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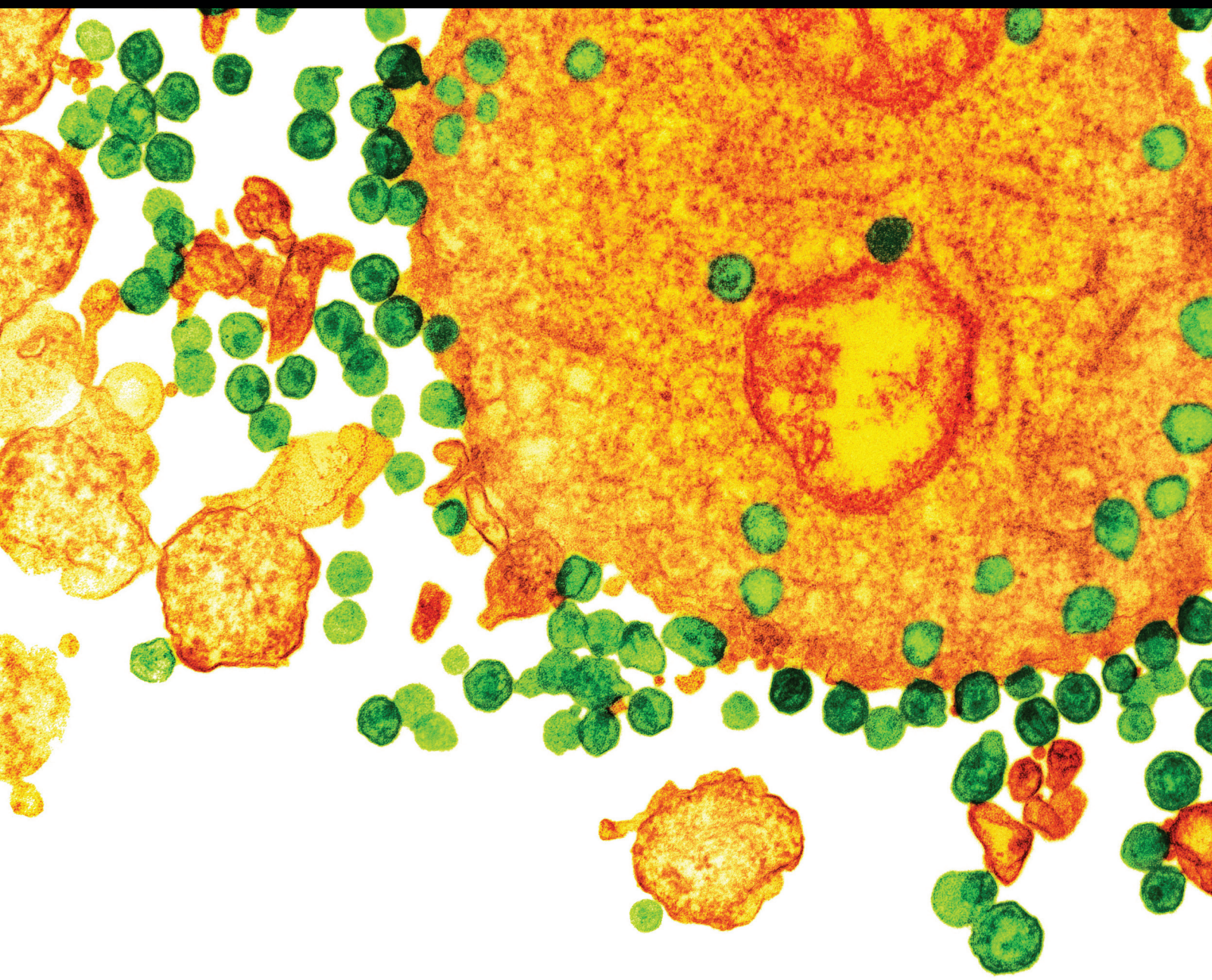
Helicobacter pylori: Infection and New Perspective for the Treatment

Lead Guest Editor: Teresa Fasciana
Guest Editors: Mara Di Giulio, Paola Di Carlo, and Ahlem Jouini



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Editorial Board

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Editorial

Helicobacter pylori: Infection and New Perspective for the Treatment

Teresa Fasciana,[1] **Paola Di Carlo**,[1] **Ahlem Jouini**,[2] **and Mara Di Giulio**[3]

[1]*Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties, University of Palermo, Palermo, Italy*
[2]*Laboratory of Epidemiology and Veterinary Microbiology, Group of Bacteriology and Biotechnology Development, Pasteur Institute of Tunis, Université de Tunis El Manar, Tunis, Tunisia*
[3]*Department of Pharmacy, "G. d'Annunzio" University Chieti-Pescara, Chieti, Italy*

Correspondence should be addressed to Teresa Fasciana; teresa.fasciana@virgilio.it

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The alarming phenomenon of antibiotic resistance in *Helicobacter pylori* suggests to pay close attention in the treatment. In particular, the clarithromycin resistance in *H. pylori* was designed by the World Health Organization (WHO) as high priority for antibiotic research in 2017 [1]. In Italy, the overall primary resistance to clarithromycin can be detected in 35.2% of cases; in France, it can be detected in 26% of cases, while in Spain, clarithromycin resistance is present in 27.2% of strains [2–4].

Natural/innovative strategies, as well as novel potentiators to restore the antibiotic susceptibility in resistant strains, could be used to improve the efficacy of *H. pylori* eradication, and they could be worthy to change attitude of medicine in dealing with the challenge known as *H. pylori* [5, 6].

Obviously, these therapeutic strategies should be used in patients infected by resistant *H. pylori* and in presence of coinfection with other pathogens responsible to develop severe gastric diseases [7].

On the basis of this evidence, the aim of this special issue was to collect research manuscripts and review manuscripts, case reports, and literature reviews with the objective to expand our knowledge in this innovative field.

In this special issue, a total of seven manuscripts were received, and five of these were accepted.

This issue confirmed that the prevalence of *H. pylori* in developing countries is high because the population lives in households with low socioeconomic status and hygiene. Therefore, in order to improve the diagnostic accuracy, should be recommended the combination of microscopy and PCR assay for effective monitoring of *H. pylori* infection in these endemic areas. PCR is a more sensitive assay to detect *H. pylori* infection than microscopy, and it is not yet considered as the gold standard assay.

Using a mathematical model and the force of infection, it is possible achieved to translate the decreasing pattern into the time-dependent decline in the hazard rate of infection and also permitted the future prediction of seroprevalence in areas with high prevalence of infection. Moreover, the model could be used to predict the future size of gastric cancer.

In experimental studies, it has been observed that the use of 52 kDa *H. pylori* membrane peptide as a vaccine has been effective to immunize against the development of gastric ulcer when used in murine models. However, the isolation and purification of such protein presents important challenges; therefore, the use of synthetic peptides designed from immunogenic proteins has become an alternative for diagnosis and prophylaxis. Since *H. pylori* causes superficial infection of the gastric tissue, the main immunity mediators are secretory IgA antibodies, which are the objective of active oral vaccination. Immunized animals produce specific serum IgG and IgA and intestinal and salivary IgAs, and after challenge, a gastric cellular and antibody response can be observed. One immunogen-derived peptide antigen of 50–52 kDa with the amino-terminal end sequence Met-Val-Thr-Leu-Ile-Asn-Asn-Glu (MVTLINNE) produced by *H. pylori* could be used

to the prophylaxis of its infection. The results showed that immunization with the MVTLINNE peptide stimulated the cellular immune response and increased proliferative response of thymus lymphocytes. In addition, MVTLINNE peptide vaccination-mediated IgA production correlated with no alterations in the gastric mucosa and scarce presence of bacilli after *H. pylori* infection. In conclusion, these results indicated that prophylactic immunization significantly reduced the number of colonizing bacteria, which was associated with healthy gastric tissue.

In the era with high percentage of resistance to clarithromycin, rifabutin, furazolidone, and tetracycline, alone or in combination, are promising candidates for rescue therapy of antibiotic-resistant *H. pylori* strains, as no definitive rescue therapy for *H. pylori* eradication is available.

In our opinion, this first year of this special issue attracted interest in a field that is growing development, and we hope that the information contained in this special issue will help to develop new strategies to prevent/treat the *H. pylori* infection.

Conflicts of Interest

The editors declare that they have no conflicts of interest.

Teresa Fasciana
Paola Di Carlo
Ahlem Jouini
Mara Di Giulio

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Research Article

Rifabutin and Furazolidone Could Be the Candidates of the Rescue Regimen for Antibiotic-Resistant *H. pylori* in Korea

Youn I Choi[1], **Sang-Ho Jeong**[1], **Jun-Won Chung**[1], **Dong Kyun Park**[1], **Kyoung Oh Kim**[1], **Kwang An Kwon**[1], **Yoon Jae Kim**[1], **Seol So**[2], **Jeong Hoon Lee**[2], **Jin-Young Jeong**[3] and **Sun-Mi Lee**[3]

[1]*Department of Gastroenterology, Gil Medical Center, Gachon University, Incheon, Republic of Korea*
[2]*Department of Gastroenterology, Asan Medical Center, Ulsan University, Seoul, Republic of Korea*
[3]*Asan Institute for Life Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul 138-736, Republic of Korea*

Correspondence should be addressed to Jun-Won Chung; drgreen@gilhospital.com

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Guest Editor: Teresa Fasciana

Background/Aim. In Korea, the rate of *Helicobacter pylori* (*H. pylori*) eradication has declined steadily as a result of increasing resistance to antibiotics, especially dual resistance to clarithromycin and metronidazole. However, microbiological culture data on drug-resistant *H. pylori* is lacking. This study evaluated the antimicrobial efficacy of candidate antibiotics against resistant *H. pylori* strains. *Methods*. After retrospectively reviewing the data from the *Helicobacter* Registry in Gil Medical Center (GMC) and Asan Medical Center (AMC), along with 4 reference strains, we selected the 31 single- or multidrug-resistant strains. The susceptibility of the *H. pylori* strains to seven antibiotics (clarithromycin, metronidazole, levofloxacin, amoxicillin, tetracycline, rifabutin, and furazolidone) and minimum inhibitory concentration were tested using the broth microdilution technique. *Results*. Among 31 antibiotic resistance strains for *H. pylori*, there were no strains resistant to rifabutin or furazolidone, which had MICs of <0.008 and 0.5 μg/mL, respectively. Only one tetracycline-resistant strain was found (MIC < 2 μg/mL). Amoxicillin and levofloxacin were relatively less effective against the *H. pylori* strains compared to rifabutin or furazolidone (resistance rates 22.6%, 1.9%, respectively). Tetracycline showed the relatively low resistance rates (3.2%) for *H. pylori* strains. *Conclusions*. Therefore, along with tetracycline which has already been used as a component for second-line eradication regimen for *Helicobacter*, rifabutin and furazolidone, alone or in combination, could be used to eradicate antibiotic-resistant *H. pylori* strains where drug-resistant *Helicobacter* spp. are increasing.

1. Introduction

Helicobacter pylori infection is responsible for the development of chronic atrophic gastritis, peptic ulcer disease, and gastric malignant neoplasms such as gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [1, 2]. *H. pylori* is recognized as a Class I carcinogen by the International Agency for Research on Cancer and the World Health Organization [1–3]. The eradication of antibiotic-resistant *H. pylori* is a global health issue [4].

However, multidrug-resistant (MDR) strains of *H. pylori* have been increasing worldwide due to the increased use of antibiotics [1, 2, 4–9]. In Korea, the rate of *H. pylori* eradication has declined steadily in recent decades as a result of increasing resistance to antibiotics, especially dual resistance to clarithromycin and metronidazole [10–12] which has resulted from the increased clinical use of macrolides and metronidazole [13, 14]. In Korea, including Incheon and Seoul where this study was conducted, the rate of resistance of *H. pylori* to clarithromycin has surpassed 15% [15–17]. Several reports suggest that 9.6% of the strains in Korea show dual resistance to clarithromycin and metronidazole [7]. Because the primary failure rate of *H. pylori* eradication has been increasing [12, 13, 18, 19], real-world antimicrobial resistance data are needed to improve therapeutic outcomes. However, there are little recent data

on *in vitro* antimicrobial effectiveness in Korea. Indeed, there is no consensus on the optimal rescue therapy for second-line eradication failure. Although the Maastricht V consensus recommended fluoroquinolone-containing therapy as first- or second-line treatment after failure of triple or nonbismuth quadruple therapy, this cannot be applied in Korea because of the increased rate of quinolone resistance [20]. Therefore, we conducted this real-world updated analysis of the *in vitro* antibacterial efficacy against MDR *H. pylori*.

The 2013 revision of the Korean Clinical Practice Guideline for *H. pylori* recommends triple therapy with a proton pump inhibitor (PPI), amoxicillin, and clarithromycin or a bismuth-based quadruple regimen if clarithromycin resistance is suspected [10, 21]. With the failure of first-line therapy, bismuth-based quadruple therapy or a regimen including two or more other antibiotics could be considered [21]. Although levofloxacin- and rifabutin-based triple therapy have been suggested for rescue therapy, there is no consensus on their use in Korea. Therefore, it is necessary to identify antibiotics effective against antibiotic-resistant *H. pylori* [22]. The Maastricht V/Florence guideline recommends culturing *H. pylori*, testing for antimicrobial susceptibility and selecting antibiotics based on the results of resistance tests [23]. However, there are limited data on antimicrobial agents that are effective against antibiotic-resistant *H. pylori* in Korea.

Therefore, this study investigated the antimicrobial activity of rifabutin, furazolidone, and other antibacterial agents as candidates for treating antibiotic-resistant *H. pylori* strains especially focusing on the multidrug-resistant *H. pylori*.

2. Patients and Methods

2.1. Institutional Review Board Approval. The Institutional Review Boards of Gil Medical Center (GMC) and Asan Medical Center (AMC) reviewed the study protocol (certification number: GAIRB2016-329).

2.2. Patient Characteristics. This study examined 4 reference strains and 31 strains isolated from patients at GMC ($n = 15$) and AMC ($n = 16$) in 2016. We retrospectively reviewed the data of cultures for *Helicobacter pylori* (*H. pylori*) up to 2016 from *Helicobacter pylori* Registry in GMC and AMC. We analyzed and tested the candidate helicobacter antibiotics including amoxicillin, clarithromycin, metronidazole, levofloxacin, and tetracycline from the strains of *H. pylori*. Patients' clinical data such as initial presentation of symptoms, reasons for endoscopy, antibiotics uses history, and reasons for cultures of *H. pylori* were retrospectively reviewed in GMC and AMC. Culture reasons for *Helicobacter* spp. for patients were as follows: (1) patients who have reported several antibiotics experiences or admission to hospital histories in 3 years, (2) first-line or second-line treatment failures, and (3) other clinically suspected medical condition of drug resistance, such as patients with old age more than 65 years who have had more chance to exposure into several antibiotics, or patients with severe comorbid conditions such as congestive heart failure, liver cirrhosis, renal failure, autoimmune disorders, pulmonary disease, and so on.

2.3. H. pylori Strain Isolation. Mucosal tissues collected from the gastric antrum of each patient were used to isolate *H. pylori*. To isolate the bacteria, the tissues were placed in an aseptic Petri dish, then crushed using a surgical knife and cultivated in Brucella broth agar, and supplemented with 5% sheep blood containing vancomycin (10 μg/mL), trimethoprim (5 μg/mL), amphotericin B (5 μg/mL), and polymyxin B (2.5 IU). These were cultured at 37°C under microventilation conditions (5% O_2, 10% CO_2, and 85% N_2). The colonies obtained from the initial cultures were confirmed to be *H. pylori* using Gram staining and biochemical methods. Each strain identified as *H. pylori* was stored at −70°C in Brucella liquid medium (Difco Laboratories, Detroit, MI, USA) containing 15% glycerol. Shortly before the subsequent experiments, they were melted, multiplied, and used.

2.4. H. pylori Antimicrobial Susceptibility Testing. The minimum inhibitory concentrations (MICs) of the following antimicrobial agents were tested: clarithromycin (Abbott Laboratories, Abbott Park, IL, USA), amoxicillin, metronidazole, tetracycline, levofloxacin, rifabutin, and furazolidone (all from Sigma Chemical Co., St. Louis, MO, USA).

2.5. Culture Conditions. To test the MICs of *H. pylori*, we used the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) [24], an internationally recognized antimicrobial susceptibility testing laboratory, using Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5% defibrinated sheep blood. The medium was sterilized by autoclaving, and each antimicrobial agent was serially diluted in medium supplemented with 5% sheep blood (Comed, Seoul, Korea), which was collected within 2 weeks of birth and cooled to 80°C. Then, the suspension of *H. pylori* strains (1×10^7 colony-forming units) was cultured in blood culture medium for 72 hours and inoculated on Mueller–Hinton agar containing an antimicrobial agent using a micropipette. This was incubated at 37°C for 3 days under microventilation conditions, and the presence of bacterial colonies was observed. Each experiment was performed in triplicate, and experiments were repeated at least three times per strain.

2.6. Antimicrobial Resistance Criteria. The MIC was defined as the minimum dilution concentration of the antimicrobial agent that did not produce bacterial colonies. The criterion for resistance to each antimicrobial agent was set to MIC >1 μg/mL, as given in the CLSI for resistance to

clarithromycin [24, 25]. The resistance criteria for antimicrobial agents were set to greater than 0.5 μg/mL for amoxicillin [7], 8 mg/mL for metronidazole [7], 4 μg/mL for tetracycline [7], 1 μg/mL for levofloxacin antibiotics [26], 0.25 μg/mL for rifabutin [7, 27], and 4 μg/mL for furazolidone [7, 28]. Resistance to two or more antimicrobials was defined as multidrug resistance (MDR) [29]. For quality control, *H. pylori* strain (ATCC 43504), which is used as a standard strain in CLSI, was selected [24].

3. Results

3.1. Characteristics of the Study Population. The mean age of the study population was 58.2 ± 10.3 years, and 41.9% (n = 13) were more than 65 years. Reasons for endoscopy of study populations were as follows: (1) patients who received endoscopy for routine health checkup (n = 15) or (2) patients with gastrointestinal symptoms such as dyspepsia, regurgitation, or pain (n = 16). The most common reason for eradication was peptic ulcer disease (n = 15, 48.4%) (Table 1).

3.2. Characteristics of the Isolated Strains of H. pylori. Of the 31 isolated strains, 9 were resistant to one antimicrobial agent and 22 (71.0%) were resistant to two or more antimicrobial agents, including 13 strains resistant to two antibiotics (41.9%), seven strains resistant to three antibiotics (22.6%), and two strains resistant to four antibiotics (6.4%). The most common combination of drug resistance was clarithromycin + metronidazole (16 strains, 51.6%) (Table 2).

Of the 31 strains, 1 (3.2%) was resistant to tetracycline and none were resistant to rifabutin or furazolidone (Table 3).

3.3. Clarithromycin MIC and Resistance in H. pylori Strains. The range of MICs for clarithromycin was very broad, from 0.03 to >128 μg/mL. Overall, the MICs of the 31 strains had two distinct peaks (Figure 1), with MIC < 0.0625 μg/mL in 37.1% of the isolates and very high MICs in others (e.g., 16, 32, 64, and 128 μg/mL). The CLSI criterion for clarithromycin resistance is MIC >1 μg/mL. [24] Of the 31 strains tested, 22 were resistant (71.1%) according to this criterion (Table 3).

3.4. Metronidazole MIC and Resistance in H. pylori Strains. The MIC for metronidazole ranged from 1 to 128 μg/mL (Figure 1). The resistance standard for metronidazole was established to exceed 8 μg/mL, which is normally used without established criteria [24]. The resistance rate according to this standard was 67.7% (21/31).

3.5. Simultaneous Clarithromycin and Metronidazole Resistance in H. pylori Strains. Of the 31 strains studied, 22 showed MDR and 16 strains (51.6%) were resistant to both clarithromycin and metronidazole, accounting for 64% of all MDR strains. Rifabutin and furazolidone had excellent antibacterial activity with no resistant strains.

3.6. Quinolone MIC and Resistance in H. pylori Strains. The MIC for levofloxacin ranged from 0.25 to 64 μg/mL. The criterion for bacterial resistance to quinolone antibiotics is

Table 1: The demographic characteristics of the patient with *H. pylori* strain (N = 31).

Characteristics	N (%)
Age, mean ± SD (years)	58.2 ± 10.3
Age >65 years, N (%)	13 (41.9%)
Men, N (%)	16 (51.6%)
Smoking, N (%)	6 (19.3%)
Drinking, N (%)	12 (38.7%)
Comorbidity	
Diabetes mellitus type 2	2 (6.4%)
Hypertension	8 (25.8%)
Liver cirrhosis	1 (3.2%)
Cerebrovascular disorders	1 (3.2%)
Thyroid cancer	1 (3.2%)
Idiopathic pulmonary fibrosis	1 (3.2%)
Pulmonary tuberculosis	1 (3.2%)
Reasons for *Helicobacter* spp. cultures[§]	
First-line or second-line empirical treatment failure	9 (29.0%)
Patients' reported antibiotic uses history in 3 years	15 (48.4%)
Other clinically suspected medical condition of drug resistance[¶]	13 (41.9%)
Reason for eradication for *H. pylori*	
Peptic ulcer disease	15 (48.4%)
Early gastric cancer	2 (6.5%)
MALToma	4 (12.9%)
Atrophic gastritis	10 (32.3%)

[¶]Other clinically suspected medical conditions of drug resistance; patients with old age more than 65 years who have had more chance to exposure into several antibiotics or patients with severe comorbid conditions such as congestive heart failure, liver cirrhosis, renal failure, autoimmune disorders, pulmonary disease, and so on. [§]Total sum of population is not 100% since duplication cases. NSAID, nonsteroidal anti-inflammatory drug; HTN, hypertension; PUD, peptic ulcer disease; EGC, early gastric cancer; MALToma, mucosa-associated lymphoid tissue lymphoma.

Table 2: Drug resistance of *H. pylori* isolates (N = 31).

No. of resistant antibiotics	Types of drug resistance	No. of strains (%)
1	CLR	3 (9.7%)
	MT	3 (9.7%)
	LVX	3 (9.7%)
2	AMX + CLM	2 (6.5%)
	AMX + LVX	1 (3.2%)
	CLM + MET	10 (32.3%)
3	CLM + MET + LVX	4 (12.9%)
	AMX + MET + LVX	2 (6.5%)
	AMX + CLM + LVX	1 (3.2%)
4	CLM + MET + TET + LVX	1 (3.2%)
	AMX + CLM + MET + LVX	1 (3.2%)
Total	—	31

AMX, amoxicillin; CLM, clarithromycin; MET, metronidazole; TET, tetracycline; LVX, levofloxacin.

Table 3: Prevalence of antibiotic resistance of *H. pylori* isolates.

	Resistant breakpoint of MIC (μg/mL)	No. of resistant strains/Total strains	Resistance rate (%)
Clarithromycin	>1	22/31	71.1
Metronidazole	>8	21/31	67.7
Levofloxacin	>1	13/31	41.9
Amoxicillin	>0.5	7/31	22.6
Tetracycline	>4	1/31	3.2
Rifabutin	>0.25	0/31	0
Furazolidone	>4	0/31	0

MIC, minimum inhibitory concentration.

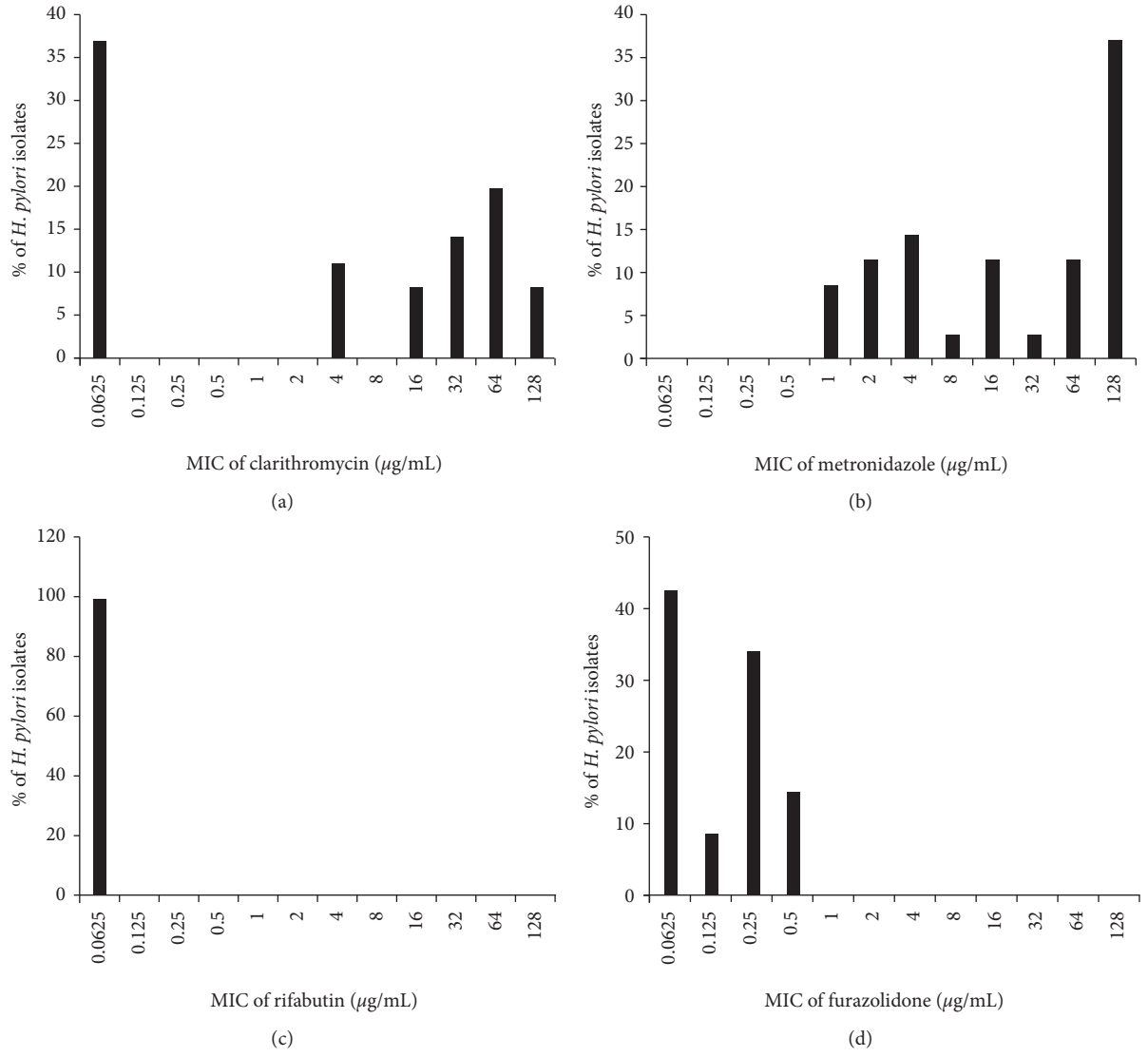

Figure 1: MIC distribution of (a) clarithromycin, (b) metronidazole, (c) rifabutin, and (d) furazolidone for *H. pylori*. MIC, minimum inhibitory concentration.

MIC >1 μg/mL [24]. Using this standard, 41.9% (13/31) of the strains were resistant to levofloxacin (Table 3).

3.7. Tetracycline MIC and Resistance in H. pylori Strains. The MIC for tetracycline ranged from <0.03 to 2 μg/mL (Figure 1). Only 1 of the 31 strains was resistant to tetracycline.

3.8. Rifabutin and Furazolidone MIC and Resistance in H. pylori Strains. The MIC for rifabutin ranged from <0.00098 to 0.0078 μg/mL and that of furazolidone from <0.03 to 0.5 μg/mL (Figure 1). The criteria for resistance are MIC >0.25 μg/mL for rifabutin and MIC >4 μg/mL for furazolidone. None of the 31 strains was resistant to either drug.

4. Discussion

In this *in vitro* analysis of antimicrobial effectiveness, we aimed to investigate which of the antibiotics were effective for multidrug resistance *H. pylori* and found that rifabutin and furazolidone had excellent potential for eradicating not only single-drug-resistant *H. pylori* but also MDR *H. pylori* through culture-based data in Korea. No strains were resistant to rifabutin or furazolidone, which had very low MICs of <0.00098 and 0.5 μg/mL, respectively, for all strains. Tetracycline also had low MICs, which were <2 μg/mL for all but one resistant strain. Therefore, along with tetracycline which has already been used as a component for second-line eradication regimen for *Helicobacter*, rifabutin and furazolidone, alone or in combination, could be used to eradicate antibiotic-resistant *H. pylori* strains. In comparison, amoxicillin and levofloxacin were only partially effective against the *H. pylori* strains in this *in vitro* study.

To our knowledge, this is the first *in vitro* antimicrobial analysis of antibiotics candidate, rifabutin, and furazolidone, in MDR *H. pylori* in Korea where clarithromycin resistance rate exceeds 15%.

Studies have explored several antibiotics as rescue therapy following the failure of first- and second-line treatment in Korea. Sung et al. evaluated the efficacy of rifabutin-based rescue therapy among patients with third-, fourth-, or fifth-line eradication failure [30]. Rifabutin-based rescue therapy had an approximately 55% eradication rate with few side effects. Jeong et al. reported that rifabutin-based therapy eradicated over 70% of *H. pylori* in third-line rescue therapy in 21 patients [31]. There might be several reasons for the variation in the eradication rate of rifabutin-based therapy in Korea. First, the sample sizes of these studies were small. Second, because *H. pylori* eradication failure is diagnosed using the rapid urease test after treatment, the causes of eradication failure were unclear. Major causes of eradication failure apart from drug-resistant *H. pylori* are loss of compliance with treatment, the density of *H. pylori* in the stomach wall, presence of CagA, and smoking. In our *in vitro* antibacterial efficacy analysis of drug-resistant *H. pylori*, rifabutin showed excellent antimicrobial activity in MDR *H. pylori*. To our knowledge, this is the first *in vitro* analysis of rifabutin in MDR *H. pylori*. Given the high rates of tuberculosis infection and antituberculosis medication use in Korea, the low rate of rifabutin-resistant *Helicobacter* spp. is interesting. Before selecting rifabutin as rescue therapy in Korea, clinicians should carefully monitor its major side effects, including rare myelosuppressive events [27, 30, 32–34], strictly limit its use to confirmed eradication cases, and monitor patient compliance closely to avoid the development of rifamycin-resistant tuberculosis [32].

Another option for third-line rescue therapy in Korea is furazolidone. In a meta-analysis, Mohammadi et al. reported an *H. pylori* eradication rate exceeding 80% and a low rate of side effects in Iran, where MDR *H. pylori* is common [35]. In a multicenter randomized controlled trial in China, where MDR *H. pylori* is also common, Xie et al. reported an eradication rate with furazolidone of up to 90% in 720 patients with *H. pylori* [36]. Despite limited data on furazolidone as a *Helicobacter* spp. treatment in Korea, Kim et al. reported a 1.5% resistance rate in first-line treatment failure patients [37]. However, their study was conducted in 2001 and recent data on furazolidone for *Helicobacter* spp. eradication in Korea are not available. Because the antibacterial resistance rate differs among countries, large multicenter population-based studies are needed in Korea. Our study showed that furazolidone has an extremely low rate of resistance in drug-resistant *H. pylori in vitro*.

Tetracycline is one component of bismuth-based quadruple therapy (PPI, bismuth, metronidazole, and tetracycline), which is effective for *Helicobacter* spp. eradication, especially in areas with high levels of clarithromycin resistance [13, 38]. In our *in vitro* study, only one strain of drug-resistant *H. pylori* was resistant to tetracycline, suggesting that tetracycline is still effective for drug-resistant *H. pylori* eradication in Korea.

This study had several limitations. First, it did not confirm the eradication rate by actually treating the patients, so it is impossible to know how the *in vitro* results will correspond to *in vivo* effects. Further studies need to confirm the eradication rate of furazolidone and rifabutin and safety in actual patients. Second, because we studied antibiotic-resistant *H. pylori* strains that were selected randomly, our result might not reflect the general prevalence of antibiotic-resistant *H. pylori* in Korea; selection bias could be an issue. Nevertheless, this was the first study of the effectiveness of rifabutin and furazolidone in Korea though *H. pylori* culture data, and almost all of the antibiotics used in clinical practice for *H. pylori* eradication in Korean were covered. Third, because the antibiotic resistance data for *H. pylori* were relatively small in this study, it should be cautious for physicians to generalize these results to a general population or other ethics. Given that the cost and time to obtain results of MIC for each antibiotic from culture data of *Helicobacter* spp., it might be important to invent and use molecular methods to evaluate the resistance of drugs directly in biopsies samples when it is impossible to isolate the strains [39].

In conclusion, this study showed that rifabutin, furazolidone, and tetracycline, alone or in combination, are promising candidates for rescue therapy of antibiotic-resistant *H. pylori* strains, as no definitive rescue therapy for *H. pylori* eradication is available. A future eradication regimen could potentially be designed based on these results.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Youn I. Choi and Sang-Ho Jeong contributed equally to this work. Dr. Youn I. Choi, Sang-Ho Jeong, Dong Kyun Park, and Jun Won Chung contributed to the study concept and design. Dr. Youn I. Choi, Seol So, Jeong Hoon Lee, Jin-Young Jeong, and Sun-Mi Lee analyzed and interpreted the data. Youn I. Choi drafted the manuscript, and Kyoung Oh Kim, Kwang An Kwon, and Yoon Jae Kim critically revised the manuscript for important intellectual content. All authors approved the final version of the manuscript.

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Research Article

Immunization with a Synthetic *Helicobacter pylori* Peptide Induces Secretory IgA Antibodies and Protects Mice against Infection

David Espinosa-Ramos,[1] Diana Caballero-Hernández,[1] Ricardo Gomez-Flores,[1] Armando Trejo-Chávez,[2] Luis Jerónimo Pérez-Limón,[1] Myriam Angélica de la Garza-Ramos,[3] Reyes Tamez-Guerra,[1] Patricia Tamez-Guerra,[1] and Cristina Rodriguez-Padilla[1]

[1]*Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Departamento de Microbiología e Inmunología, San Nicolás de los Garza, NL. C.P. 66450, Mexico*
[2]*Universidad Autónoma de Nuevo León, Facultad de Medicina Veterinaria y Zootecnia, Departamento de Patobiología, Campus de Ciencias Agropecuarias, Escobedo, NL. C.P. 66050, Mexico*
[3]*Universidad Autónoma de Nuevo León, Facultad de Odontología y Centro de Investigación y Desarrollo en Ciencias de la Salud, Unidad de Odontología Integral y Especialidades, Av. Dr. Aguirre Pequeño y Silao S/N, Monterrey, NL. C.P. 64460, Mexico*

Correspondence should be addressed to Ricardo Gomez-Flores; rgomez60@hotmail.com

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Helicobacter pylori is a spiral Gram-negative bacterium associated with inflammation of the gastric mucosa, peptic ulcer, and gastric adenocarcinoma, whose treatment has failed due to antibiotic resistance and side effects. Furthermore, because there are no vaccines effective against *H. pylori*, an appropriate vaccine design targeting conserved/essential genes must be identified. In the present study, a *H. pylori* 50–52 kDa immunogen-derived peptide antigen with the sequence Met-Val-Thr-Leu-Ile-Asn-Asn-Glu (MVTLINNE) was used to immunize against *H. pylori* infection. For this, mice received an intraperitoneal injection of 100 μg of *H. pylori* peptide on the first week, followed by two weekly subcutaneous reinforcements and further 10^9 bacteria administration in the drinking water for 3 weeks. Thymic cells proliferative responses to concanavalin A, serum levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α cytokines, and IgG1, IgG2a, IgG2b, IgG3 IgM, and IgA immunoglobulins were evaluated. Significant ($p < 0.05$) increases on lymphoproliferation and spleen weights after immunization were observed. In contrast, infection significantly ($p < 0.05$) decreased lymphoproliferation, which was recovered in immunized mice. In addition, levels of serum TH1 and TH2 cytokines were not altered after immunization, except for the significant increase in IL-6 production in immunized and/or infected animals. Moreover, immunization correlated with plasma secretory IgA and IgG, whereas infection alone only elicited IgM antibodies. Peptide immunization protected 100% of mice against virulent *H. pylori*. MVTLINNE peptide deserves further research as an approach to the prophylaxis of *H. pylori* infection.

1. Introduction

H. pylori is a Gram-negative spiral-shaped bacterium that represents the main factor for the development of human chronic gastritis, duodenal ulcer, and gastric adenocarcinoma [1–3]. Despite the decrease in the incidence of gastric carcinoma due to *H. pylori* in recent years, this disease is still the most common cause of death from cancer worldwide. In fact, it is the fourth cause of cancer cases per year, according to a 2000 report, with 945,000 new cases [1]. In developed countries, 70 to 90% of the population acquires the infection before 10 years of age and its routes of transmission are oral-oral or fecal-oral, but iatrogenia may be also involved, when performing endoscopy with a contaminated tube [4, 5]. In

addition to surgery, which includes partial gastrectomy, a wide variety of antibiotics have been proposed for the treatment of gastric ulcer accepted by the Food and Drug Administration, namely, the use of bismuth subsalicylate, metronidazole, and tetracycline, along with an antacid agent; however, this regimen can cause systemic damage, such as pseudomembranous colitis in 11% and vaginal candidiasis in excess of 10% in women under treatment [4]. Commonly, the first-line treatment consists of a 7 to 10 days regimen with a proton-pump inhibitor plus amoxicillin and clarithromycin [6]. However, antibiotics are affected by increasing levels of resistance [7, 8]. For instance, clarithromycin resistance has recently been reported in 26%, 27.2%, and 25% of patients infected by *H. pylori* in France, Spain, and Italy, respectively [9]. However, in developing countries, particularly in Mexico, resistance reaches 28.2% [10]. It has recognized that infection is strongly associated with the socioeconomic and sociodemographic conditions of the population where the variation of *H. pylori* virulence-associated genotypes could favor the development of gastrointestinal tract pathologies in infected patients [11]. Because of this, there has been an increasing interest in the development of vaccines as a prophylaxis to *H. pylori* infection [12].

In experimental studies, it has been observed that the use of 52 kDa *H. pylori* membrane peptide as a vaccine has been effective to immunize against the development of gastric ulcer when used in murine models. However, the isolation and purification of such a protein presents important challenges; therefore, the use of synthetic peptides designed from immunogenic proteins has become an alternative for diagnosis and prophylaxis. Since *H. pylori* causes a superficial infection of the gastric tissue, the main immunity mediators are secretory lgA antibodies, which are the objectives of active oral vaccination [13]. Immunized animals produce specific serum IgG and IgA, and intestinal and salivary IgA, and, after challenge, a gastric cellular and antibody response can be observed [14–16].

The aim of the present study was to evaluate the preventive effectiveness of vaccination with the MVTLINNE peptide, designed from a 52 kDa *H. pylori* immunogenic protein in a murine model.

2. Materials and Methods

2.1. Reagents, Cell Line, and Culture Media. Penicillin-streptomycin solution, L-glutamine, phosphate-buffered saline (PBS), and RPMI 1640 medium were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), *N*,*N*-dimethylformamide (DMF), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and concanavalin A (Con A) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin-streptomycin solution was referred as complete RPMI 1640 medium. *H. pylori* strain ATCC 700824 was purchased from the American Type Culture Collection (Rockville, MD) and grown on *Brucella* broth at 37°C. The strain was identified by Gram staining morphology and biochemical positive tests for catalase and urease. Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

2.2. Animals. Female BALB/c mice (20–25 g) were provided by the Bioterium of Facultad de Ciencias Biológicas at Universidad Autónoma de Nuevo León. They were kept in a pathogen- and stress-free environment at 24°C, under a light-dark cycle (light phase, 06 : 00–18 : 00 h), and given water and food *ad libitum*. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals by the National Institute of Health (Bethesda, MD) and approved by the University Ethics and Animal Care Committee.

2.3. Immunization Procedure. The immunizing peptide methionine-valine-threonine-leucine-isoleucine-asparagine-asparagine-glutamic acid (MVTLINNE) was synthesized by Genscript (Nanjing, China) with >85% purity and two modifications, acetylation at the amino terminus and amidation at the carboxyl terminus. The lyophilized peptide was stored at −20°C until immunization; for this, peptide solution was prepared at a concentration of 1 mg/mL in sterile saline. Mice were immunized by s.c. injection with the *H. pylori* peptide (100 μg in 500 μl of distilled water) in Sigma adjuvant (Sigma-Aldrich) (1 : 1) on day 0 and in incomplete Freünd adjuvant (1 : 1) on days 21 and 28. Mice were bled and spleens and thymuses were removed on day 91.

2.4. H. pylori Challenge. H. pylori was cultured on *Brucella* agar under microaerophilic conditions at 37°C in 5% O_2, 10% CO_2, and 85% N_2. *H. pylori* concentration was determined by CFU counts. Seventy days after the last immunization, mice were challenged with a *H. pylori* suspension (10^9 CFU/L) in the drinking water [17], for 21 days, after which, blood was obtained by terminal cardiac puncture, and the spleen, thymus, and stomach were aseptically removed.

2.5. T-Cell Proliferation Assay. T-cell proliferation was determined by a colorimetric technique using MTT [18]; 100 μl thymus cell suspensions (1×10^7 cells/ml) from immunized, immunized plus infected, infected, and control animals were added to flat-bottomed 96-well plates (Costar, Corning, NY), containing triplicate cultures (100 μl) of RPMI 1640 medium supplemented with 5% fetal bovine serum (unstimulated control), in the presence or absence of Con A (6.25 μg/ml), or MVTLINNE peptide (10 μg/ml) for 48 h at 37°C in 95% air-5% CO_2 atmosphere. After incubation for 44 h at 37°C with 5% CO_2, MTT (0.5 mg/ml, final concentration) was added, and cultures were additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer (100 μl), and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Bio-Tek Instruments, Inc., Winooski,

VT) at 540 nm. The lymphocyte proliferation index (LPI) was calculated as follows: LPI = A540 in resident or Con A-treated cells/A540 in untreated cells.

2.6. Plasma Cytokine and Antibody Responses. Plasma samples were evaluated for IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α levels, using the mouse Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA), and IgG1, IgG2a, IgG2b, IgG3 IgM, and IgA, using the BDTM cytometric bead array (CBA) mouse immunoglobulin isotyping kit, by flow cytometry (Accuri C6, BD Biosciences).

2.7. Mouse Gastric Tissue Histopathology. Histopathological analysis of gastric biopsies from experimental mice 21 days after infection was performed. After sacrificing, infected or immunized + infected mouse stomachs were removed and washed with sterile water. A longitudinal segment along the greater curvature from the esophagus to the stomach was used for histological examination. To determine histological alterations, tissue segments were fixed in 4% formalin solution, dehydrated, paraffin-embedded, and 3 μm sections stained with hematoxylin-eosin by using routine procedures. Sections were observed in an Olympus IX71 microscope, and the image acquisition was performed in an Infinity I camera with the Infinity Capture Software (Lumenera Co., Ottawa, ON). Sections were also stained with Warthin–Starry stain for *H. pylori* detection. All histological analyses were performed blinded by an experienced veterinarian pathologist [19].

2.8. Statistical Analysis. The results were expressed as mean ± SEM of the response of 5 animals per treatment group, from three independent experiments. Level of significance was assessed by Student's *t*-test and ANOVA.

3. Results

3.1. Effect of Immunization and Infection on Spleen Weights. Spleens were excised and weighed after animal's death. As seen in Figure 1, immunization significantly ($p < 0.05$) increased 37% spleen weights, in contrast to 12% nonsignificant increase in infected mice, as compared with untreated controls.

3.2. Effect of Immunization on Thymic Lymphocyte Proliferation. Thymus cell lymphocytes from immunized, immunized plus infected, infected, and control animals were incubated in the presence or absence of Con A or MVTLINNE peptide, and lymphoproliferative responses determined, as explained above. Con A significantly ($p < 0.05$) induced 1.2-fold increase, 1.8-fold decrease, and 1.33-fold increase in thymus lymphoproliferation from immunized, immunized plus infected, and infected, respectively, as compared with untreated control.

3.3. Plasma Cytokine Levels and Antibody Production. As shown in Figure 2, immunization and/or infection did not alter cytokines IL-2, IL-4, IL-10, IL-17, IFN-γ, and TNF-α levels; however, IL-6 significantly ($p < 0.05$) increased, as compared with untreated control. In addition, antibody isotype observed in infected and untreated control groups was IgM, whereas immunization induced IgM, IgA, IgG1, and IgG2a antibodies; immunization and infection induced IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 antibodies (Table 1).

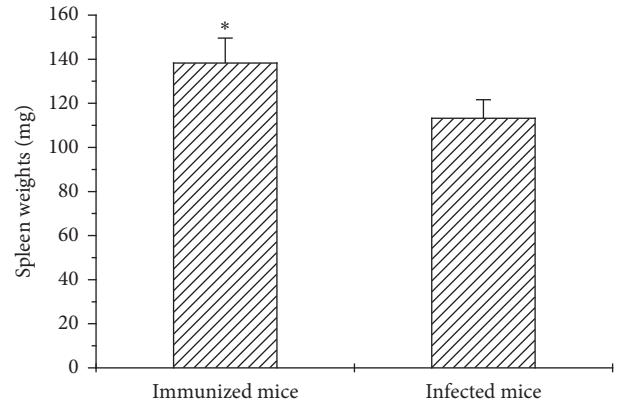

Figure 1: Spleen weights: spleens were removed and weighed after mice death, following immunization or *H. pylori* infection protocol, as detailed in the text. Data represent mean ± SEM of 5 animals per experimental group, from 3 independent experiments $^{*}p < 0.05$.

3.4. Gastric Tissue Histopathology. In infected animals, at the level of the mucosa adjacent to the esophagus, a wide ulcerative area, which is composed of cellular detritus, elongated bacteria, and some spores of unicellular parasites, is observed; in addition, at the level of the submucosa, there are discrete foci of inflammatory infiltrate of mononuclear cells mainly constituted by lymphocytes and some plasma cells (Figure 3(a)). When performing the Warthin–Starry stain, elongated *H. pylori* bacteria and spores are also observed (Figure 3(a)). The diagnosis was ulcerative gastritis with presence of bacteria in gastric epithelium. In regard to immunized and infected animals, no ulcerative, inflammatory, degenerative, or neoplastic changes nor the presence of bacteria were observed at the mucosal level; only some parasitic ovoid structures on the edge of some areas of the gastric epithelium were formed. When performing Warthin–Starry staining, these unicellular parasites were also observed. The diagnosis showed presence of few ovoid parasitic structures in the gastric epithelium. In both groups of animals, esophagus did not show pathological changes.

4. Discussion

It is estimated that 50% of the world population has been infected by *H. pylori*, a disease that although in the early stages is not considered deadly, in the long term, it leads to more serious diseases, such as cancer. In recent years, infections related to bacteria and viruses have been associated with the development of gastric diseases including cancer, chronic gastritis, and MALT lymphoma. In particular, the role of *H. pylori* and Epstein–Barr virus (EBV) in gastric

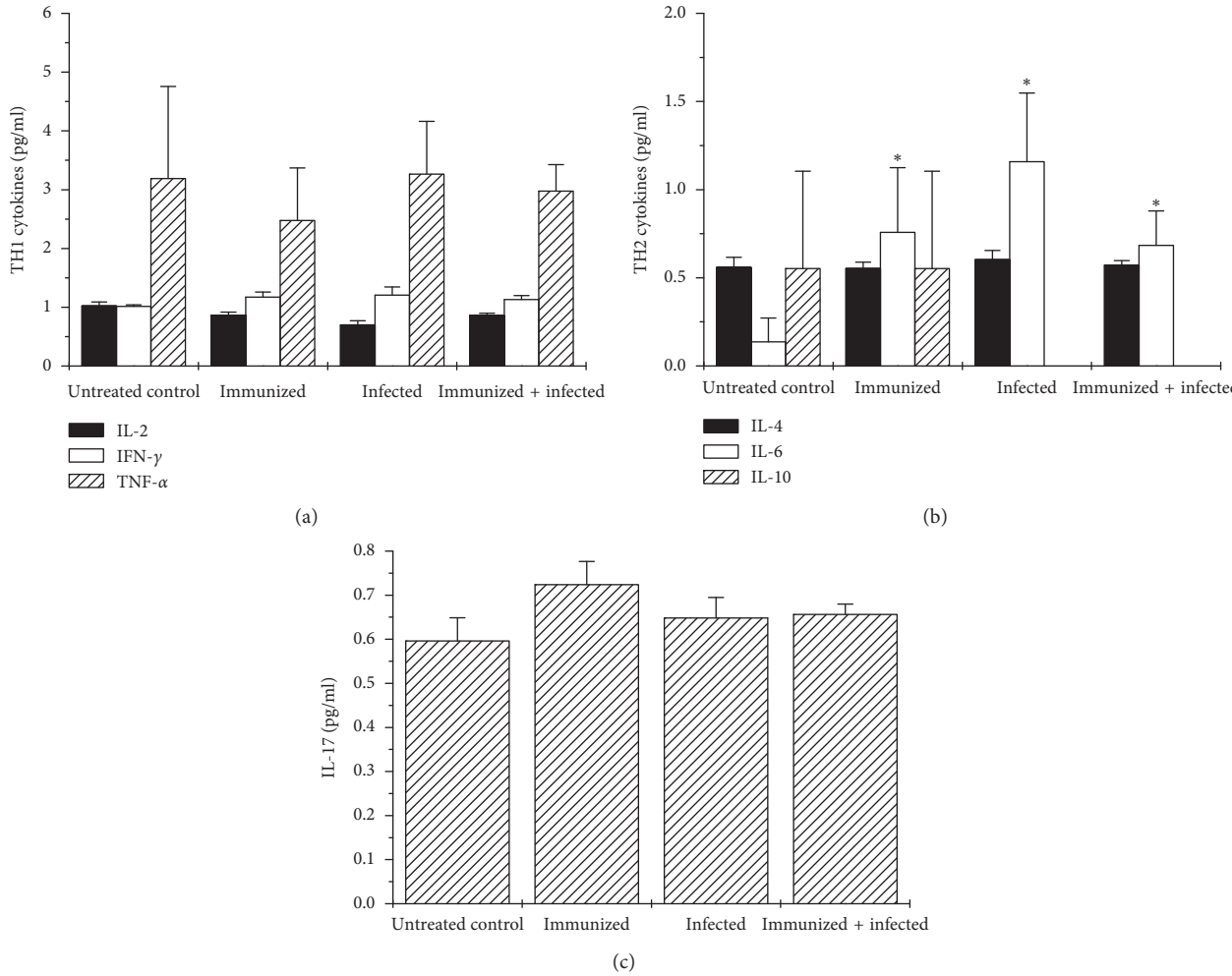

Figure 2: MVTLINNE peptide immunization increases plasma IL-6 levels. Plasma IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α levels were measured in immunized, immunized and infected, infected, and untreated control animals, as explained in the text. Data represent mean ± SEM of 5 animals per experimental group, from 3 independent experiments. $p < 0.05$, as compared with untreated control.

Table 1: Plasma immunoglobulins.

Experimental group	Antibody isotype
Untreated control	IgM
Immunized	IgM, IgA, IgG1, IgG2a
Infected	IgM
Immunized + infected	IgM, IgA, IgG1, IgG2a, IgG2b, IgG3

carcinogenesis has been evaluated. The relevance of the inflammatory response is hypothesized by recent studies showing how coinfection with *H. pylori* and EBV can cause tissue damage through inflammatory reactions or through increased contact between the CagA protein of *H. pylori* and EBV, which supports the increased activation of B cells in transit through the gastric mucosa [20]. Fasciana et al. demonstrated that the correlation of *H. pylori* and EBV is highly frequent [20]. Therefore, it is important to find new alternatives either for treatment or prophylaxis. The use of synthetic peptides designed from immunogenic proteins is considered an alternative for diagnosis and prophylaxis, but requires homogeneity in the antigenic preparation as described by Giammanco et al. [21]. In the present study, a murine model was used to determine the preventive potential of the MVTLINNE peptide, which is part of the terminal portion of a 52–55 kDa protein, identified as a homologue of citrate synthase, and has previously been described and patented for its usefulness as a diagnostic tool for *H. pylori* infection [22].

In *H. pylori* preclinical models, the evaluation focuses on the proliferation of lesions in the stomach mucosa as a result of infection, and other methods such as molecular diagnosis are also useful in the evaluation of the success of an intervention therapy [23, 24]; however, its use requires expensive equipment and is not always available. Both the low mortality rate and the chronic nature of the disease limit preclinical models of the disease. Another important aspect in a preclinical model is the selection of the animal to be used; the most frequent is the mouse and in some studies the rat. However, the strain of animals used is also important when interpreting the studies, since there are important intrinsic physiological and immunological variations that can determine the treatment outcome. In the present study,

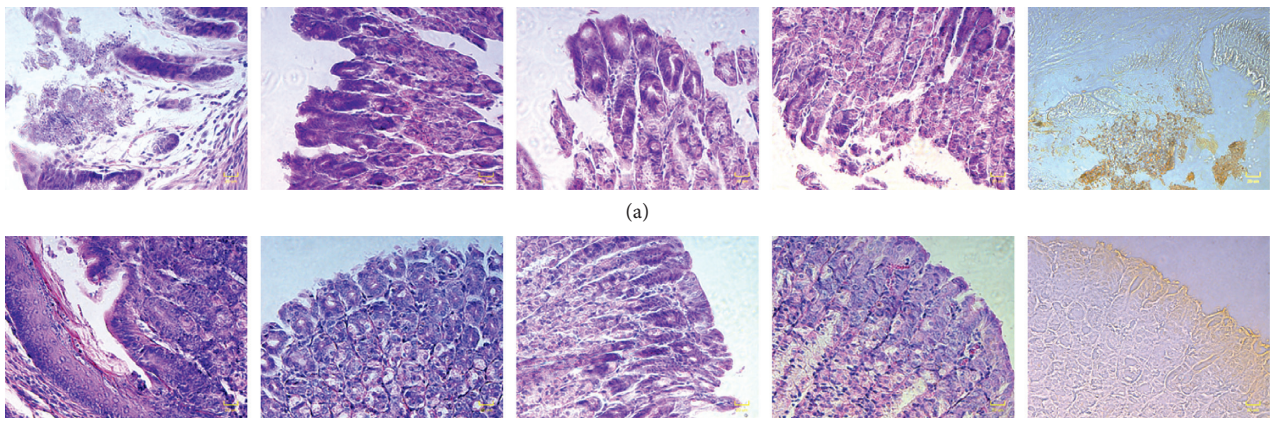

Figure 3: MVTLINNE peptide immunization protects mice from infection. Histological alterations and *H. pylori* presence in gastric tissue segments of infected or immunized + infected animals were determined, as explained in the text. (a) Infected animals and (b) immunized + infected animals (40x magnification).

an *in vivo* model of *H. pylori* was established in the BALB/c mouse; a noninvasive method of infection, administering the bacteria in the drinking water, was developed [17]. This model was used to evaluate the preventive efficacy of the administration of a synthetic peptide from a *H. pylori* protein; the MVTLINNE immunogenic peptide was administered twice, first intraperitoneally on week 1 and then subcutaneously as reinforcement; on week 4, in order to enhance the animal's immune response against infection. In this concern, the most used method for *in vivo* infection is the oral administration with a cannula of a known quantity of the bacteria [25]. However, this method requires considerable skill and carries risks for the welfare of the experimental animals; thus, an alternative method to reduce these risks was selected, in which bacteria are administered in the drinking water [17]. There is controversy regarding the time that the viability of the bacteria in water is maintained; however, several reports indicated that it is sufficient for oral infection [26, 27]. In our study, the data indicated that infected mice have immunological and histopathological response parameters consistent with infection (Figures 1–4).

Cellular immune response plays a critical role against *H. pylori* infection, which has been shown in immunodeficient mice models [28, 29]. In the present study, results showed that immunization with the MVTLINNE peptide stimulated the cellular immune response, as shown by the larger size of the spleen of immunized mice (Figure 1) and increased Con A-mediated proliferative response of thymus lymphocytes (Figure 4). *H. pylori*-mediated gastritis involves the release of cytokines from inflammatory cells, which contributes to maintain and amplify the local inflammation process. However, in this work, the analysis of TH1/TH2 cytokine profiles did not allow us to reach a conclusion about the success of the vaccination strategy, since no statistically significant differences were found between the experimental groups (Figure 2). However, there was an indication of an IL-6-mediated inflammatory response during infection and/or immunization (Figure 2). IL-6 plays an relevant role in innate and adaptive host defense by inducing IFN-γ

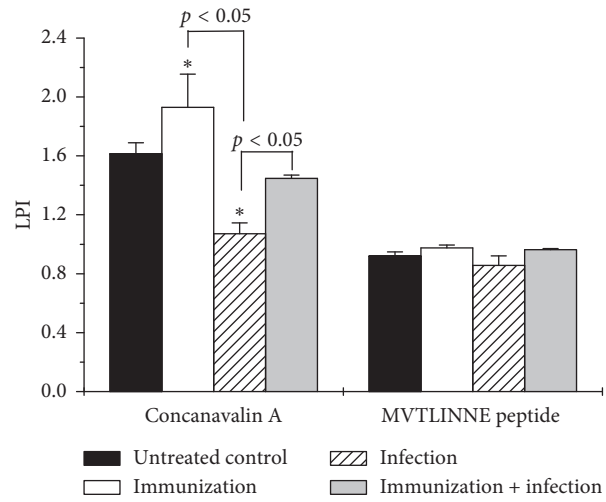

Figure 4: MVTLINNE peptide immunization stimulates Con A-mediated thymic lymphocyte proliferation. Lymphoproliferation was determined in thymus cell suspensions from immunized, immunized and infected, infected, and untreated control animals. Thymuses were surgically excised and mechanically dissociated into single-cell suspensions. Lymphocyte suspensions were then incubated in the presence or absence of Con A (6.25 μg/ml) and/or MVTLINNE peptide (10 μg/ml), and lymphoproliferation was measured by the MTT reduction assay, as explained in the text. Data represent LPI means ± SEM of triplicates from three independent experiments, $n = 5$ in each group. Untreated control optical density was 0.48 ± 0.004. $^{*}p < 0.05$, as compared with the untreated control.

production, immunoglobulin secretion, and neutrophil activation [30], and hence its involvement in protection against microbial infection *in vivo* [31]. In contrast, it was shown that IFN-γ may be involved in induction of *H. pylori*-mediated gastric inflammation [32].

It has been reported that *H. pylori* infection is associated with overexpression of IL-6 at the margin of gastric ulcer by macrophages [33, 34]. Furthermore, it was shown that

gastric epithelium significantly contributed to the antral IL-1β and IL-6 response from *H. pylori*-infected duodenal ulcer patients and asymptomatic carriers [26]. In addition, increased production of IL-6 and TNF-α in human antral mucosa cultures from *H. pylori*-infected chronic gastritis patients has been observed by others [35].

Macrophage cytokine upregulation in gastric tissues during *H. pylori* infection has been proven [34], particularly increased expression levels of IL-1, TNF-α, and IL-6; IL-6 mRNA expression in gastritis tissues was shown to correlate with *H. pylori*-mediated infection and inflammation [34–37], and serum IL-6 concentrations were related to *H. pylori*-induced gastric cancer [38]. Since inflammation plays a significant role in gastric carcinogenesis, it has been suggested that polymorphisms in genes involved in inflammatory response may partly explain why only a subgroup of patients infected with *H. pylori* develop gastric cancer. Proinflammatory cytokine genetic background is believed to play a pathogenic role in age-related diseases; conversely, genetic variations determining increased production of anti-inflammatory cytokines or decreased production of proinflammatory cytokines have been shown to be associated with successful aging. It has been reported that polymorphisms in the IL-1 and IL-10 genes could contribute to determining the background for inflammation in which *H. pylori* infection might facilitate cancer development [39].

A potential mechanism by which *H. pylori* induces IL-6 production by macrophages in chronic gastritis patients was reported to be related to heat shock protein 60 stimulation [40]. Furthermore, in the present study, serum IL-17 was not altered by immunization and/or infection, although others have reported its upregulated expression in *H. pylori*-infected human gastric mucosa [41].

Because of the marginal efficacy and antibiotic resistance in the clinics and eradication of *H. pylori* protects from damaging gastric tissues, the development of a safe and effective vaccine for humans continues to be an active research issue [25]. The use of whole bacteria may be potentially harmful, whereas recombinant vaccines became an alternative for prophylaxis; however, additional immunogenic antigens must be tested [14]. In the present study, oral vaccination with the MVTLINNE peptide induced protective IgA and IgG antibodies, as shown in Table 1. Since *H. pylori* produces an intraluminal infection, immunity may be mediated, at least in part, by secretory IgA antibodies. For instance, human breast milk IgA protects children against *H. pylori* infection [42]. Oral immunization with killed *H. pylori* was reported to induce specific IgA and IgG antibodies in mice gastrointestinal secretions and sera [25]. It is recognized that oral vaccination induces an IgA-dependent mucosal immune response that eradicates long-term infection with *H. pylori* in mice [43, 44]. Oral administration of *H. pylori* recombinant urease plus adjuvant was reported to induce protective and long-lasting protective specific IgA immunity against challenge with virulent *H. felis* [15, 16, 45].

In our study, MVTLINNE peptide vaccination-mediated IgA production correlated with no alterations in the gastric mucosa and scarce presence of bacilli after *H. pylori* infection (Figure 3(b)), as compared with untreated control (Figure 3(a)). Taken together, these results indicated that prophylactic immunization significantly reduced the number of colonizing bacteria, which was associated with healthy gastric tissue [46].

Data Availability

The experimental data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Spleen weights Student's *t*-test analysis: a Student's *t*-test analysis of spleen weights between immunized and control groups is depicted. One-way ANOVA test for peptide-immunization lymphoproliferation data: an ANOVA test for data related to the effect of peptide immunization on thymus lymphocyte proliferation, as compared with controls was performed. One-way ANOVA test for peptide-immunization and ConA-mediated lymphoproliferation data: an ANOVA test for data related to the effect of peptide immunization on concanavalin A-mediated thymus lymphocyte proliferation, as compared with controls, was performed. One-way ANOVA test for IL-6 production: an ANOVA test for data related to the effect of peptide immunization on IL-6 production, as compared with controls, was developed. (*Supplementary Materials*)

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Research Article

Application of PCR and Microscopy to Detect *Helicobacter pylori* in Gastric Biopsy Specimen among Acid Peptic Disorders at Tertiary Care Centre in Eastern Nepal

Nayanum Pokhrel,[1] Basudha Khanal,[1] Keshav Rai,[1] Manish Subedi,[2] and Narayan Raj Bhattarai[1]

[1]*Department of Microbiology, B. P. Koirala Institute of Health Sciences, Dharan, Nepal*
[2]*Department of Internal Medicine, B. P. Koirala Institute of Health Sciences, Dharan, Nepal*

Correspondence should be addressed to Narayan Raj Bhattarai; bhattarai03@yahoo.com

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Background. *Helicobacter pylori* infection is most prevalent in developing countries. It is an etiological agent of peptic ulcer, gastric adenocarcinoma, and mucosal-associated lymphoid tissue (MALT) lymphoma. Despite the development of different assays to confirm *H. pylori* infection, the diagnosis of infection is challenged by precision of the applied assay. Hence, the aim of this study was to understand the diagnostic accuracy of PCR and microscopy to detect the *H. pylori* in the gastric antrum biopsy specimen from gastric disorder patients. *Methods*. A total of 52 patients with gastric disorders underwent upper gastrointestinal endoscopy with biopsy. The *H. pylori* infection in gastric biopsies was identified after examination by microscopy and 23S rRNA specific PCR. The agreement between two test results were analysed by McNemar's test and Kappa coefficient. *Result*. *H. pylori* infection was confirmed in 9 (17.30%) patients by both assays, 6.25% in antral gastritis, 22.22% in gastric ulcer, 100% in gastric ulcer with duodenitis, 50% in gastric ulcer with duodenal ulcer, and 33.33% in severe erosive duodenitis with antral gastritis. Out of nine *H. pylori* infection confirmed patients, 3 patients were confirmed by microscopy and 8 patients by PCR. In case of two patients, both microscopy and PCR assay confirmed the *H. pylori* infection. The agreement between two test results was 86.54% and disagreed by 13.46% (p value > 0.05). *Conclusion*. We found that PCR assay to detect *H. pylori* is more sensitive than microscopy. However, we advocate for the combination of both assays to increase the strength of diagnostic accuracy due to the absence of the gold standard assay for *H. pylori* infection.

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that plays a remarkable role in the causation of gastrointestinal diseases such as peptic ulcers, low-grade B-cell lymphoma (MALT lymphoma), and gastric cancer [1, 2]. Several epidemiological studies also evidenced that *H. pylori*-infected individuals showed the incidence of gastric carcinoma [3]. The discrepancy of *H. pylori* prevalence has been shown among different population as well as in different countries. In fact, the transmission of the infection is influenced by the socioeconomic conditions. About 90% prevalence have been reported in developing nations in comparison with 50% occurrence in developed countries [4, 5]. Moreover, both gastric cancer and peptic ulcer cause more than a million deaths per year globally, thus making it an important health issue [6, 7].

Diagnostic tests for *H. pylori* include invasive and noninvasive methods with the involved techniques being either direct or indirect. Microscopy detection of the bacteria and culture is a direct method whereas demonstration of urease production and detection of stool antigen or an antibody is considered an indirect method, which is used as a response marker of infectious diseases. Advancement in molecular methods is now used as a reliable tool for diagnosis of infectious diseases due to its increasing sensitivity and specificity [8]. Due to resource constraints, diagnosis by noninvasive tests such as urea breath test or

invasive approach by bacterial culture of the biopsied tissue is not performed in our setting. Likewise, the reliability of immunological tests is always a matter of debate. In recent years, application of molecular method such as polymerase chain reaction (PCR) has revolutionized the diagnostic approaches for the detection of *H. pylori*. In addition, it also tracks the several genetic alteration in bacilli for understanding the drugs resistance characteristics [9] and coinfection of pathogens in gastric disease [10]. The molecular approach has also helped in comparative analysis between conventional methods such as microscopy and rapid urease test with PCR in resource-limited settings for effective diagnosis and treatment. In our setup with the advantage of the availability of molecular methods, we compared microscopy with PCR to see the effectiveness of each method for further evaluation of the study.

It is utmost important to identify *H. pylori* infection in gastroduodenal diseases so that the probable gastrointestinal malignancy can be prevented on time. In developing countries such as Nepal, the prevalence of *H. pylori* is notably higher in number of duodenal ulcer, gastric ulcer, and gastritis but a few data on burden of infections are available [11]. Therefore, this study has the aim to detect *H. pylori* in upper gastrointestinal endoscopic biopsy specimens by different diagnostic tools and evaluate the accuracy of *H. pylori* detecting tools in acid peptic disorder patients attending B. P. Koirala Institute of Health Sciences, Dharan.

2. Materials and Methods

2.1. Patients and Samples. This study was performed at B. P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal, from January 2017 to December 2017. Ethical clearance was obtained from the Institutional Review Committee (IRC-321/073/074) at BPKIHS. A written consent from 52 patients with symptoms of dyspepsia was taken before the biopsy specimen was collected for the study. The patient with age less than 14 years was excluded in this study. Likewise, the patients with history of long-term drugs known to cause gastritis such as steroids, anticoagulants, and lesions suggestive of malignancy on endoscopy were excluded from the study.

About 4 mm biopsy specimen from either the infected site or normal mucosa of the gastric antrum was collected. The tissue biopsy was cut with a sterile scalpel blade in a sterile Petri dish into two pieces. First specimen was preserved in normal saline and kept in a freezer at −80°C for PCR. Second tissue biopsy was processed for microscopic assessment [12]. In this study, storage of the biopsy specimens was done at −80°C which prevents the deterioration of DNA before the PCR analysis. In order to confirm the PCR inhibition, PCR-negative samples were diluted in 1 : 10 PCR grade water and PCR was repeated.

2.2. Microscopy. A smear was prepared by picking the biopsy specimen with a sterile swab and smeared onto two clean microscopic glass slides. After air-drying, the smear was fixed with uppermost flame of the Bunsen burner and allowed to cool. The smear was stained with the modified Gram-staining technique using carbol fuchsin as the counterstain. In the second glass slide, smear was fixed with methanol and Giemsa staining was performed [12].

2.3. PCR. The biopsy sample was taken out from the freezer and thawed at 37°C prior to processing the sample. The DNA extraction from the biopsy specimen was performed by using the Wizard Genomic DNA purification kit (Promega, Cat no. A1125) [13]. In brief, the biopsy was homogenized by a glass rod in nucleic lysis solution, and lysate was incubated at 65°C for 30 minutes and at 80°C for 5 minutes. After removal of protein precipitates from the lysate, the supernatant containing DNA was further precipitated in isopropanol and 70% ethanol, separately. Ethanol was gently removed, and pellet was air-dried. Finally, 100 μL of DNA rehydration solution was added to rehydrate the DNA by incubating overnight at 4°C and stored at −20°C.

The *H. pylori*-specific PCR was performed to detect 23S rRNA gene [14]. PCR master mix was prepared in 25 μl final volume which constituted 2 mM of $MgCl_2$, 0.1 mg/ml of BSA, and 0.175 μM of primer HP-23S-F (5′-AGATGGGAGCTGTCTCAACCAG-3′); 0.25 μM of primer HP-23S-R (5′-TCCTGCGCATGATATTCCC-3′); and 0.2 mM DNTP mix, 0.5 unit of Hotstar Taq polymerase (Qiagen, Cat. nr. 203605), and 2.5 μL of DNA template. PCR water was used as a negative control, and the DNA from the biopsy specimen with *H. pylori* PCR positive result was considered as a positive control. Mastercycler ProS (Eppendorf, Germany) thermocycler was used to amplify the target DNA in the samples. After the electrophoresis of the PCR product in 2% agarose gel at 5 V/cm and ethidium bromide staining, the DNA band was visualized with UV exposure. The sample was determined as *H. pylori*-positive PCR result if DNA band of length 137 bp was seen in gel.

In order to evaluate the quality of DNA extraction, the second human-specific PCR was done to assure the presence of human DNA in each sample. Human β-globin gene was the targeted to amplify using the primers KM29 and KM38 following the protocol developed by Saiki et al. [15].

2.4. Statistical Analysis. Data were entered in MS Excel 2007 worksheet and further analysed by using SPSS software version 11.5 [16] and R package [17]. Kappa coefficient (κ) was used for qualitative analysis of categorical data. McNemar's test was applied to analyse the disagreement between the tests. The chi-squared test was used to analyse the p value between the categorical data.

3. Results

Out of 52 patients enrolled in this study, majority of the patients were young adults between ages of 20 and 30 years (25%) followed by 60 to 70 years (21.15%) which has been depicted in Table 1. Female patients were, 25 (48.07%), found lesser than male patients, 27 (51.93%). Laboratory analysis of biopsy demonstrated that 9 (17.30%) patients were confirmed *H. pylori* infection as shown in Table 2.

Table 1: Demographic and laboratory analysis ($n = 52$).

Age (years)	Frequency
20–30	13 (25%)
30–40	8 (15.38%)
40–50	8 (15.38%)
50–60	7 (13.46%)
60–70	11 (21.15%)
70–80	5 (9.63%)
Gender	
Female	25 (48.07%)
Male	27 (51.93%)
Laboratory test	
Microscopy positive	3 (5.77%)
PCR positive	8 (15.38%)

Table 2: Comparison of endoscopy findings and positive *H. pylori* tests ($n = 52$).

Endoscopy finding	No. of cases (%)	*H. pylori*-positive case (%)
Normal	1 (1.92)	1 (11.11)
Gastritis	32 (61.54)	2 (22.22)
Duodenitis	6 (11.54)	1 (11.11)
Ulcers	13 (25.0)	5 (55.56)
Total	52	9

Amongst them, 3 (5.77%) cases of *H. pylori* infection were confirmed by microscopy and 8 (15.38%) cases were confirmed by PCR assay.

The spiral- or curved-shaped morphology resembling *H. pylori* was confirmed in 2 (3.84%) Gram-stained biopsies and 1 (1.9%) Giemsa-stained biopsy. We found microscopy positivity in 5.76% patients which could be due to small size of the biopsy. Out of three microscopy-positive cases, the endoscopic examination showed one case of severe erosive duodenitis + antral gastritis and one gastric ulcer + duodenal ulcer. However, one microscopy-positive case had normal mucosa in endoscopic examination. Among microscopy-positive cases, one had consumed PPI in less than two weeks prior to endoscopy with no history of antibiotic intake in any of the patients. The forty-nine microscopy-negative cases have history of PPI consumption in 30 (61.22%) patients and 5 (10.20%) patients had taken antibiotic in less than 2 weeks. The alteration in the morphology of bacteria from the spiral to coccoid form due to consumption of PPI and antibiotics can be responsible for false negativity leading to the possibility of misdiagnosis by the microscopic technique [18, 19].

Among 9 cases of *H. pylori* confirmed by biopsy analysis, endoscopic investigation showed that 32 patients had confirmed gastritis, 6 had duodenitis, and 13 had ulcers as shown in Table 2. But one patient with confirmed *H. pylori* under biopsy analysis had no abnormality in endoscopic observation.

In this study, 44 out of 52 cases had PCR-negative results. In order to rule out the false-negative *H. pylori* PCR results, human β-haemoglobin PCR was performed to assure the quality of DNA extraction. Out of three microscopically positive cases, *H. pylori* PCR was also positive in two cases. Among the PCR-positive cases, endoscopy examination showed two antral gastritis cases, two gastric ulcer cases, one gastric ulcer + duodenal ulcer case, and one severe erosive duodenitis + antral gastritis case, and two gastric ulcer + duodenitis cases. But one case of normal mucosa in endoscopy had also showed PCR-positive test result.

Overall, 9 (17.30%) patients of acid peptic disease (APD) were tested positive by either of the two methods. As yet reported elsewhere, none of the diagnostic assay is standalone and universal for disease diagnosis because of several extrinsic and intrinsic limitations [20]. On comparing the different laboratory methods used in detecting *H. pylori*, combination methods using both conventional and molecular techniques have been recommended [21]. Out of 52 patients in the present study, *H. pylori* is confirmed by PCR alone in 6 (11.53%) cases, microscopy alone in 1 (1.92%) case, and combination of PCR and microscopy in 2 (3.84%) cases. Combination of diagnostic assays has proven to be promising in detecting *H. pylori*. In this study, the combination of diagnostic assays microscopy and PCR increased the test positivity from 5.77% (3/52) to 17.31% (9/52).

Two (9.52%) in three microscopy positives and 5 (23.8%) in 8 PCR confirmed *H. pylori* positive had no history of PPI intake. In case of patient with history of antibiotic intake, both diagnostic tests had shown the *H. pylori*-negative results but *H. pylori*-positive test results were demonstrated in patients without antibiotic intake as shown in Table 3. The *H. pylori* microscopy results were 86.54% in agreement with PCR results whereas 13.46% results between both diagnostic tests were in disagreement, but kappa statistical analysis showed that the disagreement was not significant (p value = 0.14), as depicted in Table 4. The PCR is superior in diagnosing the presence of the bacteria in gastric biopsy than the microscopy. McNemar's analysis between two assays had shown an agreement of 86.54% (Kappa test, $p = 0.14$) which means diagnostic efficiency of both assays was not significantly different.

4. Discussion

Several assays have been proposed to detect the *H. pylori* infection; up to date, none of the assay is considered as gold standard for diagnosis of *H. pylori* due to the question in diagnostic precision and feasibility of the available assays [21]. In this study, microscopy and PCR were used as the diagnostic assay, in detecting the presence of the *H. pylori* bacterium in patients with acid peptic disorders using gastric mucosal biopsy. Moreover, bacterial distribution is mostly irregular and/or decreased bacterial load in the available cut specimen. In contrast to our study, Khalifehgholi et al. used Giemsa staining assay and identified *H. pylori* in 77.8% of the specimen [22]. Likewise, Siavoshi et al. found 47.9% *H. pylori* positive by Gram staining [23] and Roy et al. found 65.83% *H. pylori* positive by using modified Giemsa along with hematoxylin + eosin [24]. The results were undoubtedly higher than our findings which could be due to variation in staining techniques and sampling population. Moreover, the study conducted in Nepalese population showed that 67.5% of stomach carcinoma cases were found H. pylori positivity by histopathology and rapid urease analysis [11]. The contrasting results in the aforementioned study could be

Table 3: History of PPI and antibiotic intake in comparison with diagnostic test positive for *H. pylori*.

History of PPI intake	Microscopy positive	PCR positive
Yes ($n = 31$)	1 (3.22%)	3 (9.67%)
No ($n = 21$)	2 (9.52%)	5 (23.8%)
Total ($n = 52$)	3 (5.77%)	8 (15.3%)
p value	0.02*	0.32
History of antibiotic intake		
Yes ($n = 5$)	—	—
No ($n = 47$)	3 (6.38%)	8 (17.0%)
Total ($n = 52$)	3 (5.76%)	8 (15.3%)
p value	1	0.73

*Statistical significant at 0.05.

Table 4: Comparison of diagnostic results for *H. pylori* ($n = 52$).

Microscopy	PCR	Frequency	Agreement*	Disagreement$	Kappa test (p value)
Positive	Positive	2	86.54%	13.46%	0.14
Positive	Negative	1			
Negative	Positive	6			
Negative	Negative	43			

*Agreement between microscopy and PCR. $Disagreement between microscopy and PCR.

differences in study population enrolled and the application of diagnostic methods such as histopathological examination using hematoxylin and eosin. However, several reports on advantages of staining by other methods had been reported, and it was not used in the present study [25, 26].

PCR diagnosis by amplifying the conserved gene 23S rRNA has highest performance than other PCR assays [27]. Hence, 23S rRNA PCR was used to identify *H. pylori* infection in this study. In addition, it is efficient to rule out the other neighbouring species which were close to phylogenetic cluster of *Helicobacter* bacteria. The advent of molecular methods for diagnosis of *H. pylori* infection has proven to be a reliable tool as it amplifies the target gene by more than 10^6 fold, thereby increasing the diagnostic sensitivity and specificity, enabling better clinical management [21]. In addition, it is also capable to detect clarithromycin resistance genotype due to point mutations in the *H. pylori* 23S rRNA gene [28]. Eight (15.38%) out of 52 biopsy specimens detected 23S rRNA which was much lower than that reported by Archampong et al. in Ghana (48.4%) with *cagA* gene [29], Ruparelia et al. in Brazil (50%) with *ureA* + *ureC* gene [30], and Sugimoto et al. (44%) with 16S rRNA [31]. Hundred percent diagnostic accuracy cannot be achieved by the application of single PCR assay [31] since the genomic flexibility between strains of *H. pylori* complicates the choice of target genes [20]. Discrepancy in PCR resulting among the studies could be due to the difference in the type of target genes. In other studies, at least two types of genes were used, either a combination of two virulent genes or a conserved virulent gene. Despite advantages with application of multiple PCR, this study had only single target to amplify *H. pylori* 23S rRNA. Furthermore, other factors such as storage conditions, presence of PCR inhibitor, and repeated thawing and freezing leads to the loss of DNA in the biopsy material [32, 33]. Indeed, low bacterial load, patchy distribution of bacteria in the mucosa, and intake of PPI and antibiotics have been found to negatively influence the outcome of diagnostic tests including PCR. The PCR results were found exactly same as the previous and confirmed the absence of PCR inhibitors. However, none of the patients who had PCR positive took antibiotics for any major or minor sicknesses prior to endoscopy, and 3 (37.5%) PCR-positive cases were taking PPI in less than two weeks. In case of PCR negative, PPI was consumed by 28 (63.63%) patients and antibiotics by 5 patients (11.36%) in less than two weeks. This indicates that the growth of *H. pylori* could be inhibited by uptake of PPI less than two week, but more study with large samples are required to show the significant association.

Shetty et al. showed that diagnostic the sensitivity of microscopy was the highest (54.7%) followed by PCR (54.5%) and rapid urease test (RUT) (48.9%), whereas the culture had sensitivity (29.1%). Among different assays, the PCR had shown the highest sensitivity and specificity [34]. Due to resource constraints, the culture could not be performed and the rate of false positivity in RUT refrained us from performing this test. Moreover, Lim et al. showed that *rpoB* PCR also showed highest positive rate (53.7%) followed by *glmM* PCR (48.8%) [35]. Ruparelia et al. showed that the combination of serology and PCR had the highest sensitivity (100%) rather than RUT (81.81%) in the Indian population [30]. Moreover, recently published report on PCR diagnosis of rhinopharyngeal tumor also had the consistent results with this work [36]. Therefore, in absence of the gold standard assay for identifying *H. pylori*, the combination of diagnostic assays could be applied in order to reduce the false-negative *H. pylori* infection.

5. Conclusion

Although PCR is more sensitive assay to detect *H. pylori* infection than microscopy, it is not yet considered as the gold standard assay. Therefore, in order to improve the

diagnostic accuracy, we recommend the combination of microscopy and PCR assay for effective monitoring of *H. pylori* infection in endemic sites.

Data Availability

This is a hospital-based study. Samples were collected during the routine diagnostic procedure, and the samples were used to evaluate the performance of PCR to find their role in the implementation of diagnostics in hospital. Therefore, the data of the analysis are available upon the request from the corresponding author or head, department of Microbiology (hod.microbiology@bpkihs.edu), BP Koirala Institute of Health Sciences, Dharan, Nepal.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Estimating the Force of Infection with *Helicobacter pylori* in Japan

Taishi Kayano,[1] Ki-Deok Lee,[1,2] and Hiroshi Nishiura [1]

[1]*Graduate School of Medicine, Hokkaido University, Kita 15 Jo Nishi 7 Chome, Kitaku, Sapporo 0608638, Japan*
[2]*Department of Infectious Diseases, School of Medicine, Eulji University, Seoul 01830, Republic of Korea*

Correspondence should be addressed to Hiroshi Nishiura; nishiurah@gmail.com

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Background. Although the seroprevalence against *Helicobacter pylori* (*H. pylori*) in Japan has declined over the birth year, Japanese people have yet exhibited a relatively high risk of gastric cancer. The present study employed mathematical models to estimate the time- and age-dependent force of infection with *H. pylori* in Japan, predicting the future seroprevalence by time and age. *Methods*. We investigated the published seroprevalence data against *H. pylori* in Japan from 1980–2018. Solving the McKendrick partial differential equation model, the seroprevalence was modeled as a function of survey year and age. Maximum likelihood estimation was conducted to estimate parameters governing the time- and age-dependent force of infection. *Results*. Among all fitted models, the time-dependent and age-independent model with an exponentially decaying force of infection over years was most favored. Fitted models indicated that the force of infection started to decrease during and/or shortly after the World War II. Using the parameterized model, the predicted fraction seropositive at the age of 40 years in 2018 was 0.22, but it is expected to decrease to 0.13 in 2030 and 0.05 in 2050, respectively. *Conclusion*. The time dependence was consistent with the decline in the force of infection as a function of the birth year. The force of infection has continuously and greatly declined over time, implying the diminished transmission of *H. pylori* through the time course and small chance of persistence. These findings are critical to anticipate the future decline in gastric cancer incidence.

1. Introduction

Helicobacter pylori (*H. pylori*) is a bacterium known as the most important cause of gastric ulcer and cancer [1]. The bacterium is a helix-shaped gram-negative microaerophilic curved rod with four to six flagella at the same location, which is most commonly found in the stomach [2]. Although the exact mode of transmission has yet to be clarified, it is believed that the fecal-oral and/or oral-oral route are the most likely routes of transmission. A majority of people infected with *H. pylori* do not exhibit any clinical signs or symptoms. During the acute phase of infection, the symptoms associated with acute gastritis may occur. Moreover, chronic gastritis can lead to clinical symptoms that are associated with nonulcer dyspepsia. The long-lasting natural history of inflammation caused by chronic and atrophic gastritis is thought to be followed by carcinogenesis, and thus, the gastric cancer. The pathogenic factors of carcinogenesis such as VacA and CagA have been identified, and host genetic factors and several cytokine networks are proposed as the pathophysiological mechanism of cancer [3].

In the 21st century, the incidence of gastric cancer in Japan has continuously declined over time in all age groups. However, recent global estimates have indicated that Japanese people have yet exhibited a relatively high risk of gastric cancer [4], while the incidence of gastric cancer in developed regions have steadily declined. As an underlying explanation of the high incidence of gastric cancer, a high prevalence of *H. pylori* in the elderly in Japan has been considered as consistent with the natural history [1]. On the other hand, in many countries with decreased gastric cancer incidence over time, the seroprevalence of *H. pylori* has abruptly declined in young age cohorts [5].

A multi-institutional study across Japan, conducted by Ueda and his colleagues [6], has demonstrated a monotonic decline of *H. pylori* seroprevalence by birth cohorts. Similarly, analyzing the annual health check-up data, Hirayama

et al. [7] have shown a continuous decline in *H. pylori* seroprevalence in Japan, e.g., a decline from 46 % among those born in 1940s to 18 % among those born in 1970s. Moreover, another published clinical epidemiologic study indicated that the seroprevalence in Japanese children was less than 2% [8]. Considering that the route of transmission with *H. pylori* is likely associated with direct contact and hygienic conditions during the childhood, decreased contact with environment in early ages (e.g., reduced chance to swim in the pond and reduced chance of parent-to-child transmission via bathroom) may have occurred, leading to the decreased seroprevalence of *H. pylori* even among adults.

While numerous studies have already reported the decreased seroprevalence of *H. pylori* and also decreased incidence of gastric cancer, the hazard rate or the time- and age-dependent risk of infection with *H. pylori* has yet to be explicitly reconstructed from the seroepidemiological data. Published series of cross-sectional seroepidemiological studies in Japan offer a unique opportunity to estimate the so-called force of infection, i.e., the rate at which susceptible individuals are infected. The present study aims to devise mathematical models to estimate the time- and age-dependent force of infection with *H. pylori* in Japan, elucidating the transmission dynamics in the past and predicting the future seroprevalence by time and age.

2. Method

2.1. Epidemiological Data. We investigated the seroprevalence data against *H. pylori* in Japan from 1980–2018. Although the present study does not strictly adhere to the statement and officially acknowledged methodological details of the systematic review, the following literature review was systematically conducted, searching the MEDLINE and Web of Science databases, using the following search terms:

> "Seroprevalence OR Seroepidemic OR Seropositive OR Serological OR Serosurvey OR
>
> IgG,
>
> AND Helicobacter OR *H. pylori*
>
> AND Japan."

All titles identified by the search strategy were independently screened by two authors (TK and HN). Abstracts of potentially relevant titles were then reviewed for eligibility, and articles were selected for closer evaluation, if a description of the seroepidemiological study of *H. pylori* among the Japanese was available. Clinical and epidemiological studies that rested on laboratory methods other than serology and that offered nontractable seroepidemiological data over time or age were excluded. Before the present study, there was a narrative review article by Inoue [1], and we added to our literature those cited in the review but were missed by the above-mentioned systematic search.

2.2. Time and Age Elements. From each included paper, we extracted the information over the number of positive/negative samples by survey year and age. When the original data yielded the age information as only discrete age groups, we used the midpoint of age for modeling purpose. The birth year was calculated as survey year minus age. As the total number of samples and the count of positive samples are available, we computed the 95% confidence intervals (CI) of observed seroprevalence, using a binomial distribution.

2.3. Mathematical Model. The observed seropositive fraction represents the time- and age-specific history of the past exposure. We employ a mathematical model to capture the time- and age-dependent transmission dynamics of *H. pylori* from the seroprevalence data, and in particular, the present study jointly explores the time at which the rate of infection, t_0 started to decrease. Let $s(a,\ t)$ be the fraction of susceptible individuals at age a and year t. Assuming that everyone is born susceptible to *H. pylori* and discarding maternal antibodies, the boundary condition would be $s(0,\ t) = 1$ for any t. Let $\lambda(a,t)$ be the force of infection, i.e., the rate at which susceptible individuals experience infection, which depends on age a and year t, the susceptible individuals are depleted by

$$\left(\frac{\partial}{\partial a} + \frac{\partial}{\partial t}\right) s(a,\ t) = -\lambda(a,t) s(a,\ t). \tag{1}$$

We assume that the force of infection is separable to age- and time-components, i.e.,

$$\lambda(a,t) = f(a) g(t). \tag{2}$$

Integrating both sides of equation (1) along the characteristic line, we obtain

$$\begin{aligned} s(a,\ t) &= s(0,\ t-a) \exp\left(-\int_{t-a}^{t} \lambda(y-t+a, y)\, dy\right), \\ &= \exp\left(-\int_{t-a}^{t} f(y-t+a) g(y)\, dy\right), \end{aligned} \tag{3}$$

for $t > a$. Using the susceptible fraction, $s(a,\ t)$, the seroprevalence at age a and in year t is obtained from $1 - s(a,\ t)$. To quantify the force of infection, we impose three different parametric assumptions. First, we assume that $g(t)$ was initially a constant, λ_0, but from the year t_0 has been exponentially decreasing with year t, i.e., $g(t) = \lambda_0 \exp(-\delta(t - t_0))$, and also that the force of infection is age-independent. We have the seroprevalence, $p(a,\ t)$, parameterized as

$$p(a,\ t) = \begin{cases} 1 - \exp\left(-\int_{t-a}^{t} \lambda_0 \exp\left(-\delta\left(s - t_0\right)\right) ds\right), & \text{for } t - a \geq t_0, \\ 1 - \exp\left(-\lambda_0\left(t_0 - t + a\right) - \int_{t_0}^{t} \lambda_0 \exp\left(-\delta\left(s - t_0\right)\right) ds\right), & \text{for } t - a < t_0. \end{cases} \tag{4}$$

Hereafter, we identify this model as model 1.

Alternatively, following the year t_0, we assume that $g(t)$ has experienced a time-dependent decay that follows the Gompertz law with year t, i.e., $g(t) = \lambda_0 \exp(-\beta(\exp(\gamma(t - t_0)) - 1))$, and also that the force of infection is age-independent. We identify it as model 2 and we have

$$p(a,t) = \begin{cases} 1 - \exp\left(-\int_{t-a}^{t} \lambda_0 \exp\left(-\beta\left(\exp\left(\gamma\left(s - t_0\right)\right) - 1\right)\right) ds\right), & \text{for } t - a \geq t_0, \\ 1 - \exp\left(-\lambda_0\left(t_0 - t + a\right) - \int_{t_0}^{t} \lambda_0 \exp\left(-\beta\left(\exp\left(\gamma\left(s - t_0\right)\right) - 1\right)\right) ds\right), & \text{for } t - a < t_0. \end{cases} \tag{5}$$

As model 3, we assume that $g(t)$ has experienced a time-dependent exponential decay following t_0, $g(t) = \lambda_0 \exp(-\delta(t - t_0))$, and throughout the course of time, we also assume an age-dependent exponential decay, i.e., $f(a) = \exp(-\rho a)$. The seroprevalence of model 3 is described as

$$p(a,\ t) = \begin{cases} 1 - \exp\left(-\int_{t-a}^{t} \lambda_0 \exp\left(-\delta\left(s - t_0\right)\right) \exp\left(-\rho\left(s - t + a\right)\right) ds\right), & \text{for } t - a \geq t_0, \\ 1 - \exp\left(-\int_{t-a}^{t_0} \lambda_0 \exp\left(-\rho\left(s - t + a\right)\right) ds - \int_{t_0}^{t} \lambda_0 \exp\left(-\delta\left(s - t_0\right)\right) \exp\left(-\rho\left(s - t + a\right)\right) ds\right), & \text{for } t - a < t_0. \end{cases} \tag{6}$$

In the above-mentioned models, λ_0, δ, t_0, β, and ρ are dealt with as parameters to be estimated. To quantify the force of infection by estimating those parameters, we employed a likelihood-based approach. Given that there were $m_{a,t}$ positive individuals among the total of serum samples drawn from $n_{a,t}$ individuals in age a and year t, the likelihood function to estimate parameter θ was modeled as

$$L(\theta :\ n, m) = \prod_{a} \prod_{t} \binom{n_{a,t}}{m_{a,t}} p(a,t)^{m_{a,t}} \left(1 - p(a,t)\right)^{n_{a,t} - m_{a,t}}. \tag{7}$$

The 95% CI of parameters were computed using the profile likelihood. Once all parameters are estimated, we calculated the predicted seroprevalence as a function of time and age, especially, in years 2018, 2030, and 2050 for the exposition of the advantage of our approach to estimate the force of infection.

3. Results

In total, 10 seroprevalence studies were identified and used in the following analyses (Figure 1) [1, 5, 7–17]. Excluded seroepidemiological and clinical studies from the following analyses are a cohort study with variable timing of seroprevalence surveys that cannot be traced back from the literature [5], a clinical study that used urinary samples for testing *H. pylori* [17], and a study that missed the information of age grouping in summarizing seroepidemiological datasets [15]. All included publications measured the *H. pylori*-IgG antibody at the population level in a cross-sectional manner by age groups. The identified oldest survey took place in 1974, while the latest study was conducted in 2011. A small number of studies focused on particular age groups, especially on children, recruiting those aged from 0–11 years [9] and those at high school [14]. Figure 1 reveals that the seroprevalence drastically declined with birth year, and thus, most likely with time. The figure also indicated that the sample size was not large enough to distinguish the seroprevalence in one study from others in nearby birth cohorts, involving many overlapping confidence intervals of seroprevalence. Moreover, for each birth year, the expected value of the seroprevalence did not evidently increase according to the order of survey year, implying a limited age effect.

Fitting three different models to the identified data, we compared the goodness of fit as informed by the Akaike information criterion (AIC) (Table 1). Among all three models, including those employing the time-dependent and time- and age-dependent forces of infection, the model 1 with an exponential decay of the force of infection with time and without age dependence yielded the minimum AIC and was considered as the best fit model. Figure 2 compares the observed and predicted seroprevalence data by birth year, confirming that the observed patterns were overall well captured by the model 1. Due to the absence of age dependence, the predicted value did not vary over the vertical axis in Figure 2.

Figure 3 shows the estimated force of infection with *H. pylori* as a function of calendar time in Japan, using models 1 and 2. Qualitatively, the predicted values of models 1 and 2 were close to each other. The estimated year in which the force of infection was considered to have started to decrease was

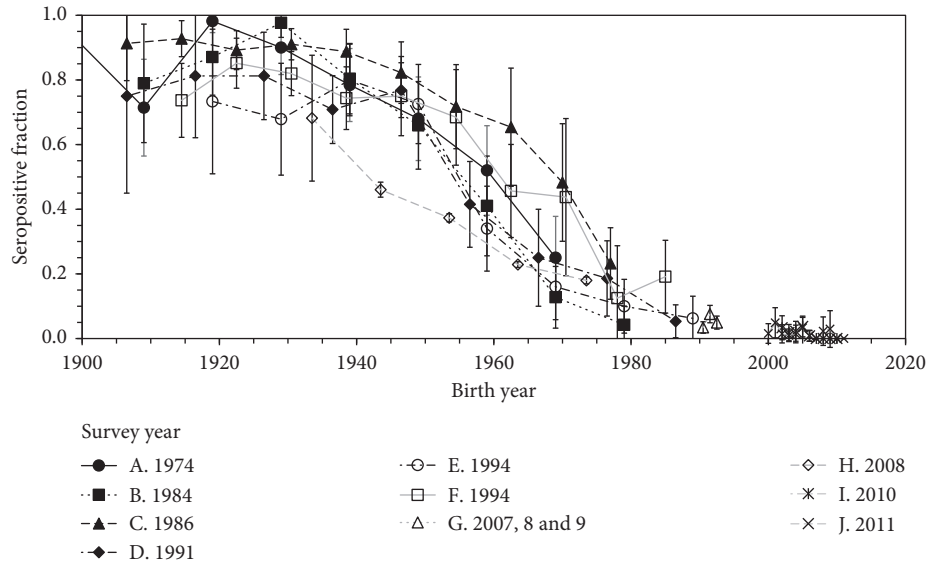

Figure 1: Seroprevalence of anti-*Helicobacter pylori* antibody in Japan by birth year. Antibody positive fraction is reviewed as a function of birth year. Same marks represent the dataset arising from an identical publication in the same survey year. Whiskers extend to lower and upper 95% confidence intervals.

Table 1: Model comparison of the time- and age-dependent force of infection to capture the transmission dynamics of *Helicobacter pylori* in Japan.

Model identity	Functional assumption	Number of parameters	AIC
Model 1	Time-dependent FOI with an exponential decay	3	937.2
Model 2	Time-dependent FOI with a Gompertz-type decay	4	3856.3
Model 3	Time- and age-dependent FOI with an exponential time-decay and exponential age-decay	4	2750.5

AIC: Akaike information criterion; FOI: force of infection.

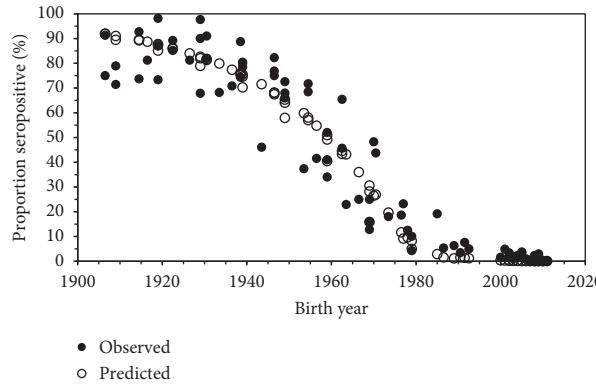

Figure 2: Comparison between observed and predicted seroprevalence against *Helicobacter pylori* in Japan by birth year. Observed data (filled marks) in various surveys are plotted by birth year and compared against model prediction (unfilled marks) that assumes time dependence in the force of infection with an exponential decay. Predictions were made as a function of survey year and age.

estimated at 1937 in model 1, while that of model 2 was 1945, the year corresponding to the end of the World War II. Estimated parameters of the best fitted model 1, λ_0, δ, and t_0 were 0.056 (95% CI: 0.048, 0.065) per year, 0.047 (95% CI: 0.045, 0.050) per year, and 1937 (95% CI: 1933, 1940), respectively.

Figure 4 shows the seroprevalence against *H. pylori* as a function of age in the past and the future in Japan. In Figure 4(a), the seroprevalence has exhibited a sigmoidal shape to increase as a function of age. While a part of the past observed data look not well aligned with the predicted values (e.g., those in 1984), the observed points with small serological samples suffered from broad uncertainty (i.e. wide confidence intervals), and the predicted values in general well captured the observed patterns of the seroprevalence data by time

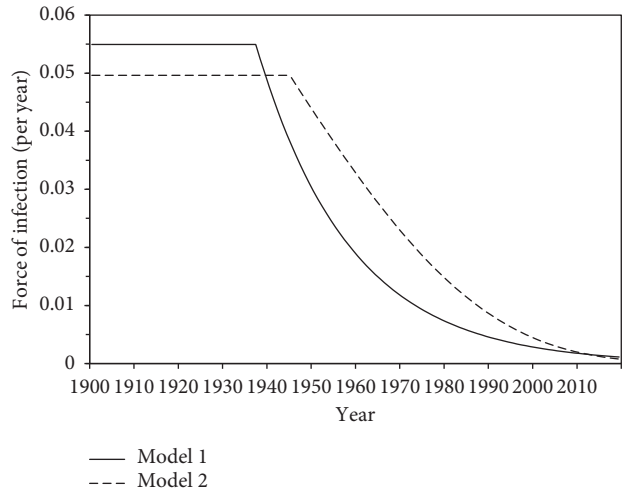

Figure 3: Estimated force of infection of *Helicobacter pylori* as a function of calendar time in Japan. Model 1 (bold straight line) is the estimate of time-dependent force of infection with an exponential decay. Model 2 (dashed line) is the estimate of time-dependent force of infection with Gompertz-type decay.

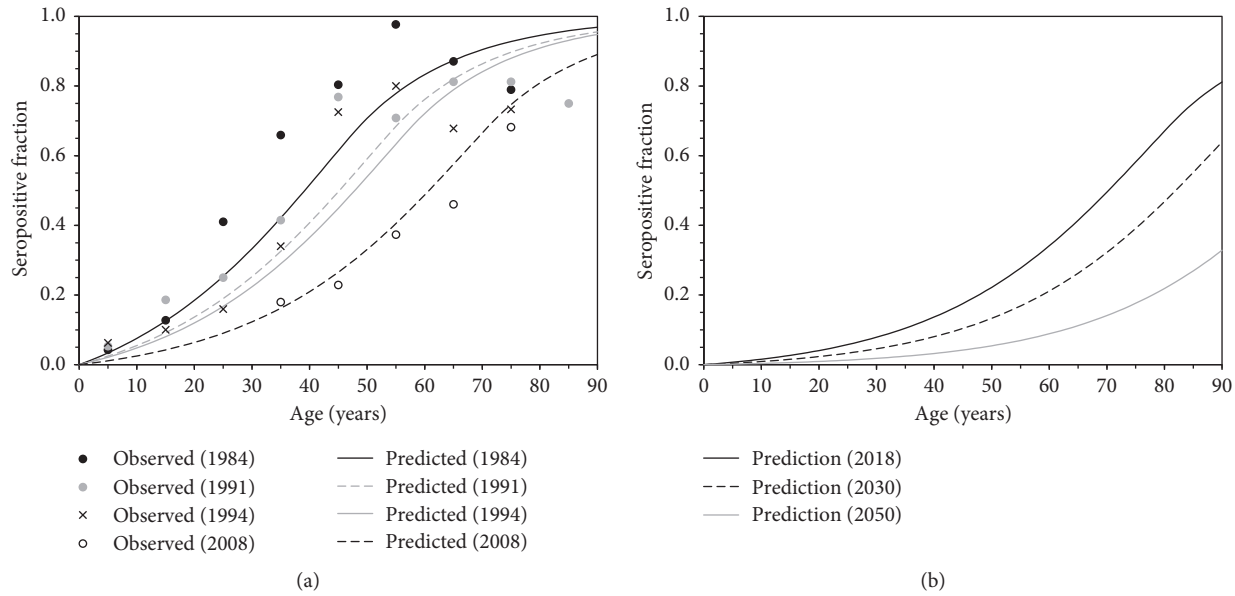

Figure 4: Prediction of the seroprevalence against *Helicobacter pylori* in the past and the future in Japan. (a) Comparison between observed and predicted seroprevalence by age and survey year. Marks represent observed data, while lines are the expected values derived from the time-dependent force of infection with an exponential decay. (b) Prediction of the future seroprevalence against *Helicobacter pylori* in Japan. Gradual right shift in the seroprevalence is captured by our time-dependent force of infection with an exponential decay.

and age. When the future seroprevalence was predicted (Figure 4(b)), the seroprevalence revealed a clear pattern of right shift over age by the year of prediction (i.e. the elevation of age at infection over future years is anticipated). For instance, the predicted fraction seropositive at the age of 40 years in 2018 would be 0.22, but it is expected to decrease to 0.13 in 2030 and 0.05 in 2050, respectively.

4. Discussion

The present study explored the long-term dynamics of *H. pylori* infection in Japan, estimating the force of infection from a total of 10 different seroepidemiological survey datasets. Fitting time-dependent and time- and age-dependent models, the time-dependent force of infection with an exponential decline was selected as the best fit model. Fitted models indicated that the force of infection started to decrease during and/or shortly after the World War II. Subsequently, the force of infection was considered to have steadily declined over time. Using the parameterized model, the age of seropositive individuals was predicted to be greatly shifted to older groups in the future, which would be more evident than in the past. To our knowledge, the present study is the first to elucidate the time-dependent

dynamics of *H. pylori* in Japan, offering predictions of seropositivity in 2030 and 2050.

The time dependence was consistent with the decline in the force of infection as a function of birth year (Figure 1) and without many variations over vertical axis, reflecting limited variations over age. Such decline with birth year has also been seen in other settings, e.g., China [18]. Using the mathematical model and the force of infection, the present study achieved to translate the decreasing pattern into the time-dependent decline in the hazard rate of infection and also permitted the future prediction of seroprevalence. The present study endorses the long-lasting notion of expert on this subject (e.g., a hypothesis by Blaser [19]): the seroprevalence continuously and greatly declines over time, implying the diminished transmission over time and small chance of persistence. These findings are critical to anticipate the future decline in gastric cancer, which have already been studied using mathematical models [20, 21].

While the time- and age-dependent model was not selected as the best model, it should be noted that the present study did not exclude the possibility that there is strong age dependence in the force of infection, especially among young children. In fact, the age effect was strongly indicated in the literature [22–26] and, as we noted from Figure 1, the uncertainty bound of seroprevalence in each birth year was very broad and the sampling error was not avoidable. Both empirical and theoretical studies clearly demonstrate that the infection mostly occurs in children [22, 23], and this notion is consistent with our age-dependent term of model 3, i.e., as a function of age, and the force of infection was considered to have decreased exponentially with the rate of 0.57 per year, indicating that the average age at infection was about 2 years. The infections would therefore perhaps be mostly seen among children, and additional studies with more precise observation with greater sample size would be essential to better identify the age dependence.

Apart from the predicted future course of the seroprevalence, it is natural to wonder how the epidemiology of gastric cancer will behave in the future. Not only the seroprevalence but also the future demographic dynamics, e.g., aging and decreasing population, would be a highly influential factor in regulating the incidence of cancer in the future. Estimating the induction period, i.e., the time from exposure to *H. pylori* to the cancer development, the future incidence of gastric cancer will become predictable by employing a mathematical modeling approach. In line with this intent, another interesting direction is to explore the epidemiological impact of eradication therapy of *H. pylori* on the gastric cancer incidence in an explicit manner.

Four limitations must be noted. First, the present study rested on the review of collected seroprevalence studies in different geographic locations. Depending on the geographic region in Japan, environmental conditions that can lead to infection can be different (e.g., urban vs. rural), but we collectively analyzed all the datasets as serial cross-sectional data in a single population. Second, even though we performed the systematic search of literature and minimized the sampling error, the sample size was small for each age group in each individual study, and the sampling error was substantial in quantifying the age effect. Third, fixed cutoffs used by a serological assay might have resulted in underascertainment of seropositive individuals, and it can sacrifice specificity, while sensitivity is ensured to be high [27]. Fourth, the force of infection is sometimes modeled as a function of the prevalence of infectious individuals, but the present study did not fully disentangle the underlying transmission dynamics by decomposing the force of infection into multiple mathematical functions.

Besides, without proper use of mathematical models, one cannot clarify the hazard rate of infection and predict the future seroprevalence [28–33]. In many countries, the decline in seroprevalence has been observed over birth cohort [1, 4, 5, 22, 34], and the present study uniquely and successfully identified the strong signature of the time dependence in the force of infection in Japan. To further predict the future size of gastric cancer, we trust that the present study substantially contributes to building the foundation for such sophisticated exercise.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Helicobacter pylori Infection and Its Risk Factors: A Prospective Cross-Sectional Study in Resource-Limited Settings of Northwest Ethiopia

Markos Negash,[1] **Habtamu Wondifraw Baynes**,[2] **and Demeke Geremew**[1]

[1]*Department of Immunology and Molecular Biology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia*
[2]*Department of Clinical Chemistry, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia*

Correspondence should be addressed to Markos Negash; markosnegash@yahoo.com

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Background. *Helicobacter pylori (H. pylori) is* implicated for the causation of gastrointestinal tract infections including gastric cancer. Although the infection is prevalent globally, the impact is immense in countries with poor environmental and socioeconomic status including Ethiopia. Epidemiological study on the magnitude of *H. pylori* and possible risk factors has priceless implication. Therefore, in this study, we determined the prevalence and risk factors of *H. pylori* infection in the resource-limited area of northwest Ethiopia. *Methods*. A prospective cross-sectional study was conducted on northwest Ethiopia among 201 systematically selected dyspeptic patients. Data were collected using a structured and pretested questionnaire, and stool and serum samples were collected and analyzed by SD BIOLINE *H. pylori* Ag and dBest *H. pylori* Disk tests, respectively. Chi-square test was performed to see association between variables, and binary and multinomial regression tests were performed to identify potential risk factors. *P* values <0.05 were taken statistically significant. *Result*. Prevalence of *H. pylori* was found to be 71.1% (143/201) and 37.3% (75/201) using the dBest *H. pylori* Test Disk and SD BIOLINE *H. pylori* Ag test, respectively. *H. pylori* seropositivity, using dBest *H. pylori* Disk tests, is significantly associated in age groups <10 years ($P = 0.044$) and married patients ($P = 0.016$). In those patients with *H. pylori* (a positive result with either the Ab or Ag test), drinking water from well sources had 2.23 times risk of getting *H. pylori* infection ($P = 0.017$), and drinking coffee (1.51 (0.79–2.96, $P = 0.025$)) and chat chewing (1.78 (1.02–3.46, $P = 0.008$) are the common risk factors. *Conclusion*. The present study discovered considerable magnitude of *H. pylori* among the dyspeptic patients in the study area. *H. pylori* infection is frequent in individuals drinking water from well sources, and thus, poor sanitation and unhygienic water supply are contributing factors. Policies aiming at improving the socioeconomic status will reduce potential sources of infection, transmission, and ultimately the prevalence and incidence of *H. pylori*.

1. Background

Helicobacter pylori (H. pylori) was the first formally recognized bacterial carcinogen. It has been etiologically associated with gastritis, peptic ulcer disease, gastric adenocarcinoma, and primary gastric lymphoma [1, 2].

Helicobacter pylori (*H. pylori*) colonizes 70–90% of the population in developing countries, whereas it is around 50% in developed countries [3–5]. In developing countries, an early childhood acquisition of *H. pylori* (30–50%) reaching over 90% during adulthood is the pattern of infection. Unless treated, colonization persists lifelong. *H. pylori* infection has been attributed to poor socioeconomic status, poor hygienic practice, and overcrowding condition [6, 7], a whole mark in developing countries.

The bacterium differs genetically, survives in harsh acidic gastric environment, and currently develops resistance for several antibiotics. Although epidemiological distribution of *H. pylori* varies globally, the magnitude of *H. pylori* has been shown to be 70.1% (Africa), 69.4% (South America), 66.6%

(Western Asia), 34.3% (Western Europe), and 37.1% (North America) [8–10].

The prevalence of *H. pylori* in the Ethiopian dyspeptic patients is similarly high to other developing countries because most Ethiopian population live in households with low socioeconomic status and hygiene [7, 11, 12]. Magnitude of *H. pylori* among the outpatient department (based on a test kit detecting Immunoglobulin G (IgG) antibodies) at the University of Gondar Hospital (UOG Hospital) was ranged between 65.7% and 85.6% [13, 14]. Besides, it is a common reason to seek primary healthcare service and accounts for 10% of hospital admissions [15, 16].

All previous prevalence researches in the study area were conducted using IgG and/or IgM antibody rapid tests which have questionable performance in detecting acute infection and distinguishing active infection from previous exposure. Hence, the current study was conducted with an aim to determine the prevalence of *H. pylori* infection among the dyspeptic patients attending the UOG hospital in northwest Ethiopia, using stool antigen as well as serum antibodies technique and assessing potential risk factors.

2. Methods

2.1. Study Design, Period, and Area. This is a facility-based cross-sectional study which was conducted on patients with dyspepsia from February to March 2016 at the University of Gondar Hospital, Gondar, Ethiopia. The University of Gondar Hospital is one of the pioneer teaching hospitals in Ethiopia conducting community-based researches, providing teaching and diagnostic services for more than 5 million inhabitants.

2.2. Study Participants and Clinical Data Collection. After informed consent was taken from the dyspeptic patients, who visited the hospital outpatient department, suspected of *H. pylori* infection, all relevant clinical and sociodemographic data were collected using a structured and pretested questionnaire by trained data collectors.

2.3. Specimen Collection and Processing. Stool and blood specimens were collected from each patient for *H. pylori* antigen and antibody tests, respectively. The blood was centrifuged until serum is separated and stored in −20°C. The stool specimens were also stored in −20°C until the tests were performed. For this study, we followed the methods of Negash et al. [17] which has been evaluated four *H. pylori* diagnostic tests in the study area.

2.3.1. SD Bioline H. pylori Ag Test (Standard Diagnostic, Inc., Korea). Principle: the SD BIOLINE *H. pylori* Ag rapid test kit result window has 2 precoated lines, "T" (Test Line) and "C" (Control Line). Both the Test Line and the Control Line in the result window are not visible before applying any samples. The "T"" window coated with monoclonal anti-*H. pylori* will form a line after the addition of the stool specimen (if there is *H. pylori* antigen). The Control window is used for the procedural control, and a line should always appear if the test procedure is performed correctly, and the test reagents are working [17].

2.3.2. dBest H. pylori Test Disk (Ameritech Diagnostic Reagent Co., Ltd., Tongxiang, Zhejiang, China). Principle: this test contains a membrane strip, which is precoated with *H. pylori* capture antigen on the test band region. The *H. pylori* antigen-colloid gold conjugate and serum sample moves along the membrane chromatographically to the test region (T) and forms a visible line as the antigen-antibody-antigen gold particle complex forms. This test device has a letter of T and C as "Test Line" and "Control Line" on the surface of the case. Both the test line and control line in the result window are not visible before applying any samples. The control line is used for the procedural control. Control line should always appear if the test procedure is performed properly, and the test reagents of the control line are working [17].

2.4. Statistical Analysis. The data were cleaned and double entered on the excel spread sheet and transported to Statistical Package for Social Sciences (SPSS). The chi-square test was performed to see association between dependent and independent variables. Binary logistic regression and multinomial regression tests were performed to identify potential risk factors of *H. pylori* infection. *P* value less than 0.05 were considered statistically significant.

3. Result

3.1. Demographic Characteristics. A total of 201 dyspeptic patients were included in the study, and serum and stool samples were analyzed by dBest *H. pylori* Test Disk and SD BIOLINE *H. pylori* Ag tests, respectively. The mean ± SD (range) age of the participants was 29.5 ± 14.85 (7–85) years with a median of 23 years. The majority (140) of the study participants were male (69%), study subjects from the urban area (141) accounted 70%, and 69 (34.3%) of the participants were married. Of 201 participants, 104 (51%) were students, 38 (18.9%) were farmers, and 23 (11.4%) were house wives (Table 1). In this study, participants who were diagnosed as positive to the *H. pylori* stool antigen test were immediately commenced appropriate therapy.

3.2. Prevalence of H. pylori with respect to Sociodemography of Participants. Accordingly, the prevalence of *H. pylori* was found to be 71.1% (143/201) and 37.3% (75/201) using the dBest *H. pylori* Ab Test Disk (95% CI: 64.2–77.6) and SD BIOLINE *H. pylori* Ag test (95% CI: 30.3–44.3), respectively (Table 2). The highest prevalence of *H. pylori* infection was seen among the males than the females (98 vs 45 by Ab test and 79 vs 27 by Ag test), and *H. pylori* is more frequent in individuals living from the urban area than rural (101 vs 42 using the Ab test and 76 vs 30 using the Ag test), respectively. Regarding the occupational status, the students are the majority groups who come up positive for *H. pylori* (both in

Table 1: Prevalence of *H. pylori* infection among the dyspeptic patients across sociodemographic characteristics at the University of Gondar Hospital Outpatient Department, $N = 201$.

Sociodemographic characteristics		Positive for the Ab test, *N* (%)	Positive for the Ag test, *N* (%)	Total, *N* (%)
Sex	Male	98 (70)	79 (56.7)	140 (69.7)
	Female	45 (73.8)	27 (44.3)	61 (30.3)
Age (years)	<10	1 (50)*	1 (50)	2 (1)
	10–19	10 (55.6)	12 (66.7)	18 (9)
	20–29	82 (67.2)	68 (55.7)	122 (60.6)
	30–39	12 (66.7)	6 (33.3)	18 (9)
	40–49	13 (92.9)	9 (64.3)	14 (7)
	50–59	12 (92.3)	5 (38.5)	13 (6.4)
	≥60	13 (92.9)	5 (35.7)	14 (7)
Residence	Urban	101 (71.6)	76 (53.9)	141 (70.1)
	Rural	42 (70)	30 (50)	60 (29.9)
Occupation	Farmer	30 (78.9)	19 (50)	38 (18.9)
	Student	73 (70.2)	64 (61.5)	104 (51.7)
	Government	14 (63.6)	8 (36.4)	22 (11)
	House wife	17 (73.9)	9 (39.1)	23 (11.4)
	Merchant	7 (87.5)	4 (50)	8 (4)
	No jobs	2 (33.3)	2 (33.3)	6 (3)
Education	Illiterate	40 (80)	24 (48)	50 (24.9)
	Primary	12 (63.2)	8 (42.1)	19 (9.5)
	Secondary	15 (62.5)	10 (41.7)	24 (11.9)
	College	76 (70.4)	64 (49.3)	108 (53.7)
Marital status	Married	56 (81.2)**	34 (49.3)	69 (34.3)
	Single	87 (65.9)	72 (54.5)	132 (65.7)
Number of siblings	0	94 (67.1)	80 (57.1)	140 (69.7)
	1–4	29 (76.3)	15 (39.5)	38 (18.9)
	5–10	20 (87)	11 (47.8)	23 (11.4)

N = number; Ag = antigen; Ab = antibody. *P value = 0.044; **P value = 0.016.

Table 2: Prevalence of *H. pylori* infection among the dyspeptic patients attending the University of Gondar Hospital Outpatient Department, $N = 201$.

Serologic tests	Prevalence of *H. pylori*			
	N	Percent (%)	SE	95% CI
dBest *H. pylori* Ab rapid test	143	71.1	3.2	64.2–77.6
SD BIOLINE *H. pylori* Ag test	75	37.3	3.5	30.3–44.3

N = number; SE = standard error; CI = confidence interval; Ag = antigen; Ab = Antibody.

the Ab and Ag tests) than others, and meanwhile *H. pylori* seropositivity, using the dBest *H. pylori* Disk tests, is significantly associated with the age groups <10 years (P value = 0.044) and married patients (P value = 0.016) (Table 1).

3.3. H. pylori Infection across Clinical Parameters and Associated Risk Factors. Clinically, the patients with heartburn, abdominal fullness, and belching had come up with positive for the *H. pylori* tests, and likewise, belching is significantly associated ($P = 0.038$), in logistic regression, with the antibody test. In those patients with *H. pylori* (a positive result with either a Ab or Ag test), drinking water from well sources had 2.23 times risk of getting *H. pylori* infection ($P = 0.017$), and drinking coffee (1.51 (0.79–2.96, $P = 0.025$) and chat chewing (1.78 (1.02–3.46, $P = 0.008$) are the most common risk factors (Tables 3 and 4).

4. Discussion

A recent study demonstrated that 65.3% of the patients were positive for *H. pylori* IgG using the immunochromatographic method [13]. This shows that the current prevalence of *H. pylori* infection based on antibodies is much lower. The current 37.3% magnitude of *H. pylori*, using the SD BIOLINE *H. pylori* Ag test, is lower than a 52.3% and 53% of report from Ethiopia [18, 19] and studies from African and Asian countries [20–22]. The variation for these findings might be the difference in the socioeconomic factors, exposure for risk factors, study settings, and essentially the variability in the diagnostic methods.

The present study revealed that *H. pylori* seropositivity has been associated with age. In developing nations, where the majority of children are infected before the age of 10, the prevalence in adults peaks at more than 80% before age 50 [23–25]. While in developed countries, evidence of infection in children is unusual but becomes more common later on adulthood. In this study, the increment in serological positivity of *H. pylori* is seen starting from children through adulthood which reaches the peak on 18–30 age groups (68 (55.7%)), but cases are becoming lower as the age gets older and older. Within any age group, infection appears to be more common in blacks and Hispanics compared to the white population; these differences are probably in part related to socioeconomic factors [26, 27].

Table 3: Prevalence of *H. pylori* infection among the dyspeptic patients across risk factors at the University of Gondar Hospital Outpatient Department, $N = 201$.

Risk factors		Positive for the Ab test, *N* (%)	Positive for the Ag test, *N* (%)	Total, *N* (%)	Multivariate OR (95% CI)	*P* value
Water source	Pipeline	111 (69.4)	86 (53.8)	160 (79.6)		
	River	27 (77.1)	15 (42.9)	35 (17.4)	2.23 (1.26–4.46)	0.017
	Well	5 (83.3)	5 (83.3)	6 (3)		
Washing hands with soap		87 (73.1)	63 (52.9)	119 (59.2)	1.04 (0.39–2.9)	0.743
Using toilet		69 (73.4)	49 (52.1)	94 (46.8)	1.80 (0.62–6.48)	0.496
Drinking alcohol		40 (65.6)	34 (55.7)	61 (30.3)	1.02 (0.48–2.9)	0.949
Drinking coffee		69 (74.2)	51 (54.8)	93 (46.3)	1.51 (0.79–2.96)	0.025
Chat chewing		4 (80)	4 (80)	5 (2.5)	1.78 (1.02–3.46)	0.008

Table 4: Prevalence of *H. pylori* infection among the dyspeptic patients across clinical parameters at the University of Gondar Hospital Outpatient Department, $N = 201$.

Clinical parameters	Positive for the Ab test, *N* (%)	Positive for the Ag test, *N* (%)	Total, *N* (%)
Heartburn	139 (70.6)	104 (47.2)	197 (98)
Epigastric pain	139 (70.9)	103 (52.6)	196 (97.5)
Abdominal fullness	133 (70.4)	101 (53.4)	189 (94)
Vomiting	51 (72.9)	41 (58.6)	70 (34.8)
Nausea	106 (71.1)	83 (55.7)	149 (74.1)
Belching	110 (71.4)	75 (48.7)*	154 (76.6)
Melena	43 (71.7)	27 (45)	60 (29.9)
Bloody vomiting	14 (87.5)	12 (75)	16 (8)

N = number; Ab = antibody; Ag = antigen. *P value = 0.038.

The increased prevalence of infection with age was initially thought to represent a continuing rate of bacterial acquisition throughout one's lifetime. However, epidemiologic evidence now indicates most infections are acquired during childhood even in developed countries [24, 28]. Most infections were acquired before five years of age with a declining incidence thereafter in one report from Ireland [29]. Thus, the frequency of *H. pylori* infection for any age group in any locality reflects that particular cohort's rate of bacterial acquisition during childhood years [28]. The organisms can be cultured from vomitus or diarrheal stools suggesting the potential for transmission among family members during periods of illness [30, 31].

The risk of acquiring *H. pylori* infection is related to the socioeconomic status and living conditions early in life. Factors such as density of housing, overcrowding, number of siblings, sharing a bed, and lack of running water have all been linked to a higher acquisition rate of *H. pylori* infection [32–34]. Our study proved that risk factors for acquiring *H. pylori* infection are most prevalent in the patients with *H. pylori* infection. Moreover, studies in the developing countries continue to show that childhood hygiene practices, and family education determines the prevalence of *H. pylori* infection [35]. In this study, illiterate individual accounts the majority (40/143 were positive for *H. pylori* Ab, and 24/106 were positive for *H. pylori* Ag tests) of *H. pylori* cases next to those who visited college. The association of *H. pylori* infection with the level of education, income, and race/ethnicity is not unique to *H. pylori,* since similar associations have been described with other chronic infections including cytomegalovirus, *herpes simplex virus-1*, and hepatitis B [36]. Studies indicated that declination of *H. pylori* infection has been attributed to economic progress and improvement in sanitation [37]. This study revealed that most (101/143 (antibody); 76/106 (antigen)) *H. pylori* positive cases are from the urban areas indicating that urbanization accompanied with poor sanitation.

The route by which infection occurs remains unknown, but multiple ways of transmission are reported [38, 39]. Person-to-person transmission of *H. pylori* through either fecal/oral or oral/oral seems most likely [31, 39]. Humans appear to be the major reservoir of infection; however, *H. pylorus has* been isolated from primates in captivity and from domestic cats [40, 41]. One report described the identification of *H. pylori* in milk and gastric tissue of sheep suggesting that sheep may be a natural host for the organism [42]. This may explain the higher infection rate that has been observed among shepherds compared to their siblings [43]. Similarly in our study, form the total *H. pylori* cases, farmers accounted the second highest proportion showing that close contact with domestic cattle may potentially result *H. pylori* transmission.

In addition to fecal/oral transmission of bacteria, contaminated water supplies in developing countries may serve as an environmental source of bacteria. In this study, majority (111/143 (antibody), 86/106 (antigen)) of *H. pylori* positive individuals use water sources from pipeline. The organism remains viable in water for several days and, using the polymerase chain reaction techniques, evidence of *H. pylori* can be found in most samples of municipal water from the endemic areas of infection [44–46]. Children who regularly swim in rivers, streams, and pools drink stream water,

or eat uncooked vegetables are more likely to be infected [47]. *H. pylori* have been cultured from diarrheal stools of children in Gambia, West Africa, where almost all inhabitants are infected by five years of age [48].

Intrafamilial clustering of infection further supports person-to-person transmission. Infected individuals are more likely to have infected spouses and children than uninfected individuals [34, 49]. A study of children in Columbia found that the risk of infection correlated directly with the number of children aged 2 to 9 in the household, while younger children were more likely to be infected if older siblings were also infected [50]. Isolation of genetically identical strains of *H. pylori* from multiple family members [51] and custodial patients in the same institution [52] and further studies support transmission among persons sharing the same living environment. In addition to the familial type of transmission that occurs in developed and other nations, horizontal transmission between persons who do not belong to a core family also appears to take place in countries where the prevalence of infection is high [49]. As revealed by studies conducted on Ethiopia and Thailand [14, 53, 54], *H. pylori* infection is higher in married individuals demonstrating that cluster living environment has an impact on *H. pylori* transmission.

At last, it should be considered that the dyspeptic patients, other than the present serum antibody and stool antigen tests, did not undergo further confirmatory tests (endoscopy with biopsy for the histology culture and/or the very least urea breath test) due to economic constraints.

5. Conclusion

The present study discovered considerable magnitude of *H. pylori* in the study area. *H. pylori* infection is frequent in individuals drinking water from well sources, and thus, poor sanitation and unhygienic water supply are contributing factors. Policies aiming at improving the socioeconomic status will reduce potential sources of infection, transmission, and ultimately the prevalence and incidence of *H. pylori* infection.

Abbreviations

Ab: Antibody
Ag: Antigen
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IRB: Institutional Review Board
SD: Standard deviation
SPSS: Statistical Package for Social Sciences
UOG: University of Gondar Hospital.

Data Availability

The dataset supporting the conclusions of this article is included within the article.

Ethical Approval

This project was ethically cleared by the Institutional Review Board (IRB) of the University of Gondar. Participation was voluntary, and informed verbal consent was taken from all adult participants and from the next of kin, caretakers, or guardians on behalf of the minors/children before inclusion to the study. Initially, the participants were briefly explained about the objectives of the study, risks, and benefits of the procedures and on voluntary participation and the right to withdraw at any stage of the study using their local language. Participants were then asked if they understood what has been explained to them. If and only if they understand the facts, implications, and future consequences of their action on themselves or their children, they would like to be part of the study. Written consent was not acquired because all the participants were recruited from the outpatient department laboratory of the Gondar University Hospital where all the participant patients were sent to undergo the *H. pylori* antibody test. The additional stool antigen test was a non-invasive procedure with minimal or no risk associated with it. Besides, the patients were benefited from the stool antigen test as it added further information on whether to commence eradication therapy by the attending physician. The result from the antibody test was collected from the laboratory record book. Official permission was also obtained from the University of Gondar Hospital before access to the record book and the conduct of the study. Therefore, considering all these facts, only the verbal agreement was acquired to be included in the study. The IRB has also evaluated the consent procedure and cleared it as sufficient. Participants who were diagnosed as positive to the *H. pylori* stool antigen test were immediately linked to the medical outpatient department of the University of Gondar Hospital for appropriate treatment and follow-up.

Conflicts of Interest

The authors declare that they have no conflicts of interest with regard to the present study.

Authors' Contributions

MN, HWB, and DG conceived the study concept and designed the study. MN and DG carried out data collection and laboratory analysis. MN, HWB, and DG supervised the data collection and laboratory analysis. MN, HWB, and DG analyzed the data and prepared the first manuscript draft. MN and DG reviewed the draft. All authors read and approved the final manuscript. All the authors are currently working at the University of Gondar.

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