Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

Initial Reverse Transcription is Essential for Reliable *Helicobacter pylori* 23S rDNA Real-Time PCR Diagnostics from Fecal Samples

Dietmar Wolff and Andreas Gerritzen

Medical Laboratory Bremen, Bremen, Germany

SUMMARY

Background: **A sensitive, non-invasive molecular test for identifying *Helicobacter pylori* (*H. pylori*) in stool samples is important in the context of a "test-and-treat strategy". The goal of this study was to elucidate the benefit of an initial reverse transcription (RT) step for the sensitivity of *H. pylori* 23S rDNA Real-time PCR in stool samples.**
Methods: **We compared a LightMix® Modular *Helicobacter* 23S rDNA PCR assay without (LightMix® PCR) and with an initial reverse transcription step (LightMix® RT-PCR) assay for rapid detection of *Helicobacter pylori* in fecal samples. For maximum sensitivity, all Lightmix® RT-PCR assays were performed in triplicate for each sample. Additionally, the LightMix® RT-PCR was compared to *H. pylori* AG ELISA.**
Results: **Direct detection of *H. pylori* in feces by Lightmix® PCR was less sensitive than LightMix® RT-PCR. Only 2 out of 44 stool samples (4.5%) from patients with diarrhea were positive for *H. pylori* in the LightMix® PCR assay. In contrast, a reverse transcription step prior Lightmix® PCR increased the assay sensitivity markedly (19 out of 44 positives, 43.2%). When re-testing 65 samples initially analyzed with *H. pylori* AG ELISA with LightMix® RT-PCR, the detection rate for *H. pylori* was similarly increased from 23.1% (15/65) with *H. pylori* AG ELISA to 43.1% (28/65) with *H. pylori* LightMix® RT-PCR. 21.5% of the 28 *H. pylori* positive samples were positive in 1/3, 32% in 2/3, and 46.5% in 3/3 triple RT-PCR approaches. When re-testing the 28 LightMix® RT-PCR positive samples by LightMix® PCR, only 17.9% (5/28) RT-PCR positive samples were positive in PCR.**
Conclusions: **LightMix® RT-PCR is much more sensitive than Lightmix® PCR. In comparison to *H. pylori* AG ELISA, the LightMix® RT-PCR shows a markedly higher sensitivity. An initial reverse transcription step is crucial for reliable *Helicobacter pylori* 23S rDNA Real-time PCR diagnostics. The triple RT-PCR approach can additionally improve the detection of *H. pylori* in fecal samples. Thus, the method presented provides a highly sensitive, noninvasive assay to detect *H. pylori* in fecal samples with potential advantages compared to other *H. pylori* detection methods currently used in routine diagnostics.**
(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2018.181002)

Correspondence:
Dietmar Wolff
MVZ Medical Laboratory Bremen GmbH
Haferwende 12
28357 Bremen
Germany
Phone: +49 421-2072203
Fax: +49 421-20727203
Email: dietmar.wolff@mlhb.de

Manuscript accepted November 21, 2018

KEY WORDS

Helicobacter, feces, reverse transcription (RT), RT-PCR

INTRODUCTION

Infection with *Helicobacter pylori* (*H. pylori*) is worldwide the most common cause of chronic gastritis with subsequent gastric cancer [1]. Recently, the Kyoto consensus report [1] defined *H. pylori* gastritis as an infectious disease, even when patients do not suffer from

overt clinical symptoms, irrespective of complications such as peptic ulcers and gastric cancer. This represents a paradigm shift, as for *H. pylori* the indication for treatment is no longer confined to patients with clinical manifestations of the infection. Because of the widespread resistance towards antimicrobial agents, an important consequence of this consensus is, as for other infectious agents, the selection of proper antibiotics to ensure eradication, including prior antimicrobial susceptibility testing [2].

Urea breath test (UBT) and immunological stool antigen test (SAT) are currently the most investigated and best recommended non-invasive tests in the context of a "test-and-treat" strategy [3]. However, no information on resistance to antibiotics can be obtained from the results of these assays. Clarithromycin is an integral part of first-line therapies to treat *H. pylori* infections [3]. Resistance of *H. pylori* to clarithromycin is mainly due to the 3 point mutations A2142G, A2143G, and A2142C in the 23s ribosomal RNA (rRNA) [4]. Molecular detection of *H. pylori* infection by real-time PCR and simultaneous clarithromycin susceptibility testing in stool samples represents a promising alternative to UBT and SAT. Several real-time PCR methods [5-12] were reported which are directed toward partial amplification of the *H. pylori* 23S rDNA gene, followed by identification of the clarithromycin resistance point mutations. The *H. pylori* 23S rRNA is a 2,967 bp long component [13] of the large subunit (50S) of the bacterial ribosome. It is transcribed from the 23S rRNA gene (23S rDNA). The *H. pylori* genome carries only two copies of the 23S rRNA gene [13]; however, one single bacterial cell is estimated to harbor about 200,000 ribosomes during fast exponential growth. Therefore, it can be postulated that there are significantly more rRNA than rDNA copies available in a *H. pylori* bacterial cell. The rRNA can be converted by reverse transcription (RT) to rDNA. None of the above mentioned real-time PCR methods employs an initial reverse transcription (RT) step to convert 23S rRNA into 23S ribosomal DNA (rDNA), which might have positive impact regarding to the subsequent DNA PCR sensitivity. This may be of diagnostic relevance as *H. pylori*'s natural habitat is the stomach, not the intestine and, therefore, is expected to be present only in low concentrations in feces.

The goal of this study was to investigate whether an initial RT-step can increase the sensitivity of *H. pylori* 23S rDNA Real-time PCR in stool samples to improve molecular *Helicobacter pylori* diagnostics.

MATERIALS AND METHODS

Forty-four fecal samples from hospitalized patients (age range 21 to 90 years) with diarrhea from northern Germany were collected at the Medical Laboratory Bremen, Germany. Another 65 fecal samples (age range 18 to 68 years) were acquired from patients for whom *H. pylori* antigen ELISA test was requested explicitly.

The *H. pylori* antigen ELISA was performed according to the manufacturer's (Standard Diagnostic, Inc., Republic of Korea) instruction. In brief, 100 µL of diluted fecal samples and controls were transferred to each well of a microtiter plate. Then, 25 µL of enzyme conjugate (mixture of monoclonal antibodies to *H. pylori* and horseradish peroxidase) was added to each well. The micro plate was covered with adhesive plate sealer and mixed well on a vibrating mixer, followed by incubation at 37°C for 60 minutes. The wells were then washed 5 times with 350 µL of washing solution, followed by the addition of 100 µL TMB-AB solution and incubated in the dark at room temperature for 10 minutes. Afterwards, 100 µL of Stopping Solution was added to each well.

The absorbance of each well was read at a wavelength of 450 nm (reference 620 nm). The individual values of the absorbance for the controls (run in duplicate) were used to calculate the mean value if $0.005 \leq$ A (neg.) ≤ 0.100 and A (pos.) ≥ 1.000. In case one of the negative controls was outside the specification, this value was discarded; however, then both absorbance values of the positive control must mandatorily meet the specification. When these specifications were not fulfilled, the test had to be repeated. The cutoff value was calculated by adding 0.100 to the mean absorbance of the negative controls [A (neg.) + 0.100 = cutoff value]. Based on the manufacturer's recommendation, the samples were considered *H. pylori* antigen negative when A (sample) < cutoff and *H. pylori* antigen positive when A (sample) ≥ cutoff.

For nucleic acid extraction, 1 g of stool sample was suspended in 400 µL of sterile water. After sedimentation of the solids, 200 µL of the supernatant and 6 µL LightMix® Modular EAV RNA Extraction Control (TIB MOLBIOL, Berlin, Germany) were used for nucleic acid extraction with NucliSENS easyMAG® system (Biomérieux, Nürtingen, Germany) according to the manufacturer's instructions. The elution volume was 50 µL.

RT-PCR, PCR, and melting curve analysis were performed on a LightCyler 480 II instrument (Roche, Mannheim, Germany) following the manufacturer's protocol. PCR reactions (LightMix® PCR) were carried out in 20 µL mixtures containing 0.5 µL LightMix® Modular *Helicobacter* 23 S rRNA reagent mix (TIB MOLBIOL, Berlin, Germany), 0.5 µL LightMix® Modular EAV control reagent mix (TIB MOLBIOL, Berlin Germany), 10 µL LightCycler 480 Probes Master (Roche, Berlin, Germany), 4 µL H_2O, and 5 µL of the template. Real-Time PCR and melting curve analysis to detect A2142G, A2143G, and A2142C mutations for clarithromycin resistance assessment were performed as described in the LightMix® Modular *Helicobacter* 23S rRNA instructions. The detection limit was 10 copies each per PCR reaction according to the manufacturer's specification. The positive control for each PCR run (1,000 copies) was considered valid with Ct (cycle threshold) values between 28 and 30. The extraction

Table 1. Detection of *H. pylori* by LightMix® PCR and LightMix® RT-PCR.

n = 44 fecal samples	PCR neg.	PCR pos.
RT-PCR neg.	25	0
RT-PCR pos.	17	2

Table 2. Detection of *H. pylori* by LightMix® RT-PCR * and *H. pylori* AG ELISA.

n = 65 fecal samples	*H. pylori* AG ELISA neg.	*H. pylori* AG ELISA pos.
RT-PCR neg.	33	4
RT-PCR pos.	17	11

*** RT-PCR was performed in triplicate.**

Table 3. Detection of 28 *H. pylori* LightMix® RT-PCR positive fecal samples by LightMix PCR *.

n = 28	PCR neg.	PCR pos.
RT-PCR neg.	0	0
RT-PCR pos.	23	5

*** PCR was performed in triplicate.**

controls of PCR reactions were considered valid with Ct values between 28 and 32. Only samples fulfilling all criteria were included into the study.

For RT-PCR reactions (LightMix® RT-PCR) LightCycler 480 Probes Master was replaced by 0.1 µL RT-Enzyme Solution (Roche, Mannheim, Germany), 4 µL RT-PCR Reaction Mix (Roche, Mannheim, Germany), and 5.9 µL H_2O. An initial RT-Step for 5 minutes at 55°C was carried out before PCR cycling.

H. pylori LightMix® RT-PCR can cross-react with *Campylobacter spp*. [12]. To avoid false positive results all *H. pylori* positive samples were retested with LightMix® Modular *Campylobacter* PCR assay (TIB Molbiol, Berlin, Germany) following the manufacturer's protocol. Only *Campylobacter* negative samples were included into the study.

RESULTS

In order to evaluate the benefit of an initial reverse transcription step to improve the sensitivity for *H. pylori* detection in clinical specimens, 44 fecal samples from patients with diarrhea were tested in parallel with *H. pylori* LightMix® PCR and *H. pylori* LightMix® RT-PCR (Table 1). The detection rate for *H. pylori* was increased from 2% (2/44) using PCR to 43% (19/44) using RT-PCR.

In the next step, we compared *H. pylori* AG ELISA with LightMix® RT-PCR. Sixty-five fecal samples, for which *H. pylori* Ag ELISA had been requested in routine diagnostics, were tested in parallel with LightMix® RT-PCR (Table 2). The detection rate for *H. pylori* was increased from 23% (15/44) using *H. pylori* AG ELISA to 43% (28/65) using LightMix® RT-PCR. The four AG ELISA positive but RT-PCR negative samples showed absorbance values near the cutoff of the test (0.11, 0.11, 0.13, and 0.24).

To further increase the sensitivity for *H. pylori* detection, Lightmix® RT-PCRs was performed in triplicate for each patient sample. 21.5% (6/28) of the 28 *H. pylori* positive samples were positive in 1/3, 32% (9/28) in 2/3, and 46.5% (13/28) in 3/3 RT-PCR tests.

Finally, to evaluate the benefit of an initial reverse transcription step, the 28 LightMix® RT-PCR positive samples were retested by LightMix® PCR (Table 3). Only 18% (5/28) RT-PCR positive samples were positive in PCR.

H. pylori positive samples produced melting curves with a melting temperature of 63°C for the wild type and melting temperatures of 59°C, 56°C and 54°C for the 23S rDNA mutations A2142G, A2143G and A2142C, respectively (data not shown).

Eleven percent (2/19) of positive patient samples with

diarrhea and 18% (5/28) positive patient samples for which *H. pylori* Ag ELISA testing was requested showed either the A2142G or the A2143G mutations as identified by melting curve analysis.

DISCUSSION

The goal of this study was to investigate whether an initial RT-step can increase the sensitivity of *H. pylori* 23S rDNA real-time PCR assay in stool samples. Using RT-PCR the detection rate could be increased more than 20-fold from 2% to 43% in stool samples from patients with diarrhea. Depending on the socio-economic development in human society, the prevalence of *H. pylori* infection was determined to have a world-wide variation ranging from as low as 18.9% in Switzerland up to 87.7% in Nigeria [14]. Under the basic assumption that diarrhea did not influence *H. pylori* infection, the detection rate of 2% in Lightmix® PCR seems to be much too low and the detection rate of 43% in LightMix® RT-PCR seems plausible in a North German population. Stool PCR is not as sensitive as stool antigen enzyme immunoassay [15] and is therefore not recommended for routine diagnostics to detect *H. pylori*. In order to investigate whether RT-PCR can increase sensitivity of *H. pylori* recovery, *H. pylori* AG ELISA and RT-PCR were tested in parallel. In comparison to *H. pylori* AG ELISA, the LightMix® RT-PCR shows a significantly higher sensitivity. The detection rate for *H. pylori* was increased nearly 2-fold from 23% to 43% with LightMix® RT-PCR. If this observation is validated by further studies, RT-PCR assay could become an important sensitive tool for screening *H. pylori* infections.

There were four AG ELISA positive but RT-PCR assay negative samples. Taking into account that all of these samples showed absorbance values near the cutoff of the test, specificity of the AG ELISA cannot be taken for granted. Under this assumption, the sensitivity of RT-PCR assay in comparison to AG ELISA would be even higher.

To evaluate the benefit of an initial reverse transcription step, LightMix® RT-PCR positive samples were retested using LightMix® PCR. Only 18% (5/28) RT-PCR positive samples were positive in PCR, which clarified the markedly better performance of RT-PCR assay.

As *H. pylori* specifically colonizes the stomach and is not an intestinal bacterium, it is present only in low numbers in stool, a factor which influences the sensitivity of *H. pylori* detection using fecal samples. In order to deal with this issue, we carried out RT-PCR in triplicate to analyze *H. pylori* positive samples near the detection limit. Triple testing seems to be crucial as 53.5% of the samples were only positive in 1/3 or 2/3 PCR tests. The disadvantage of higher costs for triple testing is in our opinion more than balanced by the benefit of a quick, reliable, and highly sensitive diagnostic approach for the patients, thus enabling an early and adequate therapy with best chances for high patient compliance.

Clarithromycin is the key antibiotic in the standard primary eradication triple therapy. Clarithromycin resistance is the most important reason for primary eradication therapy failure. Clarithromycin inhibits protein synthesis of the bacterium by interacting at the 23S ribosome subunit. There are three main point mutations in the 23S rDNA known to induce clarithromycin resistance (A2142G, A2143G, and A2142C,); all of them could be identified by melting curve analysis following PCR. Eighteen percent of the positive samples for which *H. pylori* Ag ELISA testing was requested revealed clarithromycin resistance which is in accordance to other studies [5,10,16].

CONCLUSION

The use of a highly sensitive screening method capable of simultaneously identifying both *H. pylori* infection and clarithromycin resistance with a non-invasive procedure could possibly decrease the rate of therapy failures and reduce the number of gastroscopies and biopsies. Through effective treatment, the number of gastric cancers can be reduced.

To our knowledge this is the first report to describe the importance of reverse transcription for reliable non-invasive *Helicobacter pylori* 23S rDNA real-time PCR diagnostics in fecal samples. Further studies will be necessary to determine which clinical impact this observation will be have for sensitive non-invasive molecular *H. pylori* testing.

Acknowledgment:
We gratefully acknowledge the staff, especially Andrea Kleemeyer, of the molecular biology department of our laboratory for excellent technical assistance.

Declaration of Interest:
The authors report no conflict of interests.

References:

1. Sugano K, Tack J, Kuipers EJ, et al. Kyoto global consensus report on Helicobacter pylori gastritis. Gut 2015;64:1353-67 (PMID: 26187502).

2. Mégraud F. Time to change approaches to *Helicobacter pylori* management. Lancet Gastroenterol Hepatol 2017;10:692-3 (PMID: 28781120).

3. Malfertheiner P, Megraud F, O'Morain CA, et al. Management of *Helicobacter pylori* infection - the Maastricht V/Florence Consensus Report. Gut 2017;66(1):6-30 (PMID: 27707777).

4. Versalovic J, Shortrige D, Kibler K, et al. Mutations in the 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. Antimicrob Agents Chemother 1996;40(2):477-80 (PMID: 8834903).

5. Gibson JR, Saunders NA, Burke B, Owen RJ. Novel method for rapid determination of clarithromycin sensitivity in *Helicobacter pylori*. J Clin Micobiol 1999;37(11):3746-8 (PMID: 10523591).

6. Matsumura M, Hikiba Y, Ogura K, et al. Rapid detection of mutations in the 23S rRNA Gene of *Helicobacter pylori* that confers Resistance to clarithromycin treatment to the bacterium. J Clin Microbiol 2001;39(2):691-5 (PMID: 11158129).

7. Chisholm SA, Owen RJ, Teare EL, Saverymuttu S. PCR based diagnosis of *Helicobacter pylori* infection and real-time determination of clarithromycin resistance directly from gastric biopsy samples. J Clin Microbiol 2001;39(4):1217-20 (PMID: 112830 30).

8. Oleastro M, Ménard A, Santos A, et al. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. J Clin Microbiol 2003;41(1):397-402 (PMID: 12517879).

9. Lascols C, Lamarque D, Costa JM, et al. Fast and accurate quantitative detection of *Helicobacter pylori* and identification of clarithromycin resistance mutations in *H. pylori* isolates from gastric biopsy specimens by real-time PCR. J Clin Microbiol 2003;41: 4573-7 (PMID: 14532184).

10. Schabereiter-Gurtner C, Hirschl AM, Dragosics B, et al. Novel real-time PCR assay for detection of *Helicobacter pylori* infection and simultaneous clarithromycin susceptibilty testing of stool and biopsy specimens. J Clin Microbiol 2004;42(10):4512-8 (PMID: 15472302).

11. Beckman E, Saracino I, Fiorini G, et al. A novel stool PCR test for *Helicobacter pylori* may predict resistance and eradication of infection at a high rate. J Clin Microbiol 2017;55(8):2400-5 (PMID: 28515219).

12. Redondo JJ, Keller PM, Zbinden R, Wagner K. A novel RT-PCR for the detection of *Helicobacter pylori* and identification of clarithromycin resistance mediated by mutations in the 23S rRNA gene. Diagn Microbiol Infect Dis 2018;90(1)1-6 (PMID: 291111 47).

13. Taylor DE, Ge Z, Purych D, Lo T, Hiratsuka K. Cloning and sequence analysis of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. Antimicrob Agents Chemother 1997;41(12):2621-8 (PMID: 9420030).

14. Hooi JKY, Lai WY, Ng WK, et al. Global Prevalence of *Helicobacter pylori* infection: Systematic Review and Meta-Analysis. Gastroenterol 2017;153(2):420-9 (PMID: 28456631).

15. Mishra S, Singh V, Rao GR, et al. Detection of *Helicobacter pylori* in stool specimens: comparative evaluation of nested PCR and antigen detection. J Infect Dev Ctries 2008;2(3):206-10 (PMID: 19738352).

16. Ierardi E, Giorgio F, Iannone A, et al. Noninvasive molecular analysis of *Helicobacter pylori*: Is it time for tailored first-line therapy? World J Gastroenterol 2017;23(14):2453-58 (PMID: 284 65629).