Original article

The inhibitory effect of anti-urease IgY on *Helicobacter pylori* infection in *Swiss albino* mice

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ABSTRACT

Urease inhibition with oral antibodies is a promising strategy in the treatment of Helicobacter pylori infection. This study aimed to investigate the advantages of the producing method of anti-urease IgY and its inhibitory effect on H. pylori infection on experimental animal. We started the process of producing IgY with the preparation of H. Pylori's urease. Then the eggs from the hens that were immunized with the prepared urease were collected to be used for IgY extraction. We divided 18 Swiss albino mice into 2 groups to test the inhibitory effect of the IgY. One group was fed the food containing IgY and the other was not. In the end, the mice were examined to determine the reactivity of anti-H. pylori urease IgY in the sera and the inflammation and infection status of the stomach biopsies. The ELISA results confirmed the presence of IgY in the hens' sera. The IgY from egg yolks was purified using ammonium sulfate precipitation. Pathological examination of 55.6% of the stomachs from the infected mice from the study group showed considerable reduction of inflammation in the tissue compared to those from the control group (p < 0.0001), and 22.2% of the mice from the study group was free from gastritis, based on the histopathology results. Our method of IgY purification can be promisingly applied in large-scale production. Oral administration of urease-specific IgY is successful in inhibiting *H. pylori* colonization in the inocculated mice and can be considered an alternative to antimicrobial therapy.

1. INTRODUCTION

Helicobacter pylori infection is presently a global health issue which is strongly linked to chronic gastritis, gastric and duodenal ulcers, gastric cancers and gastric mucosa-associated lymphoid tissue (MALT) lymphomas¹⁻⁵. Supported by statistical data, the infection rate in different regions in the world varies depending mainly on socioeconomic status of a country and other factors. This rate fluctuates between 20% to 50% in developed countries while it remains over 80% in developing countries, including the countries in Southest Asia⁶⁻⁸. Accumulating evidence about the mechanism responsible for *H. pylori* associated diseases showed that the bacteria secrete urease to neutralize the hostile gastric acid surrounding them, making it less acidic in order for the bacteria to invade into the gastric mucosa, colonize and release effector toxins⁹.

Treatments for *H. pylori* infections usually include antimicrobial therapy with a combined use of metronidazole, amoxicillin, clarithromycin and either bismuth or a proton pump inhibitor¹⁰. However, the bacteria in 10-15% of cases have nullified the effect of antimicrobial therapy due to the tremendous development of antibiotic resistance^{11,12}. Henceforth, prophylactic treatment with oral antibodies that inhibit the bacteria before they get colonized has become a new and promising approach in developed countries like Korea and Japan¹³. Similar to mammals, hens are capable of creating antibodies against pathogen microbes; these antibodies can be transferred from a hen's blood into the egg yolk laid by the hen itself, hence its name is IgY (yolk immunoglobulin). Technologies and procedures for antigen-specific IgY production have been described in Ko et al.'s study¹⁴. IgY has been used orally as a passive immunization method in the prevention and treatment of Escherichia coli15, Salmonella *typhymurium*¹⁶ and Rotavirus¹⁷⁻¹⁹ infections in animals and human. IgY against the urease of H. *pylori* might be effective in preventing *H. pylori* infection, and/or reduce damages caused by the bacteria on gastric mucosa. Overall, this study aims to evaluate the productivity of our IgY producing method and study on the inhibitory effect of IgY against H. pylori-derived urease on H. pylori in experimental animals.

2. MATERIALS AND METHODS

We started our study with isolating *H. pylori*'s urease from the biopsies that we took from 18 patients diagnosed with gastritis from 103 Military Hospital in Ha Noi, Vietnam. The biopsies was sent for the incubating, then we chose the samples with the highest urease activities for bacterial multiplication. After the bacteria had colonized, we extracted the urease from the culture medium. It was then processed with precipitation until reaching the maximum purification. The process of preparing urease is described in Figure 1. After each phase of the isolation process, the solution was proceeded to evaluate the activity of the urease, following the method from Icatlo et al.'s studies²⁰. The activity of the enzyme is determined based on the principle that urease hydrolyses urease into ammonia. This ammonia will create an alkaline environment, which in turn turns the phenol red pH indicator into pink, the latter is most photoadsorbed at the wavelength of 340nm. The concentration of the enzyme is determined by Bradford assay. The purification and the molecular size of the product is determined by SDS-PAGE in denaturing conditions. After being electrophoresed, the molecular weight of the enzyme will be defined by establishing a directrix graph showing the correlation between the urease standard molecular weight and the migrated distances of the proteins. The directrix graph is established

with a standard protein weighed from 10 to 120 kDa (Thermo Scientific, USA), which makes up the Y-axis, and its migrated distances, which makes up the X-axis. The molecular weight of *H. pylori* urease will be inferred from the migrated distance of the urease on the electrophoregram and the established directrix graph.



Figure 1. The process of preparing *Helicobacter pylori*'s urease from gastric biopsies.

After the urease had been purified, we used it to immunize the hens via injection at different

doses. Then we monitored the antibody titers in the hens' sera. The eggs were then harvested from the group of hens that had the highest antibody titer for the isolation and purification of IgY. We produce the IgY by ammonium sulfate precipitation and exchange chromatography methods. After the production of IgY, we used 18 *Swiss albino* mice, which were divided into 2 groups to test the inhibitory effect of the IgY by feeding them food containing the IgY. At the end of the study, we expected to get the results as followed: (1) the isolation and purification results of urease; (2) the purification results of the anti-urease IgY; and (3) the results of the treatment of *H. pylori* infected experimental animals.

2.1. Isolation and purification of IgY

The urease extracted from different samples of H. *pylori* incubation were mixed together at equal ratios, then mixed with Freund's adjuvant (at the ratio of 1 to 1 to form the antigenic suspension for the hens' immunization).

A total number of 12 hens of Luong Phuong breed, of the same maturity age and same strain weighed from 1.9 to 2.1 kgs were provided by the Experimental Animal Breeding Service, Vietnam Medical Military University. The hens were kept in natural lighting condition with an abundant food and water. They were divided into 3 groups (4 hens per group) based on the urease injection doses as follows: group 1 with a dose of 250 μ g/ml/animal; group 2 with a dose of 500 µg/ml/animal and group 3 with a dose of 750 μ g/ml/animal. The urease were injected into the hens' breasts every 3 weeks until all 5 doses of urease were completely injected in all hens. After each dose, the hens' blood was taken for ELISA for detecting the presence of immunoglobulin. The health and egg-laying status of the hens were collected every day. The eggs were collected daily, the date and the code number were also embedded on the eggs. After being collected, the eggs are kept at 4°C constanly until being used for the experiments.

The IgY from the egg yolks was extracted using ammonium precipitation and exchange chromatography methods, which are similar to methods that were described before in Ko et al.'s, Shin et al.'s and Siriya et al.'s studies^{14,21,22}. First, the yolks were separated from the white and homogenized until it turned into a suspension. Then it was diluted with distilled water (at pH 6.0) at the ratio of 1:9. Subsequently, the suspension was left overnight at 4°C for delipidation. The suspension was then filtered through a Whatman No.1 filter paper to collect the supernatant. We precipitated the supernatant, which contained IgY with 40% saturated ammonium sulfate at 4°C in 2 hours. After precipitation, the pellet was centrifuged to remove the liquid part and collect the sediment containing IgY. The sediment was then dissolved in PBS, then it was dialyzed to remove NaCl.

After being purified with ammonium sulfate precipitation, the IgY underwent another purification phase with ion exchange purification using Biologic-LP low pressure chromatography system (BioRad) with anion columns, buffer solution A (Tris-HCl pH 7.94) and buffer solution B (buffer solution A with 0.5M NaCl), at the flow speed of 1 ml per minute. All of the products at different phases were collected using an automatic collecting system, in which the volume of sample at each stage is 1 ml.

The products after each stage were all assessed using Bradford protein assay, SDS-PAGE and ELISA in order to evaluate the activity of the anti-urease IgY.

The IgY extracted from the egg yolks was divided into 3 groups and stored at room temperature, 4°C and -20°C respectively to evaluate the stability of the antibodies in different temperature conditions. The samples are collected periodically to be assessed by ELISA and *in vitro* activity assay.

2.2. Passive immunization with IgY against *H. pylori* infection in Swiss mice

The mice food was prepared by mixing the yolk protein suspension rich in anti-urease IgY with sterilized conventional nutrition-enriched mice food, which was imported from Viet-Phap Joint Stock Company (4 mg IgY per 1 gr of food, according to the concentration estimation method described by Nomura et al.¹³).

A total of 18 healthy male *Swiss albino* mice which were 4 weeks old, weighed from 16 to 18 grams were provided by the Experimental Animal Breeding Service, Vietnam Medical Military University. The mice were divided into 2 groups: group 1 (control group) and group 2 (study group) with 9 mice per each group. The mice of group 2 were fed the food containing anti-urease IgY while the mice from group 1 were only fed the conventional food. These groups are to evaluate the effect of IgY in eradicating the bacteria. At day 7, the mice of group 2 were infected in the next morning with *H. pylori* through esophageal intubation (10⁸ CFU/mL; 15 mL/kg body weight). After 9 weeks, blood



Figure 2. Timeline of the experiment on Swiss albino mice.

samples of all mice were taken for determination of anti-*H. pylori* antibody titer. All the mentioned processes are chronically displayed in Figure 2.

After 9 weeks of the experiment, the stomachs of the animals were subjected to ELISA and histopathological examination with focus on the presence of *H. Pylori* antobodies, the severity of gastritis and the presence of *H. pylori*. The gastritis status of the samples was assessed with Eosin and Hematoxylin stain, and the presence and density of *H. pylori* were evaluated with Giemsa stain.

The biopsies were chemically-fixed in 10% neutral buffer formalin. They were then embedded in paraffin blocks and cut for histology investigation using Eosin and Hematoxylin stain. The samples were stained following the flow chart (Figure 3). After being stained properly, the samples were then investigated under microscope to look for the specific histopathological images of chronic gastritis, which were then evaluated and graded based on the Updated Sydney Classification for gastritis (Table 1)²³.

Histologic	Definition —	Grade		
properties		Mild	Moderate	Severe
inflammation	Lymphocyte and plasma cell in lamina propria	1+	Chronic inflammation	Lymphocyte and plasma cell in lamina propria
Neutrophil activation	Neutrophilic infiltration in lamina propria or superficial epithelium	<1/3	Neutrophil activation	Neutrophilic infiltration in lamina propria or superficial epithelium
Glandular atrophy	Loss of corpus and antral glands	1+	Glandular atrophy	Loss of corpus and antral glands
Intestinal metaplasia	Intestinal metaplasia of mucosal epithelium	<1/3	Intestinal metaplasia	Intestinal metaplasia of mucosal epithelium
Helicobacter pylori	Helicobacter pylori intensity	1+	2+	3+

For the Giemsa stain, after being processed with paraffin, the samples were dipped in toluene to remove the paraffin for 3×5 minutes. Then they were dipped in 100°C, 95°C, 80°C and 70°C ethanol, respectively. Subsequently, the samples were washed with distilled water to remove the excess ethanol. The samples were then stained in diluted Giemsa solution for 1 hour. After that, we rinsed the samples again with distilled water to remove the excess staining solution. For differentiation, the samples were rinsed with diluted acetic acid (1 volume of acetic acid diluted with 100 volumes of distilled water) and they continued to be rinsed again with tap water in 1 minute. The excess stain was remove with 95°C ethanol, and finally the excess water was removed with xylene. After being stained with Giemsa, the samples were then investigated under the microscope with a magnification of x100 to detect the presence of *H. pylori*. The density of the bacteria is graded based on the description table from Nguyen et al.'s study, which is described in Table 2^{24} .

The antibody titer in Swiss mice were measured using ELISA, in which the samples

Table 2. Semi-quantitative grading of Helicobacter pylori density.

Grade	Symbol	Feature (in the microscopic field)
0	Hp (-)	No presence of Helicobacter pylori
1	Hp (+)	Scattered or only one small colony
2	Hp (++)	Less than 5 small colonies and/or 1 large colony
3	Hp (+++)	More than 5 small colonies and/or more than 2 large colonies



Figure 3. Hematoxylin Eosin (HE) staining procedure.

from the two groups were investigated and interpreted at the wavelength of 450 nm, which was similar to the technique of detecting the IgY antibody titer in the hens' sera.

The collected data were processed with SPSS and were stastically analyzed based on medical statistical principles, which were expressed as mean \pm SE, and a p < 0.05 was used to denote statistical significance. The data were analyzed using independent samples test. The study was also conducted under no ethical violence against animals and human's rights.

3. RESULTS

3.1. Isolation and purification of H. pylori urease

The gastroduodenoscopy and histopathology results showed that 2 of the samples were gastritis, 11 of the samples were gastroduodenitis and the last 5 samples were gastric and duodenal ulcers. After being treated at the laboratory, these samples were coded from HP01 to HP18 and were incubated for bacterial isolation and identification.

After 5 days of incubation at 37°C in microaerobic culture bags, there were bacterial colonizations in 17/18 samples (except HP10). Gram staining results indicated the presence of gram-negative bacteria, and urease, catalase and oxydase test results were all positive, which can be eligible to indicate that the bacteria from all 17

culture samples were H. pylori.

The urease enzyme activity assay of *H. pylori* was measured using optical density with DTX 880 spectrophotometer system (Beckman Coulter) at a wavelength of 340 nm. Three samples which had the highest enzyme activity of urease (which were HP13, HP16 and HP18) were then taken for bacterial multiplication. After the bacteria had multiplied, the urease was extracted from the cuture medium by ammonium sulfate precipitation. The precipitate went through dialysis to remove salts and electrophoresis on 10% polyacrylamide gel to determine the molecular weight of the urease. The electrophoresis results are showed in Figure 4(a).

The results showed that the urease after being treated with precipitation and dialysis was quite homogeneous. The darkest bands on the electrophoregram migrated at the positions of the urease subunits. The molecular weight of the subunits were calculated by establishing a directrix graph displaying the correlation between the molecular weight of the proteins and the distances that they migrated when being electrophoresed. The mentioned graph is showed in Figure 4(b).

According to the graph, the estimated molecular weight of the protein is 60.3 kDa, which is in approximate accordance with the molecular weight of the UreB subunit, theoretically (60.3 to 64.3 kDa)^{7,8,25}. Hence the isolation and purification of *H. Pylori*'s urease were considered qualified.



Figure 4. a) The electrophoregram in determining the molecular weight of the urease subunits (M: prestained protein ladder; HP13, HP16, HP18: purified urease from 3 H. pylori samples HP13, HP16 and HP18). The arrow points at the ideal molecular weight of urease, which should be between 60.3 and 64.3 kDa7,8,22 (b) The graph displays the correlation between the proteins and their migrating distances. Black line: the graph of the directrix y = -0.371x + 5.117.



Figure 5. The changes in anti-urease IgY antibody titers in the hens' sera after each dose of immunization with urease suspension. Blue line: the antibody titer in the sera of the hens from group 1; orange line: the antibody titer in the sera of the hens from group 2; gray line: the antibody titer in the sera of the hens from group 3.



Figure 6. Anti-urease IgY antibody activity in the egg yolks laid by the hens from 3 groups after the 5th dose of urease. Blue line: negative control; red line: IgY activity in the yolks from the eggs laid by group 1 hens; gray line: IgY activity in the yolks from the eggs laid by group 2 hens; yellow line: IgY activity in the yolks from the eggs laid by group 3 hens.

3.2. Immunization of hens and purification of IgY

The ELISA results of the hens' sera after each dose of the urease suspension are displayed in Figure 5. It can be seen from the graph that the hens that were immunized with a 750 μ g/ml/animal dose produced the immunoglobulin faster and more intensely.

All the eggs collected from the hens that were immunized with urease 5 times were all positive to immunoglobulin Y. According to the graph below (Figure 6), the ELISA and optical density results indicated that the eggs laid from the hens from group 3 had the highest antibody activity.

The anti-urease IgY was purified using ammonium sulfate precipitation, followed by ion exchange chromatography. Figure 7 displays the results of the finished product with 40% ammonium sulfate.

There are two protein peaks that can be seen in the chromatogram. The first peak represents

the proteins that did not bind to the column and were eluted by the buffer solution A which has a low concentration of salt. The proteins in this stage are miscellaneous and the antibody activity of these proteins is minimal. The second one represents the proteins that were released using the buffer solution B with a high concentration of salt. The proteins in this stage has a strong antibody activity reacting to *H. pylori* urease.

Purity and yield of the IgY are moniored



Figure 7. Ion exchange chromatogram of the finished IgY product after being purified by ammonium sulfate precipitation.



Figure 8. SDS-PAGE results of the finished IgY products after isolation and purification from egg yolks. M: IgY pre-stained protein ladder; (1): IgY product after purified by ammonium sulfate precipitation; (2): IgY after purified by pure water; (3): IgY binding to the column at the 2nd peak of the chromatography process.

at various stages by SDS-PAGE. The SDS-PAGE results are showed in Figure 8, in which the proteins obtained by ion exchange chromatography are the most purified, compared to those only processed with delipidation precipitation and ammonium sulfate precipitation. The sample (2) band in the figure is the protein after being purified with distilled water. Hence it revealed a considerable amount of miscellaneous proteins, as the bands from this sample got boarden and darker than the others. On the other hand, the bands from protein (1) and (3)has clearer edges as they contained more specific proteins that were purified once more with ammonium sulfate 40% precipitation and ion exchange chromatography, respectively. It can be relatively inferred from the figure that the proteins purified with ion exchange chromatography were the purest. With the molecular weight of 70 kDa and 42 kDa, which matched the molecular weight of the heavy chain and the light chain of IgY, respectively, it can be confirmed that the product from the the purification process was IgY.

3.3. Treatment of infected animals by oral administration of anti-urease IgY

Within the first 9 weeks of the experiment, the mice from both groups showed a decrease in appetite and ruffled hair within 3 days after being infected with *H. pylori*. The mice from group 1 (control group) showed constant weight loss after being affected with *H. pylori* until the experiment ended.

The average body weight (BW) of the mice from the control group decreased, being 17.5 grs at the beginning of the experiment, and 17.4 grs at the end of the experiment (with a net weight loss of 1 grs). Meanwhile, the average BW of the mice from group 2 (study group) were 17.3 grs and 19.1 grs at the beginning and the end of experiment, respectively (with a net weight gain of 1.7 grs). No significant difference in the BW of the mice from groups 1 and 2 was observed (p = 0.26); however, the weight gain of the mice from the study group may revealed a positive effect of anti-urease IgY (used as a preventive measure) on the development of *H. pylori*-infected mice.

In terms of the preventive effect of the IgY, The *H. pylori* antibodies in the mice's sera at the end of the experiment (8th week after *H. pylori* inoculation) were recorded and presented in Table 3, based on the ELISA results at the wavelength of 450 nm. The difference is statistically significant (p = 0.002).

The HE stain histopathology results revealed a significant difference between the two groups. The levels of gastritis were evaluated based on the histopathology image and the Updated Sydney System for gastritis assessment, whose results are described in Table 4. The difference is statistically significant (p = 0.004). The two figures below show the microscopic image of the HE staining of two representative mice from the two groups.

In the HE staining result of mouse 1 from

Group	Mice	ELISA	Qualitative result
	1	0.150	+
	2	0.216	+
	3	0.175	+
	4	0.176	+
Group 1	5	0.215	+
	6	0.266	+
	7	0.163	+
	8	0.133	+
	9	0.160	+
Positive prevalence			9/9 (100%)
Means \pm SE		0.18 ± 0.014	
	1	0.095	-
	2	0.147	+
	3	0.166	+
	4	0.127	+
Group 2	5	0.109	-
	6	0.128	+
	7	0.132	+
	8	0.122	+
	9	0.091	-
Positive prevalence			6/9 (66.67%)
Means \pm SE		0.12 ± 0.008	

Table 3. ELISA results at the wavelength of 450 nm.

Table 4.	Hematoxylin	Eosin (HE)	staining results.
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Group	Mice	Level of gastritis
	1	+++
	2	++
	3	+++
	4	+++
Group 1	5	++
_	6	++
	7	+++
	8	++
	9	++
Positive prevalence		9/9 (100%)
Means \pm SE		2.44 ± 0.17
	1	+
	2	+
	3	+
	4	-
Group 2	5	-
Ĩ	6	++
	7	++
	8	+
	9	++
Positive prevalence		7/9 (77.78%)
Means \pm SE		1.11 ± 0.26

Table 5. Giemsa staining results.

Group	Mice	Bacterial density
	1	++
	2	+
	3	+++
	4	++
Group 1	5	+
	6	++
	7	+++
	8	+
	9	++
Positive prevalence		9/9 (100%)
Means \pm SE		1.89 ± 0.26
	1	+
	2	-
	3	+
	4	-
Group 2	5	-
	6	++
	7	+
	8	-
	9	-
Positive prevalence		4/9 (44.44%)
Means \pm SE		0.56 ± 0.24

group 1 (Figure 9(a)), whose level of gastritis was (+++), the epithelial cells lining the ducts were atrophied, flattened or turned into intestinal epithelial cell (dysplasia). The glandular ducts at places were narrowed or collapsed, their cells' nuclei were heavily alkaline-stained. The stromal cells were developing uneven fibrosis. At certain locations, the fibrous stroma cut through the epithelium, creating groups of glandular ducts. At other places they surrounded the ducts. The tissues were densely infiltrated with lympho

cells, plasma cells and macrophages, and lymphoid follicles were even present.

On the other hand, histopathology result of mouse 4 from group 2 (level of gastritis (-)) shows a different image (Figure 9(b)). The gastric epithelium consisted of single glandular ducts that located close to one another. The ducts contained mucus in their lumen. The epithelial cells here were columnar, the cytosol contained numerous mucus granules. Lining the ducts are acidophilic, polyhedral cells lying in between with columnar cells, which were slightly alkalinestained. There were not any inflammatory signs recorded at the stroma tissues of the mucosa as well as the submucosa.

Giemsa stain to detect *H. pylori* also showed significant difference between the two groups. The results are described in the Table 5.

It can be seen from the table that all the mice from group 1 (control group) were positive to the staining method, which means the bacteria were presented in every sample from this group. Meanwhile, the mice which were fed IgY containing food from group 2 (study group) showed a noticeably less bacterial density in the samples, with only 44.44% of them were detected with *H. pylori*. This difference between 2 groups are also statistically significant (p < 0.0001).

Below is the Giemsa stained image of mouse 1 from group 1 (control group). The density of the bacteria was recorded to be less than 5 small colonies on the microscopic field, hence the grading for the bacterial density is Hp(++) (Figure 9(c)).



Figure 9. a) Hematoxylin Eosin (HE) staining result of mouse 1 - group 1 (level of gastritis (+++)); b) Hematoxylin Eosin (HE) staining result of mouse 4 - group 2 (level of gastritis (-)); c) Giemsa staining result of mouse 1 - group 1 ((bacterial density Hp(++)). The arrow indicates the bacteria colony.

4. DISCUSSION

4.1 The IgY producing method

Recently, there have been several studies that demonstrated the processes of isolation and purification of IgY from hens' egg yolks^{14,26,27}. However, to determine which method is suitable for the settings of our study depends on numerous factors, which can be listed as follows: the amount of the antibody produced and its purity, the purpose of the IgY producing process, chemicals used in the process, the settings of the laboratory and the economic application of the IgY products. The protocol developed in this study had the antiurease IgY purified in two consecutive phases, which were precipitation with 40% saturated ammonium sulfate and ion exchange. From the result of this study, we found this protocol could optimise the purification result from the beginning supernatant, compared to purification with only ammonium sulfate or delipidation with only distilled water. Based on the purification result from the chromatogram, the IgY purification process in this protocol also largely depends on the ammonium sulfate precipitation, as both the yield and purification varied greatly when the supernatant was precipitated. The final step of ion exchange also contributed significantly to the enhancement of the purification of the final IgY production. Based on the minimal steps and considerably low cost, the protocol from our study is suitable for large-scale IgY manufacturing settings so as to produce enough IgY for prophylactic treatment for *H. pylori* infection in patients. Compared to the antibodies isolated from mammals' blood, the IgY that we harvested from the hens' eggs was much higher in terms of quantity. Specifically, the estimated amount of IgY that can be produced from 1 egg fluctuates from 50 to 100 mg. One hen is capable of laying 5-7 eggs per week in average, which means around 40,000 mg of IgY per year can be extracted from the eggs. Meanwhile, one blood sample from the mammals (horses, sheep, rabbits) can provide approximately 200 mg of antibodies, but it takes almost 2-3 months to take one blood sample from the mammals. Hence, we can only obtain around 1,400 mg of antibodies per year from mammals' blood samples. Moreover, the antibodies produced from the hens' eggs do not create aggregation reactions with protein A and protein G, do not interact with rheumatoid factor and also do not trigger complement activation in mammals; while the antibodies from the mammals' blood samples are positive to all of the mentioned reactions^{28,29}. With the mentioned features, our study has pointed out that this is a potential, yet simple method to apply into large-scale separation as it can produce a high amount of low-cost antibodies that can be used in inhibitory treatment of H. pylori infections.

4.2. The inhibitory effect of IgY on model animals

Through our observation and the results of this study, it can be proved that the IgY prepared from the egg yolk of hens immunized with *H. pylori* is effective in the treatment of *H. pylori* infections. Regarding the preventive effect of anti-urease IgY, our findings were in accordance with the results published by Nomura et al.¹³ All the mice from group 2 (study group) in our study displayed a reduced activity of anti-*H. pylori* antibody, yet a complete eradication of the bacteria from gastric mucosa in the *H. pylori*-infected mice was not fully observed. Furthermore, 55.6% of the mice from group 2 got a complete bacterial eradication (HP testing results were negative), which was lower compared to that in Nomura et al.'s study $(100\%)^{13}$. Moreover, among the *H. pylori* (-) mice in our study, 40% were free of gastritis, which revealed that anti-urease IgY might help eradicate the bacteria. These results suggest that the preventive effect of the IgY-containing food helped protect the mice's gastric mucosa from the invasion of *H. pylori*; in other words, the IgY worked by eradicating the cause of gastritis. The preventive effect of anti-urease IgY was confirmed by the significant reduction of H. pylori bacterial density on the gastric mucosa, based on histological findings of the Giemsa stained gastric mucosal sections (p < 0.0001). Furthermore, the severity of gastritis in the mice from group 2 was proportionally decreased along with the decrease of H. pylori density on the gastric mucosa (p = 0.004).

It was proved by our observation in this study that the mice fed the food containing antiurease IgY (both from the control group and the *H. pylori*-infected group) had better weight gain compared to the others. This weight gain was probably due to the fact that IgY, by having a protein's nature, had provided more nutrition which helped the mice developed better. Incidentally, IgY is a protein originated from hen's egg, and in this study, it showed no immunogenicity when orally administered to the mice, manifested by causing no observable adverse effects on the animals. Generally, these findings may proved the non-toxic property of the antibody suspension containing IgY.

In general, the *H. pylori* infection rate of the mice from group 1 (control group) was higher compared to group 2 (study group). The activity of anti-*H. pylori* antibody and the severity of gastritis in the mice from this group, which is displayed via the ELISA and histopathology results, were also higher than those from the other group as well. This observation suggested that antiurease IgY had contributed to the improvement of *H. pylori*-induced gastritis as well as the decrease of urease activity at the gastric mucosa by inhibiting *H. pylori*'s urease, and thus, eliminate the survival factor for *H. pylori*, leading to a reduction of gastric mucosa's damages and making the bacteria easier to be removed.

However, in terms of the producing method of IgY, our study did not examine the *in vitro* hydrolysis color reaction between the IgY and and urease. Although we conducted the ELISA test to confirm that the anti-urease IgY can specifically attach to the urease molecules, the binding site of the antibodies on the antigens may affect the enzymatic activity of the latter, especially in larger antigens. In other words,

In further studies, the safety and toxicity characteristics of the produced IgY should be evaluated. After the toxicity and safety characteristic have been proved, we will have the foundation to continue and boarden our study and see if the IgY has a positive effect on inhibiting H. pylori colonization on human. Recently, other studies on IgY's inhibitory effect on other infections in human are conducted. IgY and its effects have recently been studied on various human infections. Nilsson et al. applied a treating combination with Azithromycin and IgY on Pseudomonas aeruginosa infections on 17 patients with cystic fibrosis up to 12 years³⁰. The culture results revealed that only 29 cultures tested positive for P. aeruginosa in the anti-biotic treated group (p = 0.028). In the IgY group (n = 17), there were only two siblings were chronically colonized with P. aeruginosa, compared to 7 patients in the control group (n = 23). From the results, IgY may add a complementary effect to antibiotic treatment in treating cystic fibrosis, which means that it can be potentially used for prophylactic treatment or combining with other antibiotics to improve the patients' prognosis.

5. CONCLUSION

In conclusion, the promising results of this study indicated that the methods of producing egg-yolk derived IgY with precipitation and exchange chromatography can be applied in large-scale production. The oral administration of IgY obtained from eggs laid by hens can provide a prophylactic approach in the management of *H. pylori* infections and *H. pylori*-associated diseases. Further studies on clinical application on infected patients are suggested.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to this research and publication.

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Ethics approval

The study was approved by the Research Committee of Can Tho University of Medicine and Pharmacy on April 1, 2017.

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