ACTA MICROBIOLOGICA BULGARICA Volume 34 / 2 (2018)

Detection and Identification of *Helicobacter pylori* from Patients and Comparison of Different Methods

Mariyana Milashka[1], Stefan Milashki[2], Ralitsa Popova[2*], Bilyana Borisova[3], Magdalena Bonovska[4]

[1]*Military Medical Academy, Sofia, Bulgaria*
[2]*National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria*
[3]*Faculty of Dental Medicine, Medical University - Sofia, Bulgaria*
[4]*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

Abstract

The aim of this study was to determine the most appropriate method for identification of *Helicobacter pylori* following isolation from gastric mucosal biopsy of patients with clinical signs of gastritis or duodenitis. Microbiological analysis and polymerase chain reaction (PCR) were found to be the most appropriate and highly reliable in diagnosing the presence of *H. pylori* in the gastrointestinal mucosa in patients with clinical symptoms of gastritis or ulcer. The microbiological analysis of 41 gastric mucosal biopsy specimens from patients with clinical symptoms of gastritis or ulcer revealed *H. pylori* in 36 patients (87.80%), mixed *H. pylori* and *C. jejuni* culture in four patients (9.75%) and presence of lactic acid bacteria in just one patient (2.45% of cases).

Keywords: *Helicobacter pylori,* gastric mucosa, gastritis, PCR

Резюме

Целта на изследването е да се определи най-подходящия метод за идентификация на *Helicobacter pylori*, както и изолиране на причинителя чрез биопсия на стомашна мукоза от пациенти с клинични признаци на гастрит и дуоденит. Установи се, че микробиологичния анализ и полимеразно-верижната реакция (PCR) са най-подходящи и високо надеждни методи за доказване наличието на *H. pylori* в стомашно чревната мукоза при пациенти с клинични симптоми на гастрит или язва. Микробиологичният анализ на 41 проби биопсичен материал от пациенти с клинични признаци на гастрит или язва показаха присъствие на *H. pylori* при 36 пациенти (87.80%), комбинация от *H. pylori* и *C. jejuni* при четири пациента (9.75%) и млечно кисели бактерии при един пациент (2.45% от случаите).

Introduction

Chronic gastritis in humans and monogastric animals is chronic inflammation of the gastric mucosa that progressively leads to various degrees of damage to the gastric glands and impaired secretion of gastric enzymes, causing a range of non-specific symptoms in patients. Chronic gastritis is a lifelong disease, which is either atrophic or nonatrophic, studied seriously since 1982 with detection of *Helicobacter pylori* as the causative agent but still with some unknowns, considering the etiology and some dietetic (Yordanov, 2017), pathogenetic and autoimmune factors (Lee *et al*., 2016). The understanding of the etiology of chronic gastritis changed completely following the discovery of the *H. pylori* infection and its role in causing the condition.

H. pylori is mainly transmitted via the fecal-oral route with an infection dose of 10^4 - 10^{10} bacterial cells (Graham et al., 2004) and is causally associated with a high percentage of chronic gastritis cases (Kusters *et al*., 2006), which can cause ulcer of the duodenum as a result of increased secretion of hydrochloric acid (Waldum *et al*., 2016), and is associated with autoimmune gastritis and pernicious anemia (de Vries and Kuipers, 2010). *H. pylori* is also a major co-factor in the etiology

* Corresponding author:
e-mail: authorr.ilinkina@gmail.com

of gastric carcinoma and is associated with mucosa-associated lymphoid tissue (MALT) lymphoma in humans (Chang *et al.*, 1999; Ruggiero 2012; Hagymási and Tulassay, 2014). It has been reported that the metabolic activity of *H. pylori* in the duodenal or gastric mucosa causes degradation of the endogenous glycoprotein lactoferrin known for its antimicrobial, anti-inflammatory and immunostimulating activity. It is due to the impairment of the dynamic equilibrium between the macroorganism and its microflora that the typical signs of gastritis or ulcer occur (Baveye *et al*., 1999; Go and Growe, 2000). Other researchers have suggested an association between *H. pylori* infection and gastric carcinoma as a logical result of the etiopathogenetic relationship of *H. pylori:* chronic gastritis, accounting for the high prevalence (up to 25%) of cancers in patients with gastric or duodenal ulcer (Gracham *et al,.* 1991; Fennety *et al.,* 1992; Sipponen *et al*., 1992). Now, we know that the rate of progression and type of gastritis, as well as the acid-secretory status of the patient, are important for determining the diseases associated with the causative agent of gastritis *H. pylori* (Ruggiero, 2012; Yordanov, 2017).

In Bulgaria, *H. pylori*was first isolated in 1990 (Tomov *et al*., 1990). This led to a series of studies on the biological role of *H. pylori* and the clinical characteristics of the diseases associated with it (Katzarov, 2001, Boyanova *et al*., 2009; Yordanov, 2017). It has also been determined that a diet involving regular intake of honey and Bulgarian yogurt could provide better protection against virulent strains (Yordanov, 2017).

From a healthcare and economic perspective, the importance of early diagnostics and identification of *H. pylori* as the main etiological agent of chronic gastritis, gastric and duodenal ulcers and a key co-factor in the etiology of gastric carcinoma in humans, calls for comparative analysis of the methods used for identification of *H. pylori* and approbation of the most appropriate one. Thus, the aim of this study was to detect and isolate *H. pylori* from gastric mucosal biopsy specimens from patients with clinical signs of gastritis or ulcer using several different methods: the rapid urease test, the culture method, light microscopy, electron microscopy, histological analysis and PCR.

Materials and Methods

Sampling

A total of 1050 direct samples from *H. pylori* agar culture were Gram-stained. Thirty isolate specimens were prepared for electron microscopy and 41 mucosal biopsy specimens for histological diagnosis. A reference strain of *H. pylori*, LMG 8775 from the collection of NDSRVMI, Sofia, was used in this study.

Rapid H. pylori urease disc test PLAST-RHp

This test was used for direct detection of *H. pylori* ex tempore in gastric biopsy specimens, followed by microbiological procedures. Forty- one gastric mucosal biopsy specimens were tested for the presence of *H. pylori* using urease test disks.

Culture method

Biopsy material from Urea Indole Broth (Bio Merieau 55752) was transferred into Basal broth (HiMedia) for enrichment in microaerophilic conditions at 37°C for 24h. From the broth, subcultures were made on Müller-Hinton agar with 10% sheep blood (pH 7.4). Following incubation for 72h at 37°C, direct Gram-staining was done. The slides were observed under an Olympus microscope (100x/1.25 magnification).

Electron microscopic identification of H. pylori

Mucosal biopsy specimens were incubated for 2h at 4°C in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and for another 2h at 4°C in 1% osmic acid, followed by dehydration through a graded alcohol series of propylene oxide. The samples were then embedded in Durcupan epoxy resin capsules. Following ultra-thin (5 nm) sectioning and negative staining with uranyl acetate and lead citrate, the samples were observed under an electron microscope. The investigations were performed on a JEM 1200 EX electron microscope at a 80 kV accelerating voltage and an instrument expansion of 40,000 to 75,000.

DNA extraction and PCR

The DNA extraction procedure was as previously described using Reagent B (Sirakov, 2012) and Isolate II Genomic DNA kit (Bioline, UK). The primers and method were based on Lage et al., (1995) and the amplification product was 294 bp. PCR amplification was performed using Bio X short Master Mix, (Bioline, UK) in a Techne TC-412 thermocycler (UK, Cambridge, Techne Dux Ford). The amplified fragments were visualized by 2% agarose gel electrophoresis (ME Sea Kem) and 10 mg/mL ethidium bromide staining; 120 V, 50 mA for 30 min, and documented with a VisiDoc-It imaging system.

Statistical data

Statistical data processing was performed with Statistics 7.0, Stat Soft Inc., USA, 2004.

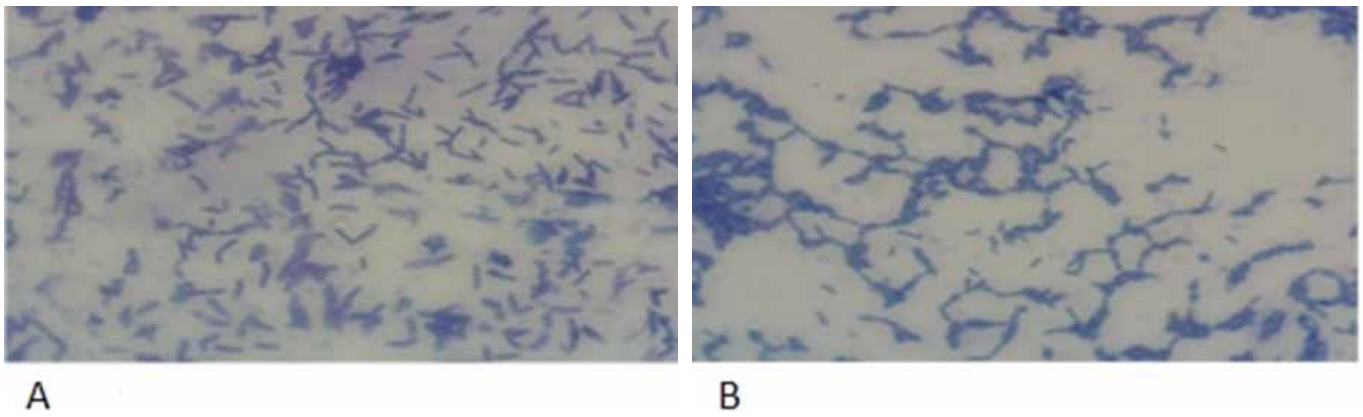

Fig. 1. (A) Reference strain *H. pylori* LMG 8775, aerobic vibroid, Gram-negative bacteria. (B) Iisolate from a gastric mucosal biopsy specimen from a patient with clinical gastritis after treatment procedures.

Results

Thirty-six isolates of *H. pylori* were microbiologically obtained from 41 gastric mucosal biopsy specimens from patients with clinical signs of gastritis. Representative results are shown in Fig. 1.

The electron microscopy results showed epithelial cells with almost completely lost integrity of the outer nuclear membrane. The inner membrane was also disrupted. The cytoplasm was observed to be highly vacuolated, with bacteria in some vacuoles. An electron micrograph of a portion of intestinal mucosa revealed that the bacterial cells were located between broken collagen fibers and apoptotic fibroblasts.

All positive mucosal specimens from patients tested with rapid urease test were further tested by PCR. Figure 2 shows PCR results from a 24-h broth culture from gastric mucosal biopsy specimens from patients with signs of gastritis or ulcer and presence of specific *H. pylori* DNA.

By direct PCR from biopsy materials, 36 samples showed a positive result for *H. pylori*. The same samples gave positive results in the case of bacterial isolates as well. Thus, the microbiological analysis of 41 gastric mucosal biopsy specimens from patients with symptoms of gastritis or ulcer identified *H. pylori* in 36 patients. In 4 patients, there was a mixed culture of *H. pylori* and *C. jejuni* and in just one patient there were lactic acid bacteria in the culture.

Discussion

In human medicine, the methods for identification of bacterial pathogens include invasive methods (microbial culture, rapid urease test, direct Gram-staining, polymerase chain reaction (PCR); and non-invasive ones: serological assays (ELISA), histopathological methods etc. Some of these diagnostic methods are slow, with low sensitivity and specificity, whereas others are not appropriate for patients who have undergone certain therapeutic procedures (Mullis *et al*., 1987; Lage *et al.*, 1995; Boyanova *et al.,* 1996; Chen *et al.,* 1997; Moayyedi *et al*., 1998; Tiveljung *et al.,* 1998; Yoshida *et al*., 1998). The rapid urease test is based on the urease enzyme that is produced by viable *H. pylori* cells (Boyanova *et al*., 1996; Mihova *et al.*, 1999), whereas the microbiological/cultural method includes several steps of enrichment in basal broth, subculture on Müller-Hinton agar in microaerophilic conditions and thermostatic incubation for 3-5 days (Oliveri *et al*., 1993; Hachem *et al*., 1995).

In order for a PCR procedure to be successful, it is necessary to carefully plan the reaction components as well as their concentrations (Lage *et al*., 1995; Mihova *et al*., 1999; Sirakov, 2016). The methods used in the study accurately diagnosed patients with clinical signs of gastritis or gastric/duodenal ulcer with microbial presence by modern molecular methods (PCR).

The positive results obtained in our study using PCR are in agreement with those reported by other authors (Lage *et al*., 1995), who tested a different number of patients (from 86 to 104 ones) with clinical signs of dyspepsia. Among them, 31 and 57 patients, respectively, were diagnosed with chronic gastritis, with 33 ones having active chronic gastritis. Unlike their study, we performed PCR analysis after microbiological analysis, isolation and identification of the bacterial pathogen and the positive PCR results obtained using the *ure* C primers were fully in accordance with the culture-based and electron microscopy results. The same authors came to the conclusion that the PCR assay is at least as sensitive as the culture methods for detection of *H. pylori*. Similar data have also been reported by oth-

ers (Yoshida *et al.*, 1998, Tiwari *et al*., 2005), who studied 106 and 78 patients, respectively, including 80 and 12 ones diagnosed with gastritis, and 20 and 4 ones without gastric disorders, whose biopsy specimens were used as control. In 90% of the cases from the first group and in 50% of those from the second one, microbiological analysis showed presence of *H. pylori*. The PCR analysis gave positive results in 87.5% of the patients with chronic gastritis and in 60% of the control ones. From the first group, 82% were *H. pylori*-positive, whereas in the second group, just 40% of the samples have been confirmed by Rapid Urease Test. In our study, besides microbiological and PCR analysis, electron microscopy was also used to confirm the final results.

There are different methods for identification of *H. pylori* (McNulty *et al*., 2011). The analysis of different methods for diagnosis of *H. pylori* has pointed out the rapid urease test as the most accurate, followed by PCR, histological analysis, the stool antigen test and serological analysis. Overall, the biopsy-based methods are preferred over other assays and are recommended to be used in combination with the non-invasive methods for confirmation of *H. pylori* infection (Khalifehgholi *et al*., 2013). In Bulgaria, the methods for isolation and identification of *H. pylori* have been studied and various new techniques have been adapted for confirmation of its presence in patients with clinical signs of gastritis or gastric ulcer (Boyanova *et al*., 1996; Katzarov 2001; Mihova *et al*., 1999).

Conclusions

Based on the results from the present study, microbiological analysis and PCR could be considered the most appropriate and highly reliable in detecting the presence of *H. pylori* in the gastroin-

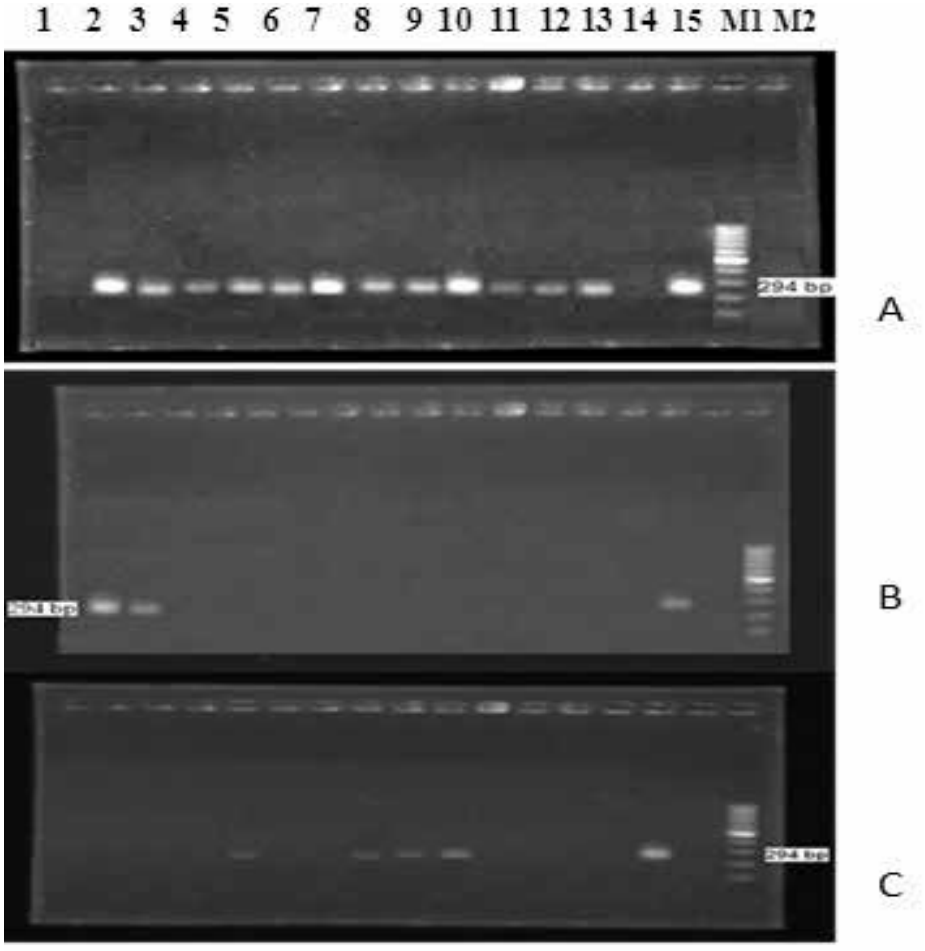

Fig. 2. PCR amplicons from gastric mucosal biopsy specimen from a patient with gastritis. A: 1-13, gastric mucosa samples; 14, negative control (DNA-free buffer); 15, positive control (*H. pylori* reference strain); M1, 100-bp molecular size marker; B: 2, *H.* reference strain; 3-15, gastric mucosa samples; M1, negative control; M2, 100-bp marker; C: 1-14, gastric mucosa samples; 15, positive control; M1, negative control; M2, 100-bp marker. Statistical analysis did not show a statistically significant difference between PCR and microbiology ($p < 0.001$), but was found amongst them and other methods included in the study.

testinal mucosa of patients with clinical symptoms of gastritis or ulcer.

The use of Urea Indole Broth for transport of mucosal biopsy specimens, enrichment in Basal Broth in microaerophilic conditions and subculture on selective Müller-Hinton agar with 10% blood (pH 7.4) is a prerequisite to preserving the viability of *H. pylori*.

The results from the microbiological analysis of 41 gastric mucosal biopsy specimens from patients with clinical symptoms of gastritis or ulcer showed presence of *H. pylori* in 36 patients (87.80%), mixed culture of *H. pylori* and *C. jejuni* in 4 ones (9.75%) and lactic acid bacteria in just one patient (2.45% of the total number of cases).

References

Baveye, S., E. Elass, J. Mazurier, G. Spik, D. Legrand (1999). Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin. Chem. Lab. Med*. **37:** 281-286.

Boyanova, L., I. Stancheva, D. Todorov (1996). Comparative analysis of three urease tests for identification of *H. pylori* in gastric biopsy specimens. *Eur. J. Gastroenterol. Hepatol*. **8:** 911-914 (in Bulgarian).

Boyanova, L., R. Markovska, D. Yordanov, M. Marina, K. Ivanova, S. Panayotov, G. Gergova, I. Mitov (2009). High prevalence of virulent *Helicobacter pylori* strains in symptomatic Bulgarian patients. *Diagn. Microbiol. Infect. Dis*. **64:** 374-380.

Chang, C., L. Chen, J. Yang, J. Lin, K. Chang, J. Wang (1999). Isolation of a *Helicobacter pylori* protein, FldA, associated with mucosa-associated lymphoid tissue lymphoma of the stomach. *Gastroenterology* **117:** 82-88.

Chen, T., F. Chang, S. Lee (1997). Serodiagnosis of *Helicobacter pylori* infection: comparison and correlation between enzyme-linked immunosorbent assay and rapid serological test results. *J. Clin. Microbiol.* **35:** 184-186.

de Vries, A. C., E. J. Kuipers (2010). *Helicobacter pylori* infection and nonmalignant diseases. *Helicobacter* **15:** 29-33.

Fennety, M. B., J. C. Emerson, R. E. Sampliner, D. L. McGee, L. J. Hixson, H. S. Garewal (1992). Gastric intestinal metaplasia in ethnic groups in th southwestern United States. *Cancer Epidemiol. Biomarkers Prev.* **4:** 293-296.

Go, M., S. Growe (2000). Virulence and pathogenicity of *Helicobacter pylori*. *Gastroenterol. Clin. North. Am*. **29:** 649-670.

Gracham, D., H. Malaty, D. Evans, D. Evans, P. Klein, E. Adam.(1991). Epidemiology of *Helicobacter pylori* in an asymptomatic population in the United States. Effect of age, race, and socioeconomic status. *Gastroenterology* **100:** 1495-1501.

Graham, D., A. Opekun, M. Osato, H. El-Zimaity, C. Lee, Y. Yamaoka, W. Qureshi, M. Cadoz, T. Monath (2004). Challenge model for *Helicobacter pylori* infection in human volunteers. Gut **53:** 1235-1243.

Hachem, C., J. Clarridge, D. Evans, D. Graham. (1995). Comparison of agar based media for primary isolation of *Helicobacter pylori*. *J. Clin. Pathol*. **48:** 714-716.

Hagymási, K., Z. Tulassay (2014). *Helicobacter pylori* infection: new pathogenetic and clinical aspects. *World J. Gastroenterol*. **20:** 6386-6399.

Lee, H. J., J. M. Park, Y. M. Han, H. K. Gil, J. Kim, J. Y. Chang, M. Jeong, E. J. Go, K. B. Hahm (2016). The role of chronic inflammation in the development of gastrointestinal cancers: reviewing cancer prevention with natural anti-inflammatory intervention. *Expert Rev. Gastroenterol. Hepatol.* **10:**129-139.

Katzarov, K. S. (2001). PhD thesis "Etiopathogenetic dependencies between *H. pylori* infection and gastric cancer" Military Medical Academy, Department of Gastroenterology, Sofia (in Bulgarian).

Khalifehgholi, M., F. Shamsipour, H. Ajhdarkosh, N. Daryani, M. Pourmand, M. Hosseini, A. Ghasemi, M. Shirazi (2013). Comparison of five diagnostic methods for *Helicobacter pylori. Iran. J. Microbiolol*. **5:** 396-401.

Kusters, J. G., A. van Vliet, E. Kuipers (2006). Pathogenesis of *Helicobacter pylori* infection. *Clin. Microbiol. Rev*. **19:** 449-490.

Lage, A., E. Godfroid, A. Fauconnier, A. Burette, J. Butzler, A. Bollen, Y. Glupczynski (1995). Diagnosis of *Helicobacter pylori* infection by PCR: comparison with other invasive techniques and detection of *cagA* gene in gastric biopsy specimens. *J. Clin. Microbiol.* **33:** 2752-2756.

McNulty, C. A., P. Lehours, F. Megraud (2011). Diagnosis of *Helicobacter pylori* infection. *Helicobacter* **16:** 10-18.

Moayyedi, P., M. Dixon (1998). Any role left for invasive test? Histology in clinical practice. *Gut* **43:** 51-55.

Mihova, A., A. Boyanova, K. Katzarov (1999). *Helicobacter pylori*, Ed. "Ciela", Sofia

Mullis, K., F. Faloona (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzimol*. **155:** 335-350.

Oliveri, R., M. Bugnoli, D. Armellini. et al., (1993). Growth of *Helicobacter pylori* in media containing cyclodextrins. *J. Clin. Microbiol*. **31:** 160-162.

Ruggiero, P. (2012). *Helicobacter pylori* infection: what's new. *Curr. Opin. Infect. Dis*. **25:** 337-344.

Sipponen, P., K. Seppala (1992). Gastric carcinoma; Failed adaptation to *Helicobacter pylori*. *Scand. J. Gastroenterol. Suppl*. **193:** 33-38.

Sirakov, I. (2012). PhD thesis, Investigation of *Caprine herpesvirus* 1 infection. Bulgarian Food Safety Agency (in Bulgarian).

Sirakov, I. (2016). Nucleic acid isolation and downstream applications, in: Larramendy, M., S. Soloneski (Eds), Nucleic Acid. InTech publisher, Rijeka. pp. 1-26.

Tiveljung, A., K. Borch, J. Jonasson, S. Mårdh, F. Petersson, H. J. Monstein (1998). Identification of Helicobacter gastric biopsies by PCR based on 16S rDNA sequences; a matter of little significance for the prediction of *H. pylori*-associated gastritis? *J. Med. Microbiol*. **47:** 695-704.

Tiwari, S., A. Khan, K. Ahmed, I. Ahmed, F. Kauser, M. Hussain, S. Ali, A. Alvi, A. Habeeb, Z. Abid, N. Ahmed, C. Habibullah (2005). Rapid diagnosis of *Helicobacter pylori* infection in dyspeptic patients using salivary secretion: a noninvasive approach. *Singapore Med. J.* **46:** 224-228.

Tomov, A., Gr. Mechkov, D. Takov, E. Tzvetkova, P. Petrov, E. Savov, K. Ivanova (1990). Isolation and microbiological characteristic of first *Campylobacter pylori* strains in

Bulgaria. *Epidemiol., Microbiol. Infect. Dis.* **27:** 14-18 (in Bulgarian).

Waldum, H., P. Kleveland, O. F. Sordal (2016). *Helicobacter pylori* and gastric acid: an intimate and reciprocal relationship. *Therap. Adv. Gastroenterol*. **9:** 836-844.

Yordanov, D. (2017). PhD thesis "*Helicobacter pylori* - virulence factors, resistance and seroprevalence" Medical University: Sofia, Department of Medical Microbiology. Sofia (in Bulgarian).

Yoshida, H., K. Hirota, Y. Shiratori, T. Nihei, S. Amano, A. Yoshida, O. Kawamata, M. Omata (1998). Use of gastric juice-based PCR assay to detect *Helicobacter pylori* infection in culture-negative patients. *J. Clin. Microbiol*. **36:** 317-320.