could only assign one sample as a new positive sample, lowering the DOB cutoff to 2.4%, as recommended in some studies [25–27]. Besides poor test performance and high cut-off values, the most plausible explanation is volunteer obedience, as some may have used PPIs or antibiotics despite

claiming otherwise. The alternative that some *H. pylori* strains lack the gene encoding urease is unlikely. Urease genes are not essential for *H. pylori* survival, although they are required for the colonization of the mouse stomach and chronic infection [28–30]. This idea could be tested experimentally by amplifying and sequencing the urease genes.

Twenty-nine volunteers who were negative according to SAT were positive according to UBT and NPCR. The most plausible explanation for this is that the threshold for SAT is lower compared to NPCR [15,18,19,31,32], together with an uneven distribution of *H. pylori* antigen in stool, as repeated SAT testing occasionally yields different outcomes [19].

The most interesting inconsistency was that there were samples positive according to SAT (the least sensitive method) but negative according to NPCR (the most sensitive method). In this study, there were two such samples; however, in the previous study, we were unable to detect *H. pylori*-specific DNA in seven samples [19]. We initially explained this as due to the interaction between the antibody present in the SAT and an antigen similar to *H. pylori* that originated from another bacterium [32]. However, the most credible explanation is DNA sequence polymorphism or divergence at primer annealing sites. We reisolated fresh DNA from freezer-stored stool samples and repeated NPCR in the presence of the S solution in both reactions. The S solution serves as an amplification enhancer, thereby lowering the stringency of primer annealing. In addition, we used different NPCR variants, such as a one-vial modification of the original 148 bp NPCR or amplification with degenerated primers (one vial, panA, Table 1). Indeed, in repeated and variant experiments, we were able to amplify DNA from all samples. Two were positive in the repeated NPCR test for the 148 bp amplicon, and four in the one-vial test (Table 1). Sequencing of these PCR products revealed sequences that can be assigned to *H. pylori* in two cases (17A and 15K; Figure 1). In three instances (1A, 30L, and 20A), we were unable to distinguish between *H. canis*, *H. pylori*, and *H. saquini*. In one case (18A), the sequence of the PCR product was closely related to the DNA of a new species of *Helicobacter* that caused the death of a turtle (*Malacochersus tornieri*) [33]. The last case (19A) required the use of degenerate, so-called panA primers (Table 1), which were intended to amplify DNA from all species of the genus *Helicobacter*. Sequencing revealed a sequence related to *H. kumamotonensis* and *H. hepaticus*. Apparently, some inconsistencies between the SAT and NPCR are attributable to sequence divergence at the primer annealing site.

A common problem with NPCR is inhibition of amplification during the first PCR reaction, which is related to substances present in stool samples, such as hemoglobin and its degradation products, polysaccharide complexes, heavy metals, or proteins [34]. This problem can be eliminated by repeated DNA isolation and lowering the DNA load in the first amplification reaction. The other obstacle to routine diagnostics is the increased risk of contamination, which can be neglected by the rules used in forensic laboratories. Testing for a short 148 bp amplicon is frequently accompanied by PCR product formation in negative controls, even when filter tips are used. The main reason for this is the spray effect when opening the tubes, which can be suppressed by adding oil to the first PCR reaction. In this study, further measures included having the individual operations performed in three different rooms. The first was reserved for DNA isolation, the second for pipetting PCR reactions in a laminar flow box (biohazard), and the third for electrophoresis and DNA analysis. Also, pipettes and centrifuges were explicitly designated for each operation and cleaned and sterilized every three months. Instead of polystyrene ice boxes, we used autoclavable plastic boxes. Two different centrifuges were used, one for mastermixes and the first PCR reaction, and another one exclusively for the second PCR reaction. With this regimen, *H. pylori*-specific DNA could be reliably identified.

Strictly speaking, this study is based on a volunteer cohort rather than a clinically defined patient cohort. As we mentioned above, 40% of them had various digestive

problems, and 30% were treated by a gastroenterologist after testing positive for *H. pylori*. Moreover, the choice of cohort, whether patients or volunteers, should not affect the sensitivity of *H. pylori* detection, at least when comparing SAT and NPCR. In a previous study, we compared these methods and the ratio of positive SAT/NPCR was 58/106 (54.7%) in the patient cohort and 35/66 (53.0%) in the volunteer cohort [19].

5. Conclusions

Any *H. pylori* identification method has its pros and cons, and the concept of a gold standard is not the correct solution. In practice, due to its cost and ease of handling, the method of choice for initial screening is SAT; however, in cases of negative results and persistent digestive problems, we recommend UBT. NPCR may be a useful complementary method, especially in discordant or clinically suspicious cases. Apparently, a combination of these three methods allows us to bypass the drawbacks of any particular assay used for non-invasive *H. pylori* diagnostics.

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Informed Consent Statement: Written informed consent, in accordance with the guidelines of the Ethical Commission of the Ministry of Health of the Slovak Republic, was obtained from each patient and volunteer.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

UBT	Urease breath test
SAT	Stool antigen test
NPCR	Nested polymerase chain reaction
16S rRNA	RNA for the small ribosomal subunit
RUT	Rapid urease test
PPI	Proton pump inhibitor
BMI	Body Mass Index
DOB	Delta Over Baseline
95%CI	95% confidence interval

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