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Original Article

Antibiotic resistance of *Helicobacter pylori* in Mongolia

Mandkhai Bolor-Erdene[1], Bira Namdag[2], Yoshio Yamaoka[3], Sarantuya Jav[4]

[1] Department of Physiology and Molecular Biology, Ach Medical University, Ulaanbaatar, Mongolia
[2] Department of Gastroenterology, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia
[3] Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Oita, Japan
[4] Department of Molecular Biology and Genetics, School of Pharmacy and Biomedicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia

Abstract

Introduction.The resistance of *Helicobacter pylori* to recently available antibiotic treatment regimens has been recognized as a growing problem. Therefore, the aim of this study was to determine the prevalence of antibiotic resistance among *H. pylori* strains isolated from Mongolians. Methodology. All gastric biopsy specimens were obtained during upper gastrointestinal endoscopy from patients referred for the exploration of dyspepsia. The urease positive samples by rapid urease test were cultured according to standard microbiological procedures and *H. pylori* were grown under microaerophilic conditions on selective Pylori agar. *H. pylori* antibiotic sensitivity was examined using E-test. In addition, the mutations of the corresponding gene were studied by GenoType HelicoDR DNA strip testing. Results. Three hundred twenty patients, 216 female and 104 male in the ages range of 18 to 83 years were included in this study. Rapid urease test yielded positive results for 65.9% (211/320). Among them, we have successfully obtained 72% *H. pylori* isolates. The antibiotic resistance rates were 35.5% for clarithromycin, 68.4% metronidazole, 23.0% amoxicillin, 25.0% tetracycline, 28.2% erythromycin and 14.5% nitrofuranton. Resistance for 2 drugs was 34.5% and that of 3 drugs was observed in 14.5% of isolates. The most prevalent mutation was A2147G followed by A2146G and D91Y. The prevalence of *H. pylori* infection increased among Mongolian population and the prevalence of resistance of *H. pylori* is very high to metronidazole, and moderate to clarithromycin. Conclusion. The data on antimicrobial susceptibilities provided by the present study is may assist the clinicians on the effectiveness of treatment regimens.

Key words: *Helicobacter pylori*; antibiotic resistance; multidrug resistance; Mongolia.

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Introduction

Helicobacter pylori is a Gram-negative bacterium associated with various digestive diseases, such as gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric cancer [1]. Current recommendations for the management of *H. pylori* infection were elaborated by the European Helicobacter Study Group (EHSG) and presented in Maastricht IV/Florence Consensus Report in 2012 [2]. Treatment regimens containing a PPI and combination of 2 or more antibiotics, including amoxicillin (AMX), clarithromycin (CLR), metronidazole (MNZ) or tetracycline (TET) are considered to be most efficacious [3]. However, the cure rate of *H. pylori* has been decreasing progressively, primarily due to increased resistance to antimicrobial agents. Increased resistance to CLR is a growing worldwide problem [4]. Prevalence of bacterial resistance to antibiotics varies in different geographic areas and it has been correlated with the consumption of antibiotics in the general population [4,5]. For example, MNZ resistance varies from 10 to 80% among geographic regions [6-9]. In developing countries antibiotic resistance is considered to be higher than in developed countries. CLR is found to be one of the most effective antimicrobial agents used for the treatment of *H. pylori* infection. CLR resistance in *H. pylori* is due to point mutations in the *rrl* gene encoding 23S rRNA, with three major mutations described: A2146C, A2146G, and A2147G [4]. In Mongolia, no domestic guidelines are available for the treatment of *H. pylori* because of insufficient domestic data. The aim of this study was to determine the prevalence of antibiotic resistance among *H. pylori* strains isolated from Mongolians.

Methodology

Patients

A total of 320 consecutive patients, who visited Shastin hospital and SonginoKhairkhan district hospital in Ulaanbaatar for upper endoscopy during 2011-2014

were enrolled in the study. All subjects provided informed consent, and the study protocol was approved by the Ethics Committee at Mongolian National University of Health. The information provided in the pathology reports or patients' files were recorded for each patient, which included patient's hospital ID number, age, gender, medical history, clinical diagnosis based on endoscopy according to Sydney classification 1994, and previous treatment. Patients who received bismuth compounds, antibiotics during the 4 weeks before endoscopy were not included in the study. Other exclusion criteria also included regular use of nonsteroidal anti-inflammatory drugs including acetylsalicylic acid, malignancy, and severe liver diseases.

Endoscopic study

All patients included in the study underwent upper gastrointestinal endoscopy. Two sets of gastric biopsy specimens were obtained from the antrum and body in all patients and one set was tested for *H. pylori* using rapid urease test and the other specimen was used for *H. pylori* culture.

Isolation of H. pylori

Biopsy specimens from the antrum and body were used for *H. pylori* culture. Biopsy specimens were macerated and homogenized in meat liver dextrosa broth and a 250 µL aliquot was inoculated on selective Pylori Agar (Biomerieux, Marcy-l'Etoile, France). Incubation was performed in microaerophilic (5% O_2, 15% CO_2, and 80% N_2) conditions (Genbox and Genbagmicroaerophilic. Biomerieux, Marcy-l'Etoile, France) at 37°C for a maximum of 5-7 days. Colonies were identified as *H. pylori* according to standard criteria including Gram-negative bacteria with typical cell morphology, and positive reactions to catalase, oxidase, and urease.

Antimicrobial susceptibility testing

The susceptibility of *H. pylori* isolates to CLR, MNZ, AMX, TET, nitrofurantoin (NIF) and erythromycin was examined by E-test strip (Biomerieux, Marcy-l'Etoile, France). The bacterial suspensions were spread onto Mueller-Hinton II agar plates (Becton Dickinson, New Jersey, USA) supplemented with 7% defibrinated sheep blood by sterile cotton swabs. After drying, each E-test strip of the corresponding antibiotic was placed on separative plate and all plates were incubated in anaerobic jar for 3 days at 37°C under microaerophilic conditions (GENbox microaer, Biomérieux, Marcy-l'Etoile, France). Minimum Inhibitory Concentration (MIC) was defined as the point of intersection of the elliptical inhibition zone with the E-test strip. The breakpoints used to classify strains as susceptible or resistant were as follows: AMX, erythromycin and NIF; MIC ≤1 mg/L = susceptible and ≥1 mg/L = resistant; CLR; MIC ≤0.25 mg/L = susceptible, = 0.5 intermediate and ≥1 mg/L = resistant; MNZ; MIC ≤4 mg/L = susceptible and ≥8mg/L = resistant; and TET; MIC ≤2 mg/L = susceptible and ≥4 mg/L = resistant [10].

PCR Method

Amplification of bacterial DNA was performed using hot-start DNA polymerase (Hain Lifescience, Nehren, Germany). Biotinylated primers were used for this study and were provided in the amplification kit (Hain Lifescience, Nehren, Germany). Polymerase chain reaction (PCR) for a single mixture had a final volume of 50 µL containing 35 µL primer/nucleotide mix (PNM), 5 µL 10 ×polymerase incubation buffer, 2 µL of 1.5mM $MgCl_2$, 3 µL of nuclease free water 0.2 µL Thermo-Start Taq DNA polymerase (1-2 units were added to each tube), and 5 µL DNA template. PCR was performed with a thermal cycler (Applied Biosystem, Foster city, USA). In protocols, the denaturation cycle was 1 cycle at 95°C for 15 min, followed by 10 cycles at 95°C for 30 s and at 58°C for 2 min. Then, 20 cycles were composed of a first step at 95°C for 25 s, a second step at 53°C for 40 s, and a third step at 70°C for 40 s. The PCR ended with 8 min at 70°C. Hybridization was performed using the TwinCubator at a temperature of 45°C. The denaturation solution was mixed with 20 µL of the amplified sample and submitted to the usual protocol for hybridization.

GenoTypeHelicoDR Analysis

Confirmation of isolates as *H. pylori,* antimicrobial susceptibility, and mutational analysis to clarithromycin was performed using the GenoTypeHelicoDR kit (Hain Lifescience, Nehren, Germany). The kit employs the use of reverse hybridization performed using hybridization trays and Twin-Cubator (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. Briefly, 20 µL of amplified DNA was denatured and added to biotinylated probes on the strip and the hybrids formed were detected by enzyme linked immunosorbent assay (ELISA) upon addition of enzyme conjugate and substrate. Four gyr87 wild type probes (gyr87WT1–gyr87WT4) and one mutant probe (gyr87MUT), one wild type probe (gyr91WT1), and three mutant probes (gyr91MUT1–gyr91MUT3) were used for detecting

Table 1. Age distribution of *Helicobacter pylori* infected patients.

	N	*H. pylori* positive (n)	Percent	95%CI
Age (years)	320	211		
18-29	56	39	69.6	58.8-72.9
30-39	72	48	66.6	54.3-68.8
40-49	74	50	67.5	60.6-74.5
50-59	76	49	64.4	55.3-69.6
≥ 60	42	25	59.5	47.7-62.4
Gender				
Female	216	138	63.8	57.3-70.3
Male	104	73	70.2	61.4-78.9

fluoroquinolone resistance at position 87 and 91, respectively. For CLR, one wild type probe (23SWT) and three mutant probes (23SMUT1–23SMUT3) were used for detecting resistance. There were designated conjugate control (CC), amplification control (AC) and *H. pylori* (HP). The presence of a band at CC and AC meant that the conjugate control and amplification control were in the right frame while at HP implied presence of *H. pylori* according to the manufacturer's instruction (Hain Lifescience, Nehren, Germany).

Statistical analysis

Statistical analysis was performed using Chi-square and Fisher's exact tests. Null hypotheses of no difference were rejected if *p*-values were less than 0.05.

Results

Patients

Three hundred twenty patients including 216 female and 104 male, with median age of 43.7 years ranged from 18 to 83 years who underwent upper gastroendoscopy were recruited in this study**.** Relationship between *H. pylori* infection, age and gender of the patients are presented in Table 1. CLO test yielded positive results for 65.9% [95% CI 60.7-71.0] (n = 211).

Antimicrobial susceptibility testing

We have successfully obtained 152 (72%) *H. pylori* isolates from 211 CLO test-positive samples. Antibiotic susceptibility patterns of the 152 *H. pylori* strains was determined by E-test method

Out of 152 strains, 56 were from male and 96 from female patients. Table 2 shows the antimicrobial susceptibility rate in Mongolia. The antibiotic resistance rates were 35.5% for CLR, 68.4% MNZ, 23.0% AMX 25.0% TET, 28.2% erythromycin and 14.5% nitrofurantoin. Overall, 35.5% (n = 54) patients found to have positive cultures for *H. pylori* strains that were fully resistant to CLR. Only 2 patients had an isolate that demonstrated intermediate resistance to CLR (MIC = 0.50). The prevalence of CLR resistance in male and female was 32.1% vs. 37.5%. In general, there was a higher prevalence of resistant isolates in female compared to male patients. However, this did not reach statistical significance (p = 0.301). The frequency of CLR resistance was found in patients with gastritis (n = 23, 42.5%), stomach erosion (n = 13, 24%), gastric ulcer and atrophic gastritis (n = 7, 12.9%) and nodularity (n = 4, 7%). CLR resistance rate within the age group of 18-39 years was higher than other age groups of male patients. However, female was similar in all age groups in terms of CLR resistance rate.

Overall, 68.4% (n =117) *H. pylori* culture-positive patients had isolates that were fully resistant to MNZ. Male subjects were more likely to carry MNZ resistant *H. pylori* isolates than female subjects. However, this did not reach statistical significance (p = 0.543) MNZ resistance rate within age group of 30-39 years was higher than other age group of male. Female was 40-59 age group was higher than other age group. However, this did not reach statistical significance (p = 0.605).

Table 2. Antimicrobial susceptibility Pattern.

Agent	No. of Susceptible Strains	No. of Resistant strains	Resistance (%)
clarithromycin	98	54	(35.5)
metronidazole	48	104	(68.4)
amoxicillin	117	35	(23.0)
tetracycline	114	38	(25.0)
erythromycin	109	43	(28.2)
nitrofurantoin	130	22	(14.5)

Table 3. Probes hybridized on the DNA strip of the GenoType HelicoDR test for detection of mutations in the *rrl* and the *gyrA genes.*

Probes	Codon	Nucleotides	Associated phenotype*
23S-WT	2146 and 2147	AA	CLA-S
23S-MUT1	2146	A2146G	CLA-R
23S-MUT2	2146	A2146C	CLA-R
23S-MUT3	2147	A2147G	CLA-R
gyr87- WT1	N87	AAC	FQ-S
gyr87- WT2	N87	AAT	FQ-S
gyr87- WT3	T87	ACT	FQ-S
gyr87- WT4	T87	ATT	FQ-S
gyr87- MUT	N87K	AAA	FQ-R
gyr91-WT	D91	GAT	FQ-S
gyr91-MUT1	D91N	AAT	FQ-R
gyr91-MUT2	D91G	GGT	FQ-R
gyr91-MUT3	D91Y	TAT	FQ-R

* CLA, clarithromycin; FQ, fluoroquinolone; S, susceptible; R, resistant.

Table 4. Distribution of clarithromycin resistant strains in respect to age and gender.

	A2147G	
	Positive n (%)	**Negative n (%)**
Gender		
Male	13 (72.2)	5 (27.8)
Female	19 (57.6)	14 (42.4)
Total	32	19
Age group (years)		
18-29	8 (72.7)	3 (27.3)
30-39	6 (40)	9 (60)
40-49	4 (57.1)	3 (42.9)
50-59	11 (91.7)	1 (8.3)
60 <	3 (50)	3 (50)
Total	**32**	**19**

Table 5. Relation between *rrl* gene point mutation and clarithromycin resistance rate of *H. pylori.*

CLA Susceptibility	Number (%)	A2146G MUT1	Number (%)	A2146C MUT2	Number (%)	A2147G MUT3	Number (%)
Resistance	51 (53.7%)	+	4 (8%)	+	0	+	32 (62.7%)
		-	0	-	0	-	19 (37.3%)
Sensitive	44 (46.3%)	+	0	+	0	+	0
		-	0	-	0	-	44 (100%)
Total	**95**						**95**

CLA, clarithromycin.

Analysis of GenoType HelicoDR test

The MUT and WT probes were designed from the mutations observed in the resistant strains such as mutations in the *rrl* gene encoding the 23S rRNA for the CLR-resistant strains. Probes hybridized on the DNA strip of the GenoType HelicoDR test for detection of mutations in the *rrl* genes and *gyrA* listed in Table 3 [11]. The method of GenoType HelicoDR for 23S rNA (rrl) genotyping had been used to analyze the qualifying full growth of 95 samples.

Out of 95, 64 (67.3%) *H. pylori* isolates hybridized with wild type probe of *23S rRNA* gene of CLR. All CLR resistance isolates had at least one of the three common point mutation in 23SrRNA gene, while none of the CLR susceptible isolates had this mutation.

Table 4 shows the distribution of MUT3 in 51 CLA-R strains isolated from consecutive patients based on age and gender. Nineteen out of 32 (59.3%) MUT3 positive strains and fourteen out of 19 MUT3 negative strains isolated from the female population had this point mutation. Eleven out of 32 of the MUT3 positive strains were isolated from the most frequent age group i.e. 50-59 (Table 4). There was no significant relation between gender (p = 0.301) and age with this mutation.

Overall, the most frequent mutation was A2147G (MUT3 profile), observed in 32 strains (62.7%) of the mutated alleles. The frequency and rate of mutations encoding clarithromycin resistance in *H. pylori* isolates is shown Table 5. Eight percent of CLR resistance isolates (4 out of 51 isolates) had A2146G point mutation. We did not detect A2146C (MUT2 profile) in any of clarithromycin resistant strains. MUT3 was the most frequently detected mutation in clarithromycin resistant strains (p = 0.001).

Ten fluoroquinolone resistant strains were associated with N87K mutation. Additionally, D91N responsible for resistance to fluoroquinolone was detected in 4 (4.2%), D91G was 6 (6.3%) and D91Y was 2 (2.1%) strains (Table 6).

Rate of multidrug resistance

Of the 152 strains, 16 (10.5%) showed no resistance to any antibiotics. Table 7 shows that rate of multidrug resistance in *H. pylori* in Mongolia. Resistance for one drug was observed in 28.2% (95% CI 25.7-43.2) isolates, for two drugs 34.5% (95% CI 25.7-43.2) and for 3 drugs was 14.4% (95% CI 8.6-19.7). Within the strains tested for susceptibility to all 6 antibacterial agents, *H. pylori* resistance for 4 or 5 drugs was detected in 5-6% whilst all 6 agents resistance was observed in one strain. The incidence of multiple drug resistance of *H. pylori* isolates is listed in Table 7. The first choice of treatment for *H. pylori* infection was CLR + MNZ for 9.4% and CLR + AMX for 5.6% cases. The second choice and alternatives to these medications were MNZ + AMX 20.7% and MNZ + TET had 9.4% resistance rate. It has been shown that a popular use of MNZ + AMX combination to eradicate *H. pylori* affects the result of treatment negatively.

Discussion

This is the first study exploring the antibiotic resistance pattern of *H. pylori* strains isolated from Mongolian population. In Mongolia, no domestic guidelines are available for the treatment of *H. pylori* because of insufficient data.

Although the prevalence of *H. pylori* in developed countries is decreasing, gastric colonization by *H. pylori* remains widespread in developing countries.

Table 6. Genotypes detected by the GenoType HelicoDR test for *rrl and gyrA* genes in *H. pylori* strains.

Genotyping	Total strains (*n* =95)	Codon	Nucleotides
***23S rRNA* gene (*rrl*)**			
WT	64	2146 and 2147	AA
MUT1	**4**	2146	A2146G
MUT3	**32**	2147	A2147G
***gyrA* gene**			
Codon 87			
WT1	58	N87	AAC
WT2	22	N87	AAT
MUT	10	N87K	AAA
Codon 91			
WT	73	D91	GAT
MUT1	4	D91N	AAT
MUT2	6	D91G	GGT
MUT3	2	D91Y	TAT

WT, wild-type allele; MUT, mutated allele.

Table 7. Rate of multidrug resistance in *H. pylori*.

Agent	Resistance frequency	Percent%	95%CI
1 drug	**43**	**28.2**	**[21-35.3]**
AMX	1		
ERY	1		
NIF	2		
CLR	4		
MNZ	35		
2 drugs	**53**	**34.5**	**[25.7-43.2]**
AMX+TET	3		
CLR+AMX	3		
CLR+ERY	9		
CLR+MNZ	5		
CLR+NIF	3		
CLR+TET	2		
MNZ+AMX	11		
MNZ+ERY	7		
MNZ+NIF	5		
MNZ+TET	5		
3 drugs	**22**	**14.4**	**[8.6-19.7]**
CLR+MNZ+TET	6		
CLR+MNZ+ERY	7		
CLR+MNZ+AMX	2		
CLR+MNZ+NIF	2		
MMZ+TET+NIF	2		
CLR+TET+NIF	1		
MNZ+AMX+ERY	2		
4 drugs	**9**	**6**	**[5.2-6.7]**
CLR+MNZ+TET+ERY	3		
CLR+AMX+TET+ERY	1		
CLR+MNZ+AMX+ERY	1		
CLR+MNZ+AMX+NIF	1		
MNZ+AMX+TET+ERY	1		
MNZ+AMX+TET+NIF	2		
5 drugs	**8**	**5**	**[4.2-5.8]**
CLR+MNZ+TET+ERY+NIF	2		
CLR+MNZ+AMX+TET+ERY	4		
CLR+MNZ+AMX+ERY+NIF	2		
6 drugs	**1**	**0.9**	

AMX: amoxicillin, ERY: erythromycin, MNZ: metronidazole, CLR: clarithromycin, TET: tetracycline, NIF: nitrofurantoin.

Infection with *H. pylori* can be diagnosed by a variety of tests and can often be successfully treated with antibiotics [12]. The prevalence of *H. pylori* strongly varies between developing and developed countries, where the prevalence among adult is typically around 80-90% and <40% respectively [13]. In this study, we performed 320 endoscopies in patients with upper gastrointestinal symptoms and confirmed that the 65.9% had gastric *H. Pylori* infection using rapid urease test.

Antimicrobial resistance varies by geographical region and is highly influenced by patterns of antimicrobial use within a population [4,5,14]. The key determinants of the outcome of eradication therapy for *H. pylori* infection are compliance and the presence of pretreatment antibiotic resistance of the isolate [15]. In this study we reported the susceptibility of *H. pylori* strains from Mongolia, a country with a high prevalence of infection, to the most commonly used antibiotics. Our results revealed that rate of antimicrobial susceptibility were 35.5% for CLR, 68.4% for MNZ, 23% for AMX, 25.0% for TET, 28.2% for ERY and 14.5% for nitrofurantoin. CLR is one of the core antibiotics used in PPI triple régimen [3,16]. Many researchers found that resistance to CLR is critical to the effectiveness of *H. pylori* eradication with triple therapy [17]. Prevalence of cLR resistance has been studied widely. It ranges from close to nil to 25% [10]. In Asian countries, high prevalence of CLR resistance was detected in Japan (40.7%) whereas the

lowest (2.1%) prevalence rate was seen in Malaysia [18]. In the present study, resistance rate to CLR was 35.5%. In fact, the Maastricht III guidelines on *H. pylori* infection management recommend that CLR should not be used when resistance to the antibiotic exceeds 15–20% [19]. Therefore, CLR-based triple therapy might not be helpful to eradicate *H. pylori* in Mongolia.

The assay used in this study was designed to target the presence of A2147G, A2146G, and A2146C mutations associated with CLR resistance [11]. We detected that most frequent mutation was A2147G (MUT3 profile) in our studied isolates. Also, the strips in the GenoTypeHelicoDR assay are designed to generally target fluoroquinolones. The codons N87 and D91 are recognized as the most important target sites for ciprofloxacin binding. The N87H, N87I, N87K, and N87Y as well as D91G, D91N, and D91Y mutations in *gyrA* have been reported in fluoroquinolone-resistant *H. pylori* strains [20]. The assay used in this study was designed to depict N87K, D91N, D91G, and D91Y, which have been frequently reported. N87K was the most prevalent mutation (10/95; 10.5%) associated with fluoroquinolone resistance.

In this study, the prevalence of MNZ resistance was high (66.4%). Whereas MNZ resistance has been reported in 10–50% of all adult patients infected with *H. pylori* in developed countries, virtually all strains from developing countries have been found to be MNZ resistant [21], which is in agreement with our data that reports prevalence of MNZ resistance in 66.4% strains. In general, the high prevalence of MNZ resistance in developing countries is probably because of the frequent use of MNZ derivatives for the treatment of protozoal infections and gynecological problems [22], which is also common in Mongolia.

In the present study, AMX resistance was 23%. China, India, Mexico, and Italy have also reported variable prevalence rates for AMX resistance, i.e., 41.2%, 65%, 19.4%, and 45% respectively [23,24]. Similarly, it is difficult to explain variation of AMX-resistance in different parts of the country as well as throughout the world. The prevalence of AMX resistance was probably a result of indiscriminate use of this antimicrobial agent, because of the lack of clearly defined guidelines for the management of *H. pylori*-associated dyspepsia and other infections.

Multidrug resistance of *H. pylori* is occasional and found in individual countries or regions, for example in Taiwan [25]. A Bulgarian study reported that triple resistance to the evaluated agents was uncommon and detected in 3.5% of the untreated adults and 13.6% of the treated adults. Five *H. pylori* strains were resistant to AMX, MNZ and CLR, two of them exhibiting quadruple resistance. Resistance to four of the five antibacterials tested was found in 0.7% of the untreated and 1.8% of the treated adults [26]. Quadruple resistance of *H. pylori* has not been reported in Europe and the USA in last 5 years, although a study from India reported this type of resistance in 2.6% of isolates [27]. In the present study, resistance for 1 drug was observed in 28.2%, for 2 drugs was 34.5% and that for 3 drugs was observed in 14.4% of isolates. While *H. pylori* resistance for 4 or 5 drugs was detected in 5-6% strains.

Conclusions

This study shows that the prevalence of *H. pylori* infection was high among Mongolian population. We also report prevalence of resistance was high to MNZ and moderate to CLR. In addition, multidrug resistant strains were frequently found. CLR and fluoroquinolones resistance was mainly associated with A2147G and N87K mutations, respectively. We also demonstrates the need for continuous monitoring of the antimicrobial susceptibility in *H. pylori* to determine optimal treatment regimen.

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Corresponding author

Sarantuya Jav
Department of Molecular Biology and Genetics, School of Pharmacy and Biomedicine, Mongolian National University of Medical Sciences, Zorig Street, Post office-48, Post box-111, Ulaanbaatar 14210, Mongolia.
Phone: 976-99092771
Fax: 976-11319065
Email: sarantuya.j@mnums.edu.mn

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