Journal of Microbiological Methods 163 (2019) 105653

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

# Development of a loop-mediated isothermal amplification assay for rapid *Helicobacter pylori* detection

Saori Horiuchi[a,b,*], Ryuichi Nakano[a], Akiyo Nakano[a], Naokuni Hishiya[a,c], Kenji Uno[c], Yuki Suzuki[a], Ayako Tanouchi[a], Naoki Kakuta[a], Takashi Masui[d], Noriko Jojima[b], Hisakazu Yano[a]

[a] Department of Microbiology and Infectious Diseases, Nara Medical University, 840 Shijo-Cho, Kashihara, Nara 634-8521, Japan
[b] Department of Public Health Nursing, Nara Medical University, 840 Shijo-Cho, Kashihara, Nara 634-8521, Japan
[c] Department of Infectious Diseases, Minami-Nara General Medical Center, 8-1 Fukugami, Yoshino-gun, Oyodo, Nara 638-8551, Japan
[d] Department of otolaryngology - Head and Neck Surgery, Nara Medical University, 840 Shijo-Cho, Kashihara, Nara 634-8521, Japan

ARTICLE INFO

*Keywords:*
*Helicobacter pylori*
Loop-mediated isothermal amplification
Rapid detection

ABSTRACT

Infection with *cagA*-positive *Helicobacter pylori* is associated with gastric cancer. Molecular techniques are vital for accurate *H. pylori* diagnosis. We developed a loop-mediated isothermal amplification (LAMP) for detecting the *H. pylori cagA* gene and evaluated its use for clinical diagnosis. A LAMP primer set was designed to recognize the homologous regions of *cagA* gene sequences of 6 *H. pylori* strains. LAMP sensitivity was evaluated with serial dilutions of *H. pylori* ATCC 43504 and fecal specimens; specificity was evaluated with *H. pylori* ATCC 49396 and CIP 104086. The LAMP sensitivity for *H. pylori* specimens was $10^{-1}$ cfu/tube (reaction time, 37 min), which was 10-fold more sensitive than polymerase chain reaction. LAMP was also highly sensitive and rapid for fecal specimens. It detected *cagA* gene from ATCC 49396 and CIP 104086. The findings suggest LAMP can be used for diagnosing and screening of *H. pylori* infections to decrease gastric cancer incidence.

## 1. Introduction

*Helicobacter pylori* has been classified as carcinogenic to humans (group I carcinogen) by the International Agency for Research on Cancer (IARC) (Park et al., 2018); over 50% of the global human population is reported to be infected (Mungazi et al., 2018). The *H. pylori cagA* gene is one of the virulence factors encoded on the *cag* pathogenicity island. This pathogenicity island also encodes the system that translocates *cagA* to the inside of epithelial cells and causes inflammation (Ulloa-Guerrero et al., 2018). Chronic infection with *cagA*-positive *H. pylori* strains in the stomach is associated with a significantly increased risk of gastric diseases, including chronic atrophic gastritis and gastric cancer (Ekström et al., 2001). In East Asia, *H. pylori cagA*-positive strains comprise 90% or more of all infecting *H. pylori* strains (Ito et al., 1997), and the estimated age-standardized rate of gastric cancer is very high (22.7–41.8 cases per 100,000 of the population) (Suzuki and Mori, 2016). The infection ratio of *cagA*-positive strains is higher than that of *cagA*-negative strains worldwide, except in the East Asia region (Kamogawa-Schifter et al., 2018). Early eradication of *H. pylori* has been considered to reduce the risk of developing gastric diseases (Asaka et al., 2010); therefore, early detection and treatment of *H. pylori*, especially *cagA*-positive strains, are required.

*H. pylori* infection can be diagnosed by invasive testing such as that involving immunohistochemical staining, the rapid urease test, and culture and noninvasive testing such as the respiratory urea breath test, stool antigen detection, and serological analysis (Bazin et al., 2018). Although the level of the strategy of these tests has been increasing, their sensitivity, cost effectiveness, merits, and demerits differ from each other, such that the use of a single test for diagnosing *H. pylori* infections is not advocated (Elwyn et al., 2007). Therefore, molecular methods are important for diagnosing *H. pylori* infections more accurately and effectively (Bakhtiari et al., 2016). Fecal testing is an accurate and noninvasive tool for assessing the status of *H. pylori* infection before and after treatment (Lehmann and Beglinger, 2003). Therefore, polymerase chain reaction (PCR) has been used earlier for detecting pathogenic genes of *H. pylori* from biopsy and fecal specimens (Patel et al., 2014).

Loop-mediated isothermal amplification (LAMP) is a nucleotide acid amplification method that is highly sensitive, specific, and rapid (Nakano et al., 2015; Notomi et al., 2000). In addition, LAMP is less expensive than other molecular diagnostic methods because it can amplify the target DNA sequence under isothermal conditions of

*Abbreviation:* IARC, International Agency for Research on Cancer; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction

* Corresponding author at: Department of Public Health Nursing, Nara Medical University, 840 Shijo-Cho, Kashihara, Nara 634-8521, Japan.
*E-mail address:* saorih@naramed-u.ac.jp (S. Horiuchi).

https://doi.org/10.1016/j.mimet.2019.105653
Received 6 March 2019; Received in revised form 10 June 2019; Accepted 10 June 2019
Available online 11 June 2019
0167-7012/ 

60–68 °C, with simple incubators, such as a water bath or block heater, instead of thermal cycler, and the results are observable with the naked eye (Mori and Notomi, 2009; Kuboki et al., 2003).

Therefore, LAMP has been considered as a valuable tool for diagnosing infectious diseases and has already been applied to influenza and tuberculosis diagnosis in the clinical setting (Imai et al., 2006; Jayawardena et al., 2007; Notomi et al., 2000).

To our knowledge, our study is the first to develop a LAMP assay for detecting the *H. pylori cagA* gene. We aimed to evaluate the possibility of applying this LAMP assay to clinical diagnostics.

## 2. Materials and methods

### 2.1. Bacterial strains and DNA extraction

Serial dilutions of *H. pylori* ATCC 43504 cells (RIKEN BRC, Tsukuba, Japan; $10^{-2}$ - $10^{5}$ cfu per tube) were used to determine the most optimal temperature for and sensitivity of the LAMP assay for the *H. pylori cagA* gene. *H. pylori* ATCC 49396 and CIP 104086 cells (RIKEN BRC) were used to determine the specificity of the LAMP assay. The total bacterial DNA from the serial dilutions of *H. pylori* ATCC 49396 cells and from *H. pylori* ATCC 49396 and CIP 104086 cells was extracted by heating the cells at 100 °C for 10 min. The *H. pylori* ATCC 43504, ATCC 49396, and CIP 104086 cells had been cultured in sheep blood agar under microaerophilic conditions ($O_2$, 6–12%; $CO_2$, 5–8%) at 37 °C for 4 days.

### 2.2. Preparation of fecal specimens

In accordance with Declaration of Helsinki, a fecal sample was collected from a healthy volunteer who did not have *H. pylori* infection; written informed consent was obtained for participation in this study. *H. pylori* ATCC 43504 cells were cultured and added to human feces to obtain serial dilutions of fecal specimens ($10^{-2}$ - $10^{5}$ cfu per tube). Subsequently, DNA was extracted from these fecal specimens by using the ISOSPIN Fecal DNA kit (Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions.

The protocol was approved by the Ethical Review Committee of Nara Medical University (project identification code no. 1917).

### 2.3. Primer design

The LAMP primer set was designed using the Primer Explorer V5 software (http://primerexplorer.jp/v5_manual/index.html) to recognize the homologous regions of the *cagA* gene sequences of the following 6 *H. pylori* strains: ATCC 43526, ATCC 43579, ATCC 49503, NCTC 11637, NCTC 11637, and SS1 (GenBank accession numbers AF001357, AB015414, AB015415, AB015416, AF202973, and KR154757, respectively). The primer set 5′ → 3′; consisted of 5 primers: 2 outer primers, i.e., F3 (5′ → 3′; GCTAGTTTGTCAGCGAAAC) and B3 (5′ → 3′; TGACAAAGGAACGCTTCC); 2 inner primers, i.e., FIP (5′ → 3′; TCATTGATTGCTCCATTCTGGATATTAGACAATTACGCTACTAACAGC) and BIP (5′ → 3′; CGACCGGTATGCTAACGCACCGCAACTATCTTATCA TTCACG); and a loop primer, i.e., LB (5′ → 3′; AAAAACCCTGAGTGGC TCAAG).

### 2.4. LAMP assay

The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan). The total volume of the reaction mixture for the LAMP assay was 25 μl, which comprised the following (Nakano et al., 2015): 2.6 μl primer mixture containing the 2 outer primers (F3 and B3, 5 pmol each), the 2 inner primers (FIP and BIP, 40 pmol each), and the loop primer (LB, 20 pmol), which could accelerate the LAMP reaction; 6.9 μl distilled water; 1.0 μl *Bst* DNA polymerase; 12.5 μl 2× reaction mix; and 2 μl DNA template.

DNA amplification was performed under isothermal conditions at an optimal temperature of 64 °C for 60 min, following which the termination reaction was performed at 80 °C for 5 min. A Loopamp real-time turbidimeter (LoopampEXIA, Eiken Chemical Co.) was used for observing the progress of DNA amplification.

### 2.5. PCR assay

PCR assays were performed as described previously (Russo et al., 1999) with the forward primer AATACACCAACGCCTCCAAG (5′ → 3′) and reverse primer TTGTTGCCGCTTTTGCTCTC (5′ → 3′), which are specific for the *H. pylori cagA* gene. The number of thermal cycles was modified to 35.

### 2.6. Determination of LAMP assay sensitivity

To evaluate LAMP assay sensitivity for detecting the *H. pylori cagA* gene, serial 10-fold dilutions of genomic DNA ($10^{-2}$ - $10^{5}$ cfu per tube) extracted from cultivated *H. pylori* ATCC 43504 cells and fecal specimens were tested by LAMP and PCR. Subsequently, the results of both these assays and reaction times were compared.

### 2.7. Determination of LAMP assay specificity

To evaluate LAMP assay specificity for detecting the *H. pylori cagA* gene, the total bacterial DNA extracted from cultivated *H. pylori* ATCC 49396 and CIP 104086 cells was analyzed with LAMP.

## 3. Results

### 3.1. Sensitivity of the H. pylori cagA gene-specific LAMP assay with H. pylori cell specimens

The LAMP assay could help detect the target gene at $10^{-1}$ - $10^{5}$ cfu per tube of the *H. pylori* ATCC 43504 cells within 37 min. The reaction time for $10^{5}$ cfu per tube was the shortest, i.e., only 16 min, and that for $10^{-1}$ cfu per tube was 37 min (Fig. 1A). The detection limit of PCR was $10^{0}$ cfu per tube (Fig. 1B); the assay duration was more than 120 min. Thus, LAMP (detection limit, $10^{-1}$ cfu per tube) was found to be 10 times more sensitive than PCR (detection limit, $10^{0}$ cfu per tube).

### 3.2. Specificity of the H. pylori cagA gene-specific LAMP assay

The LAMP assay could amplify the target gene within 18 min and 19 min when the total bacterial DNA extracted from *H. pylori* ATCC 49396 and CIP 104086 cells, respectively, was analyzed.

### 3.3. Detection of the H. pylori cagA gene in fecal specimens

The LAMP assay for fecal specimens could amplify the target gene even at $10^{-1}$ cfu per tube within 44 min, whereas the detection limit of PCR was $10^{0}$ cfu per tube and the assay duration was more than 120 min. DNA amplification for $10^{5}$ cfu per tube of the specimen with the LAMP assay was observed within 20 min, which was the shortest reaction time observed in the assay for fecal specimens.

## 4. Discussion

In our current study, we developed a rapid, simple, and sensitive LAMP assay. The LAMP primer set designed was considered to be able to detect the *H. pylori cagA* gene specifically and effectively because the primers were designed to homologous regions of the *cagA* gene of 6 *H. pylori* strains, including type strains.

The detection limit of this LAMP assay with *H. pylori* ATCC 43504 cell specimens was $10^{-1}$ cfu per tube; thus, this assay was 10 times more sensitive than the previously reported conventional PCR analysis,

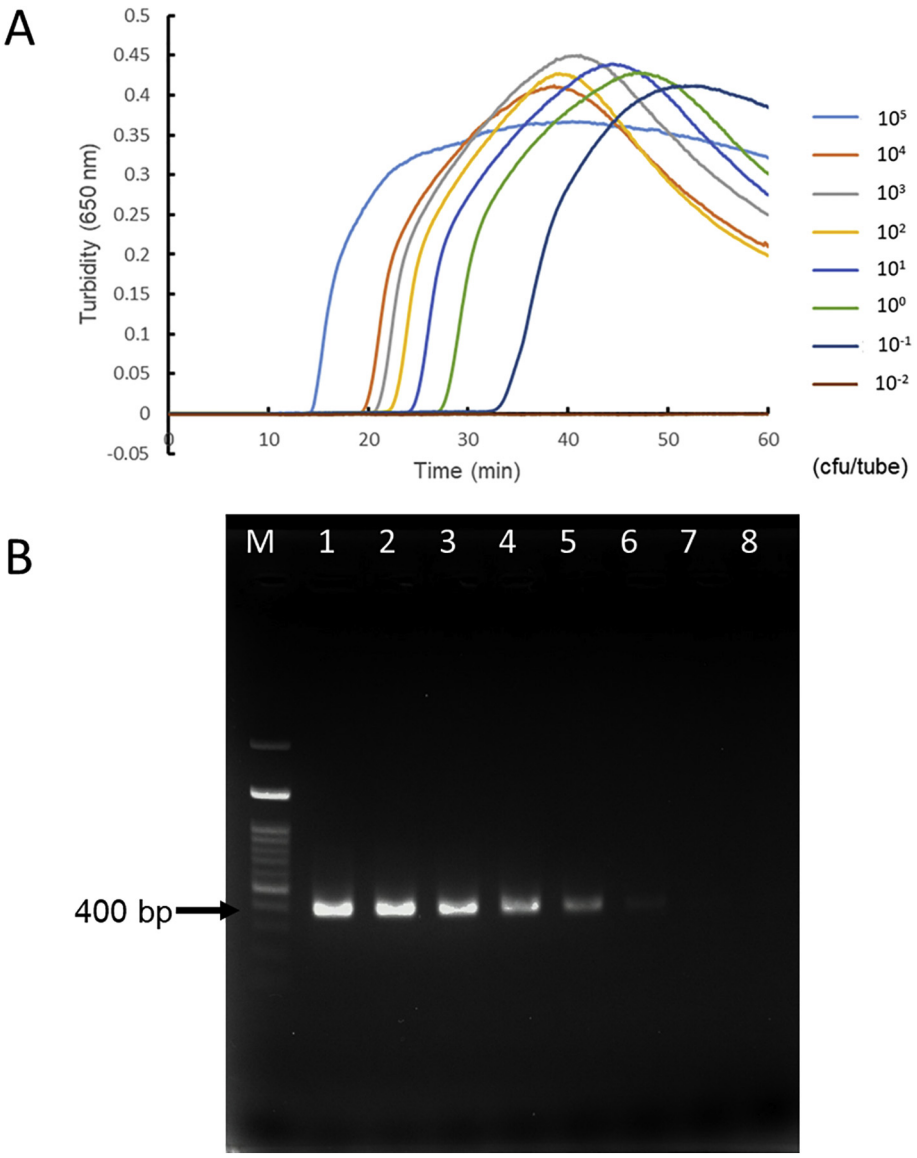


**Fig. 1.** Sensitivities of the LAMP and PCR techniques for detecting the *H. pylori cagA* gene.
(A) Detection of the *H. pylori cagA* gene by using LAMP.
(B) Detection of the *H. pylori cagA* gene by using PCR. M: Marker (100-bp DNA ladder), 1: $10^5$ cfu/tube, 2: $10^4$ cfu/tube, 3: $10^3$ cfu/tube, 4: $10^2$ cfu/tube, 5: $10^1$ cfu/tube, 6: $10^0$ cfu/tube, 7: $10^{-1}$ cfu/tube, and 8: $10^{-2}$ cfu/tube.

which had a detection limit of $10^0$ cfu per tube. The sensitivity of our LAMP assay was $10^3$ times higher than that of the LAMP assay for *H. pylori* ATCC 26695 cell specimens, which previously developed for detecting the *H. pylori glmM* gene ($10^2$ cfu per tube) (Minami et al., 2006). Although the sensitivities of LAMP and PCR are affected by the target strains, the sensitivity of LAMP is generally 10–100 times higher than that of PCR (Chaouch et al., 2018; Nakano et al., 2015). This higher sensitivity is attributable to the fact that LAMP can help amplify target DNA with high efficiency under isothermal conditions, without the results being significantly affected by the non-target DNA present (Notomi et al., 2000). The reaction times of our LAMP assay, with 16 min being the shortest reaction time and 37 min being the longest, were shorter than those of conventional PCR. The specificity of this LAMP assay was confirmed by detection of the target gene in the other *H. pylori* strains, i.e., *H. pylori* ATCC 49396 and CIP 104086, as well. Thus, our findings suggest the potential of our LAMP assay for detecting the *H. pylori cagA* gene, which is much more sensitive and rapid than previously developed LAMP assays and conventional PCR, and for application to the clinical diagnosis of *H. pylori* infections.

The high sensitivity and rapidity of this LAMP assay were maintained even for the fecal specimens; LAMP was more sensitive and rapid than PCR even for these samples. Therefore, the application of a fecal test involving LAMP, for clinical diagnosis of *cagA*-positive *H. pylori* infections, would contribute to improving the screening, diagnosis, and treatment of these infections, even in the developing countries (Fernández-Soto et al., 2019). LAMP could also be applied to the epidemiological study and monitoring of *H. pylori cagA*-positive strains, and be useful for the assessment of the risk of gastric diseases resulting from *cagA*-positive *H. pylori* infection.

To summarize, such testing would aid in early detection that is noninvasive, rapid, and highly sensitive and selective detection of *H. pylori cagA*-positive strains. Although this LAMP assay was found to be specific, a limitation of this study is that only a few bacterial strains were tested for evaluating LAMP specificity. Further studies using different *H. pylori* strains and clinical specimens like gastric biopsy and fecal specimens from patients are required to investigate the possibility of expanding the application of this assay.

## 5. Conclusion

In this study, we developed a highly sensitive, rapid, and simple LAMP assay for detecting the *H. pylori cagA* gene in fecal samples. This noninvasive fecal test involving LAMP can be applied to clinical diagnosis. Therefore, using this method in regions with a high frequency of people infected with *cagA*-positive *H. pylori* strains would help improve the diagnosis and treatment of *H. pylori* infections and reduce cases of gastric diseases, including gastric cancer in the world.

## Declaration of Competing Interests

The authors have no conflicts of interest directly relevant to the content of this article.

## Funding

This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI [grant number JP17K17546]; this funding aided in collection and analysis of data and writing of this report.

## Acknowledgements

We are grateful to M. Nakano for assistance with technical supports in our study.

## References

Asaka, M., Kato, M., Graham, D.Y., 2010. Strategy for eliminating gastric cancer in Japan. Helicobacter 15, 486–490. https://doi.org/10.1111/j.1523-5378.2010.00799.x.

Bakhtiari, S., Alvandi, A., Pajavand, H., Navabi, J., Najafi, F., Abiri, R., 2016. Development and diagnostic evaluation of loop-mediated isothermal amplification using a new gene target for rapid detection of *Helicobacter pylori*. Jundishapur. J. Microbiol. 9, e28831. https://doi.org/10.5812/jjm.28831.

Bazin, T., Nchare Mfondi, A., Julie, C., Émile, J.F., Raymond, J., Lamarque, D., 2018. Contribution of genetic amplification by PCR for the diagnosis of *Helicobacter pylori* infection in patients receiving proton pump inhibitors. United European Gastroenterol J 6, 1267–1273. https://doi.org/10.1177/2050640618787055.

Chaouch, M., Mhadhbi, M., Limam, S., Darghouth, M.A., Benabderrazak, S., 2018. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Theileria annulata* targeting the cytochrome B gene. Iran. J. Parasitol. 13, 225–234.

Ekström, A.M., Held, M., Hansson, L.E., Engstrand, L., Nyrén, O., 2001. *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. Gastroenterology. 121, 784–791. https://doi.org/10.1053/gast.2001.27999.

Elwyn, G., Taubert, M., Davies, S., Brown, G., Allison, M., Phillips, C., 2007. Which test is best for *Helicobacter pylori*? A cost-effectiveness model using decision analysis. Br. J. Gen. Pract. 57, 401–403.

Fernández-Soto, P., Gandasegui, J., Carranza Rodríguez, C., Pérez-Arellano, J.L., Crego-Vicente, B., García-Bernalt Diego, J., López-Abán, J., Vicente, B., Muro, A., 2019. Detection of *Schistosoma mansoni*-derived DNA in human urine samples by loop-mediated isothermal amplification (LAMP). PLoS One 14, e0214125. https://doi.org/10.1371/journal.pone.0214125.

Imai, M., Ninomiya, A., Minekawa, H., Notomi, T., Ishizaki, T., Tashiro, M., Odagiri, T., 2006. Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian influenza virus infection. Vaccine. 24, 6679–6682. https://doi.org/10.1016/j.vaccine.2006.05.046.

Ito, Y., Azuma, T., Ito, S., Miyaji, H., Hirai, M., Yamazaki, Y., Sato, F., Kato, T., Kohli, Y., Kuriyama, M., 1997. Analysis and typing of the *vacA* gene from *cagA*-positive strains of *Helicobacter pylori* isolated in Japan. J. Clin. Microbiol. 35, 1710–1714.

Jayawardena, S., Cheung, C.Y., Barr, I., Chan, K.H., Chen, H., Guan, Y., Peiris, J.S., Poon, L.L., 2007. Loop-mediated isothermal amplification for influenza a (H5N1) virus. Emerg. Infect. Dis. 13, 899–901. https://doi.org/10.3201/eid1306.061572.

Kamogawa-Schifter, Y., Yamaoka, Y., Uchida, T., Beer, A., Tribl, B., Schöniger-Hekele, M., Trauner, M., Dolak, W., 2018. Prevalence of *Helicobacter pylori* and its *CagA* subtypes in gastric cancer and duodenal ulcer at an Austrian tertiary referral center over 25 years. PLoS One 13, e0197695. https://doi.org/10.1371/journal.pone.0197695.

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D.J., Suzuki, H., Sugimoto, C., Igarashi, I., 2003. Loop-mediated isothermal amplification for detection of African trypanosomes. J. Clin. Microbiol. 41, 5517–5524. https://doi.org/10.1128/jcm.41.12.5517-5524.2003.

Lehmann, F.S., Beglinger, C., 2003. Current role of *Helicobacter pylori* stool tests. Digestion. 68, 119–123. https://doi.org/10.1159/000074682.

Minami, M., Ohta, M., Ohkura, T., Ando, T., Torii, K., Hasegawa, T., Goto, H., 2006. Use of a combination of brushing technique and the loop-mediated isothermal amplification method as a novel, rapid, and safe system for detection of *Helicobacter pylori*. J. Clin. Microbiol. 44, 4032–4037. https://doi.org/10.1128/JCM.00898-06.

Mori, Y., Notomi, T., 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. 15, 62–69. https://doi.org/10.1007/s10156-009-0669-9.

Mungazi, S.G., Chihaka, O.B., Muguti, G.I., 2018. Prevalence of *Helicobacter pylori* in asymptomatic patients at surgical outpatient department: Harare hospitals. Ann. Med. Surg. (Lond). 35, 153–157. https://doi.org/10.1016/j.amsu.2018.09.040.

Nakano, R., Nakano, A., Ishii, Y., Ubagai, T., Kikuchi-Ueda, T., Kikuchi, H., Tansho-Nagakawa, S., Kamoshida, G., Mu, X., Ono, Y., 2015. Rapid detection of the *Klebsiella pneumoniae* carbapenemase (KPC) gene by loop-mediated isothermal amplification (LAMP). J. Infect. Chemother. 21, 202–206. https://doi.org/10.1016/j.jiac.2014.11.010.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, e63.

Park, J.Y., Forman, D., Waskito, L.A., Yamaoka, Y., Crabtree, J.E., 2018. Epidemiology of *Helicobacter pylori* and *cagA*-positive infections and global variations in gastric cancer. Toxins (Basel). 10, e163. https://doi.org/10.3390/toxins10040163.

Patel, S.K., Pratap, C.B., Jain, A.K., Gulati, A.K., Nath, G., 2014. Diagnosis of *Helicobacter pylori*: what should be the gold standard? World J. Gastroenterol. 20, 12847–12859. https://doi.org/10.3748/wjg.v20.i36.12847.

Russo, F., Notarnicola, M., Di Matteo, G., Leoci, C., Caruso, M.L., Pirrelli, M., Caradonna, M., Morandi, L., Di Leo, A., 1999. Detection of *Helicobacter pylori cagA* gene by polymerase chain reaction in faecal samples. Eur. J. Gastroenterol. Hepatol. 11, 251–256.

Suzuki, H., Mori, H., 2016. Different pathophysiology of gastritis between East and West? An Asian perspective. Inflamm. Intest. Dis. 1, 123–128. https://doi.org/10.1159/000446301.

Ulloa-Guerrero, C.P., Delgado, M.D.P., Jaramillo, C.A., 2018. Structural analysis of variability and interaction of the N-terminal of the oncogenic effector *CagA* of *Helicobacter pylori* with phosphatidylserine. Int. J. Mol. Sci. 19, e3273. https://doi.org/10.3390/ijms19103273.