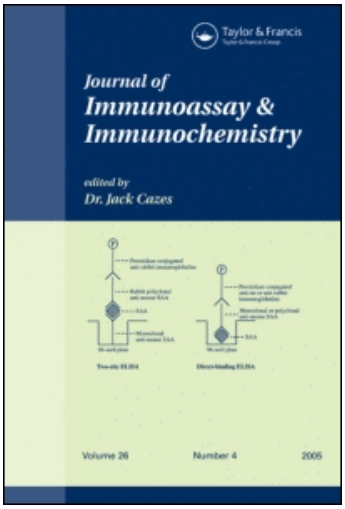

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Abstract: Consecutive triple doses of 1×10^8 CFU/mL of a pathogenic *H. pylori* strain isolated from stomach of Egyptian patients with severe gastritis were used to establish infection in BALB/c mice model. White Leghorn hens were immunized with *H. pylori* whole cell lysate (HpLysate) antigen and with a highly reactive 58-kDa *H. pylori* (Hp58) antigen. Two months later, IgY antibodies (IgY-HpLysate & IgY-Hp58) were purified from egg yolk and its efficacy was evaluated in the adopted model. Microbiological culture and immunohistochemical staining revealed that *H. pylori* infection was inhibited 1 week after oral passive immunization in 70% of infected BALB/c mice with a significant decrease ($p < 0.05$) in the degrees of gastritis. In conclusion, we have adapted BALB/c mice model for human *H. pylori* pathogenic strain and oral passive immunization with specific IgY antibodies to the 58-kDa antigen inhibited active *H. pylori* infection and decreased gastritis.

Keywords: 58-kDa antigen, BALB/c mice, *H. pylori*, IgY antibodies, Passive immunization

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INTRODUCTION

Helicobacter pylori, the most common cause of gastritis and gastric ulcers plays a pivotal role in the development of gastric carcinomas.[1–3] There have been tremendous efforts to evaluate numerous therapeutic regimens for eradication of *H. pylori* infections. Successful treatment of *H. pylori* infections most often employs antibiotic therapies.[4,5] However, antimicrobial therapeutic cures of *H. pylori* infections do not lead to immunity from reinfection.[6] Moreover, the large-scale use of antibiotics leads to the emergence of antibiotic-resistant strains,[7,8] which increase failure-of-therapy and relapse rates.[9]

Consequently, it is important to seek new therapies for a wider means of preventing *H. pylori* infection without drug resistance problems.[10] Furthermore, a stable and reliable *H. pylori* infection animal model would be necessary for evaluating vaccine efficacy and helpful for understanding the pathological mechanism of the organism.[11] Oral administration of specific antibodies is an attractive approach to establish protective immunity against gastrointestinal pathogens in humans and animals.[12] Chicken egg yolk was recognized as an inexpensive and alternative source for production of specific immunoglobulin Y (IgY).[13] The usefulness of egg yolk IgY antibodies has been assessed for therapeutic application by passive immunization therapy through oral ingestion of IgY, as in fortified food products for prevention of some intestinal infections, such as those caused by enterotoxigenic *Escherichia coli*,[14] *Salmonella typhimurium*,[15] and rotavirus.[16] Recently, we have identified a highly reactive and specific 58-kDa antigen in whole cell lysate antigenic extract of *H. pylori* and evaluateed its diagnostic potential.[17] The present work aimed to establish a stable and reliable model of *H. pylori* infection in BALB/c mice using a human pathogenic strain of *H. pylori* to produce specific IgY antibodies from egg yolk of hens immunized with *H. pylori* antigens and to evaluate its efficacy in passive immunization therapy for inhibition of *H. pylori* infection and gastritis.

EXPERIMENTAL

H. pylori Pathogenic Strain

A pathogenic strain of *H. pylori* was isolated from stomach of selected Egyptian patients with pathologically confirmed severe gastritis (all males, aged 35–52 yr). Ground biopsy specimens were inoculated onto both selective (Pylori agar; bioMérieux) and nonselective (Chocolate agar supplemented with IsoVitaleX; bioMérieux) media within 24 h. The cultures

were incubated at 37°C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2 and 99% relative humidity) provided by a CO_2 incubator (Heraeus Instruments, Germany) and checked for growth at days 5 and 7.[18] The microorganism was identified as *H. pylori* using the standard methods on the basis of colony morphology, gram stain, and the production of urease, catalse, and oxidase enzymes[19] and further confirmed by the Western blot using rabbit antiserum against whole cell lysate of *H. pylori*.[17] The human pathogenic *H. pylori* strain was adapted in a group of BALB/c female mice (6 week-old and weighing ~20 g) for 2 weeks. Mice were given 0.25 mL of 0.2 M $NaHCO_3$ to neutralize acidity, then the bacterial inoculum was administered as a single dose of 1×10^8 CFU/mL in 0.15 mL of sterile saline by gastric intubation with a blunt needle attached to a syringe under light anesthesia with halothane (Halocarbon, River Edge, NJ, USA). The bacteria was then isolated from stomach of mice and grown again onto both selective and nonselective media.

Adaptation of BALB/c Mice for *H. pylori* Infection

For BALB/c mice adaptation to colonize experimentally inoculated *H. pylori* and develop severe gastritis that mimic human gastritis, several dose regimens of mouse-adopted *H. pylori* strain were evaluated. Groups of mice (n = 10 mice/group, each ~20 g) were administered single, double, and triple oral doses each dose of 1×10^8 CFU/mL mouse-adapted *H. pylori* strain per day. The double doses were administered as alternative doses at 1 and 3 days and the triple oral doses were administered as alternative doses at 1, 3, and 5 days. Age and weight matched groups of non-infected mice were used as controls. Mice were not allowed to eat for 24 hr before infection as well as before killing 2 weeks, 4 weeks and 8 weeks after the last dose of infection. The stomach was dissected along the grater curvature and divided into three strips for *H. pylori* culture (described before), histopathology, and immunohistochemical analyses. Longitudinal segments including the antrum and corpus plus a piece of attached intestine were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 4 μm.

Histopathological Examination

The antral portion of stomach of BALB/c mice was quickly removed and used for histological examinations. Formalin-fixed tissue was processed routinely in paraffin and stained with hematoxylin and eosin (H & E)

for examination by light microscopy. For evaluation of gastritis, the H & E-stained sections were scored based on the degree of infilterating lymphocytes, plasma cells, and neutrophils. Gastric mucosal injury was classified and scored on a scale of 0, 1, 2, or 3, according to the updated Sydney system.[20]

Indirect Immunoperoxidase Staining

Four-μm paraffin section of the tissue was deparaffinized and re-hydrated through descending grades of alcohols and water. After washing, 4–6 drops of H_2O_2 were applied. Then normal goat serum diluted (1:5) with 1–4% bovin serum albumin (BSA) in tris-buffered saline (TBS, pH 7.2) was applied and incubated for 60 min. Excess liquid was wiped away and specific IgG rabbit serum to HpLysate or to the 58-kDa *H. pylori* antigen[17] was then applied an incubated for 1 hr. After washing, horseradish peroxidase conjugated goat antibody to rabbit immunoglobulins (Sigma, CA, USA) diluted 1:500 in 1.5% BSA/TBS and incubated for 60 min. After stopping the reaction, the tissue section was counter stained with Mayer's hematoxylin for 2 min. Slides were examined under light microscope at powers of X100, X400, and X1000 for the immunoreactivity.

Preparation of *H. pylori* Whole Cell Lysate Antigen

Bacterial cells were harvested, washed 3 times in phosphate buffered saline (PBS, pH 7.2), and disrupted by sonication for 3 times at 4°C, 15 sec each at 47 kHz using Bransonic ultra cleaner (B-1200 E-1, Branson Ultrasonic Corporation, Danbury, Connecticut, USA). Cellular material was removed by centrifugation at 12,000 rpm for 10 min at 4°C, and the supernatant (*H. pylori* whole cell lysate; HpLysate) was collected. The protein content was determined and aliquots were frozen at −70°C until used. The reactivity of HpLysate antigen was confirmed using western blot based on specific IgG anti-sera developed in White New Zealand rabbits weighing ~3 kg as previously described.[17]

Purification of the 58-kDa *H. pylori* Antigen

The target antigen (Hp58) was cut and electroeluted from polyacrylamide gels at 200 volts for 3 h at 4°C in a dialysis bag (Sigma) according to Attallah et al.[17] The protein content of a sample of the antigen was determined before the remainder was stored at −20°C.

Immunization of Hens with *H. pylori* Lysate and Purified 58-kDa Antigen

White Leghorn hens (25 weeks-old strain Hyline) were kept in conventional facility and immunized intramuscularly with 200 μg/mL of HpLysate (group 1, n = 8) and with purified Hp58 antigen (group 2, n = 8) using an equal volume of Freund's complete adjuvant (Difco Laboratories). Another group of non-immunized hens (group 3, n = 5) was used as a control group. Each hen was injected at four different sites (250 μL per site) of the leg muscle. Three booster injections, with Freund's incomplete adjuvant were given at 2-weeks intervals following the first injection. One month later, eggs were collected daily for 1 month and stored at 4°C.

Isolation and Purification of IgY Antibodies

Isolation of IgY-HpLysate and IgY-Hp58 antibodies from immunized hens and normal IgY antibodies (IgY-N) from non-immunized hens was carried out according to the method described by Akita and Nakai.[21] In brief, egg yolk was separated from the white, and mixed with an equal volume of distilled water for 30 min, followed by the addition of 0.15% (w/v) λ-Carrageenan (Wako Pure Chemical Laboratory, Osaka, Japan). After centrifugation at 10,000-x *g* for 30 min at 20°C, the water-soluble fraction was collected and filtered through a Whatmann filter paper (No. 1) to remove solid lipid materials. The resulting IgY-containing filtrate was further purified by salt precipitation using 19% (w/v) sodium sulfate. The isolated water protein fraction of immunoglobulin converted to powder by using Savant AES 1010 automatic environmental SpeedVac with VaporNet (Ramsey, Minnesota, USA). The dried antibodies were stored in a desiccator at room temperature until use.

Reactivity of Hen IgY Antibodies Using ELISA

After optimization of reaction condition, polystyrene microtiter ELISA plates (Costar, Corning Life Sciences, Acton, MA) were coated with 2.5 μg/mL of HpLysate or 1.25 μg/mL of purified Hp58 antigen overnight. The plates were then blocked using 0.5% (w/v) non-fat milk dissolved in coating buffer, pH 9.6 for 60 min. The plates were washed three times with PBS-T20. After washing, plate incubated with different isolated IgY antibodies diluted 1: 200 in the PBS-T20 with constant shaking for 2 hr. After washing, the plates were incubated at 37°C for 1 hr with diluted goat anti-chicken IgG alkaline phosphatase conjugate (Sigma). The plates were then washed and the reaction was visualized by addition of para-Nitrophenyl phosphate substrate (Sigma) and the

absorbance was read at 405 nm using using $\sum$ 960 microplate autoreader (Metreiteck, Axiom, Burstadt, Germany).

SDS-PAGE and the Western Blot

The SDS-PAGE resolved whole cell lysate antigen of *H. pylori* was electrotransferred onto the nitrocellulose filters (0.45 μm pore size, Sigma) in protein transfer (BioRad Laboratories, CA, USA) according to the method of Towbin et al.[22] The nitrocellulose filter was blocked using 5% (w/v) non-fat milk dissolved in 0.05 M TBS, containing 0.15 M NaCl, pH 7.4 for 30 min. The blots were washed three times (15 min each) in TBS, pH 7.4. After washing, NC incubated with isolated IgY antibodies diluted in the blocking buffer with constant shaking overnight. After washing, the blots were incubated at room temperature for 2 hr with diluted goat anti-chicken IgG alkaline phosphatase conjugate (Sigma). The blots were then washed and the reaction was visualized by soaking in premixed BCIP/NBT alkaline phosphatase substrate (ABC Diagnostics, New Damietta City, Egypt).

Passive Immunization of BALB/c Female Mice Against *H. pylori* Infection

Groups (10 mice per group) of the adapted female BALB/c mice (6 week-old and weighing ~20 g) were randomly divided into: Treated Groups (1); each infected mouse passively immunized with 0.7 g of IgY-HpLysate powder reconstituted in 0.5 mL PBS, Treated Groups (2); each infected mouse passively immunized with 0.7 g of IgY-Hp58 powder reconstituted in 0.5 mL PBS, and Control Groups; either untreated infected mice or infected mice passively immunized with 0.7 g of IgY-N powder reconstituted in 0.5 mL PBS. The IgY-HpLysate antibodies were administered 1 day after infection and the IgY-Hp58 antibodies were administered 1 day, 1 week, 4 weeks, or 12 weeks after infection using an oral feeding needle. Two months later, the *H. pylori* infection was assessed by culture, immunohistochemical staining, and histopathological examination as described above. Mice were not allowed to eat for 24 h before each infection as well as before killing.

Statistical Analysis

Data were expressed as mean ±SD and were analyzed by using the statistical analysis program package Instate Software for Science,

version 2.3 (Graphpad Software, Inc., San Diego, CA). P values <0.05 were considered significant.

RESULTS

Adaptation of *H. pylori* Infection in BALB/c Mice and Assessment of Gastric Colonization Using Culture, Immunohistochemical Staining and Histopathology

The triple oral dose regimen of *H. pylori* infection showed the highest rate of positivity in culture and immunohistochemical staining of gastric tissues of BALB/c mice at all intervals and the maximum rate was obtained after 8 weeks of infection, (Table 1). Immunohistochemical staining revealed a significant difference between infected mice and controls.

Table 1. Assessment of gastric colonization from oral infection with different doses of mouse-adapted *H. pylori* strain

Time post infection	Oral dose regimen*	% Detection (infected mice/total)	
		Microbiological culture**	IHC staining***
Two weeks			
	Single	20% (2/10)	40% (4/10)
	Double	30% (3/10)	40% (4/10)
	Triple	50% (5/10)	50% (5/10)
	Control	0 (0/10)	0 (0/10)
Four weeks			
	Single	40% (4/10)	60% (6/10)
	Double	40% (4/10)	70% (7/10)
	Triple	60% (6/10)	80% (8/10)
	Control	0 (0/10)	0 (0/10)
Eight weeks			
	Single	60% (6/10)	70% (7/10)
	Double	80% (8/10)	80% (8/10)
	Triple	90% (9/10)	90% (9/10)
	Control	0 (0/10)	0 (0/10)

*Mice (10 per group) received either single, double or triple oral doses each of 1×10^8 CFU/mL mouse-adopted *H. pylori* strain and killed 2 or 4 or 8 weeks after oral infection. Control were non-infected mice.

**All mice showing positive results for microbiological culture were positive for *H. pylori* antigen detection in stomach tissues using immunohistochemical staining.

***Immunohistochemical staining using specific antibodies to HpLysate.

Table 2. Histopathological findings in gastric mucosa from BALB/c mice 8 weeks after oral infection with different doses of mouse-adapted *H. pylori* strain

Oral dose regimen*	No. of mice	Severity of gastritis**							
		0		+		++		+++	
		No.	%	No.	%	No.	%	No.	%
1×10^8 CFU	10	4	40	6	60	0	0	0	0
2×10^8 CFU	10	3	30	6	60	1	10	0	0
3×10^8 CFU	10	1	10	3	30	4	40	2	20
Controls	10	10	100	0	0	0	0	0	0

*Mice (10 per group) received either single, double or triple oral doses each of 1×10^8 CFU/mL mouse-adopted H. pylori strain as described in details under experimental section and killed 8 weeks after oral infection. Controls were non-infected mice.

**0 (no gastritis); + (mild gastritis); ++ (moderate gastritis) and +++ (severe gastritis).

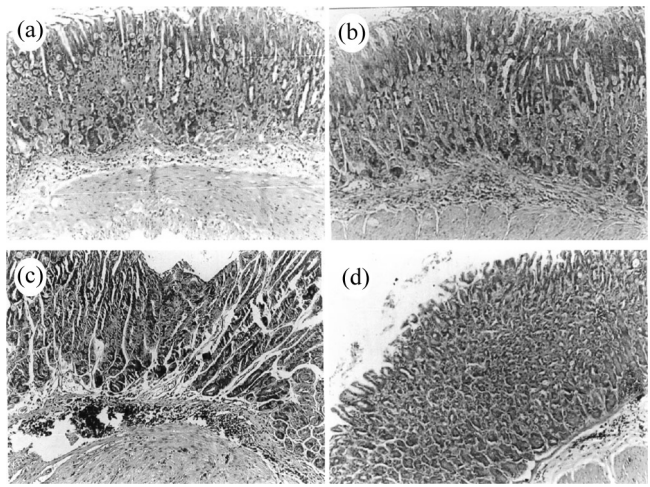

Figure 1. Histopathological examination of gastric mucosa of *H. pylor*i infected mice with different dose regimen of infection in comparison with the non infected mice. (a) Little sub-mucosal oedema with scattered inflammatory cells and congested dilated vessels within the basal pits of fundic mucosa using single dose (H&E, X400). (b) Scattered acute and chronic inflammatory cellular infiltrate on the sub-mucosa with congested capillaries at the base of the crypts of fundic mucosa using double doses (H&E, X400). (c) Dense round cells infiltrate in the sub-mucosa with oedema, congested vessels and scattered inflammatory cells in the crypts of fundic mucosa using triple doses (H&E, X400). (d) Gastric mucosa of non-infected mice showing small inflammatory foci at the base (H&E, X400).

H. pylori antigens were observed at three sites: on the surface of gastric mucosa, the upper and lower parts of the gastric pits and inside the gastric cells. On the other hand, non-infected mice as controls, give negative culture and negative immunohistochemical staining (data not shown). Regarding histopathology, the inflammatory changes were restricted to the fundic mucosa, had patchy distribution, and were concentrated in the sub-glandular portion of mucosa. The degrees of gastritis between 3 methods were statistically significant ($p < 0.05$). Mice infected with single dose of infection showing mild inflammatory changes (+) in 60% while 40% were free from gastritis. The severity of inflammation had increased in mice received 2 doses, where 10% had moderate (++) gastritis. However, 90% of mice infected with triple oral dose of infection had severe inflammatory changes, (Table 2). Numerous foci of chronic inflammatory cells were present in the sub-glandular area with extension into lamina properia and the underlying sub-mucosa using single dose of *H. pylori* (Figure 1a) in comparison with gastric mucosa of non infected mice, Figure 1d. The area of gastritis was characterized by dilated vessels

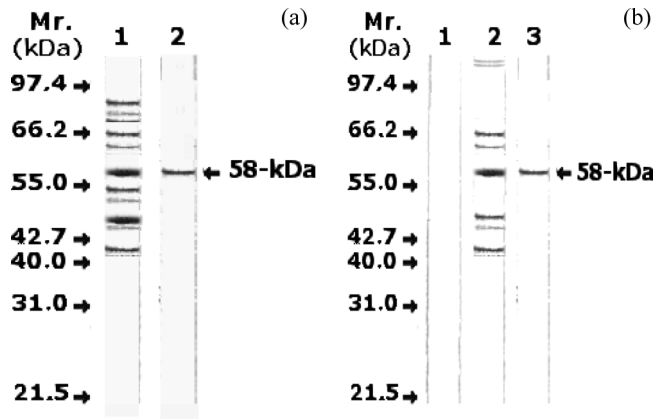

Figure 2. (a) SDS-PAGE analysis of the 58-kDa purified antigen. Lane 1: whole cell lysate of *H. pylori* and Lane 2: the 58-kDa purified antigen. Samples were resolved on 12% polyacrylamide gel and Coomassie blue stained. (b) Western blot analysis of *H. pylori* whole cell lysate towards the developed egg yolk IgY antibodies. Lane 1: immunostained with egg yolk IgY antibody obtained from non-immunized hen; lane 2: immunostained with egg yolk IgY antibody (IgY-HpLysate) obtained from hens immunized with whole cell lysate; lane 3: immunostained with egg yolk IgY antibody (IgY-Hp58) obtained from hens immunized with 58-kDa purified antigen. Molecular weight markers (not shown but indicated by arrows) include Phosphorylase B (97.4-kDa), Bovine serum albumin (66.2-kDa), Glutamate dehydrogenase (55.0-kDa), Ovalbumin (42.7-kDa), Aldolase (40-kDa), Carbonic anhydrase (31-kDa), Soybean trypsin inhibitor (21.5-kDa).

congestion and sub-mucosal oedema using double doses of *H. pylori*, Figure 1b. The mucosa showed relative thinning and atrophy at the central pit of with lymphoid follicle formation at the sub-mucosa and excess mucous secretion of surface crypts using triple doses of *H. pylori*, Figure 1c.

Development of Egg Yolk IgY Antibodies and Its Reactivity by ELISA and Western Blotting

The whole cell lysate of *H. pylori* (HpLysate) and the 58-kDa antigen purified from the HpLysate using gel electro-elution (Figure 2a) were used to develop egg yolk IgY antibodies in chicken. Several reactive bands were identified by IgY-HpLysate antibody; however, the IgY-Hp58 antibody identified the 58-kDa immunoreactive band in whole cell lysate using the western blot. No reactive band was identified in whole cell lysate by egg yolk antibodies from Non-immunized hens (IgY-N), Figure 2b. The developed IgY-HpLysate and IgY-Hp58 antibodies from immunized hens showed significant high ELISA reactivity ($p < 0.001$) towards the purified 58-kDa antigen and the whole cell lysate of *H. pylori* in comparison with normal IgY antibodies (IgY-N) from non-immunized hens using ELISA, Figure 3.

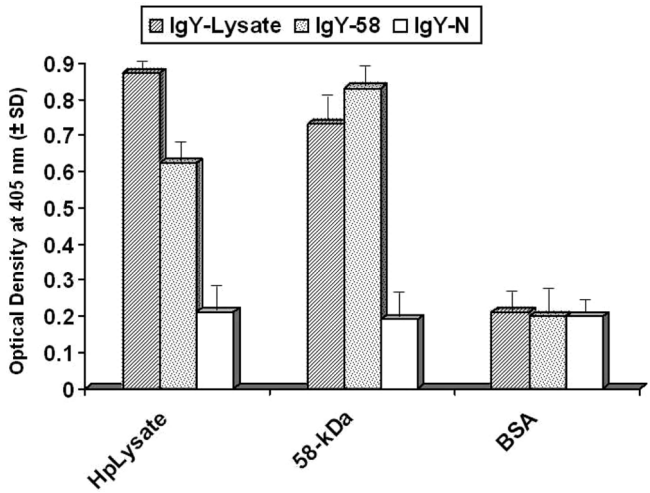

Figure 3. Reactivity of the developed IgY-HpLysate and IgY-Hp58 antibodies from immunized hens using ELISA. The developed IgY-HpLysate and IgY-Hp58 antibodies showed high reactivity ($p < 0.001$) towards the purified 58-kDa antigen and the whole cell lysate of *H. pylori* (HpLysate) in comparison with normal IgY antibodies (IgY-N) from non-immunized hens. All antibodies showed no reactivity towards BSA; non specific antigen.

Assessment of *H. pylori* Infection After Passive Immunization with the Generated Egg Yolk IgY Antibodies to Whole Cell Lysate

The percentage of *H. pylori* reduction (~67%) in mice passively immunized with the IgY-HpLysate antibodies were significantly ($p < 0.05$) higher as assayed by microbiological culture than that of control mice passively immunized with the IgY-N antibodies after one day of infection. Also, immunohistochemical staining revealed a significant difference ($p < 0.05$) between mice passively immunized with the IgY-HpLysate antibodies and controls. The severity of inflammation was lower in mice passively immunized with the IgY-HpLysate antibodies after one day of *H. pylori* infection. Moreover, the degree of gastritis in passively immunized mice was significantly ($p < 0.05$) lower than the control. On the other hand, there was no significant difference in the severity of gastritis between mice passively immunized with IgY-N and untreated infected mice as controls.

Table 3. Assessment of *H. pylori* infection in mice passively immunized with IgY-Hp58 antibodies at different times after oral infection with *H. pylori*

Passive immunization after infection	Groups*	Cure (cured or non-infected mice/total)	
		Microbiological culture (%)	IHC staining**
1 day			
	Treated	50%*** (10/20)	50%*** (10/20)
	Control	0 (0/10)	0 (0/10)
1 week			
	Treated	70%*** (14/20)	70%*** (14/20)
	Control	0 (0/10)	0 (0/10)
4 weeks			
	Treated	50%*** (10/20)	50%*** (10/20)
	Control	20 (2/10)	0 (0/10)
12 weeks			
	Treated	50%*** (5/10)	40%*** (4/10)
	Control	10 (1/10)	0 (0/10)

*Control: mice passively immunized with IgY-N at 1 day, 1 week, 4 weeks and 12 weeks after infection with 3×10^8 CFU/mL; Treated: mice passively immunized with IgY-Hp58 1 day, 1 week, 4 weeks and 12 weeks after infection with 3×10^8 CFU/mL. All were killed 8 weeks after passive immunization.

**Immunohistochemical staining using specific antibodies to the 58-kDa antigen.

***Significantly different from the control mice ($p < 0.05$).

Assessment of *H. pylori* Infection After Passive Immunization with the Generated Egg Yolk IgY Antibodies to the 58-kDa Antigen

Culture

The percentages of *H. pylori* reduction in mice passively immunized with the the IgY-Hp58 antibodies were significantly ($p < 0.05$) higher as assayed by *H. pylori* culture than that of control mice passively immunized with the IgY-N in all experiments. However, the highest effect of passive immunization was achieved in *H. pylori* infected mice that orally administered the IgY-Hp58 after 1 week with higher recovery rate, (Table 3). No significant difference was shown between percentages of *H. pylori* reduction in mice passively immunized with IgY-N and the untreated mice as controls (data not shown).

Immunohistochemical Staining

Immunostaining revealed a significant difference ($p < 0.05$) only between mice passively immunized with the IgY-Hp58 and controls, (Table 3).

Table 4. Histopathological findings in *H. pylori* infected mice after different intervals of passive immunization (PI) with IgY-Hp58

Time of PI after infection	Groups*	Severity of gastritis			
		0% (No./T.)	+% (No./T.)	++% (No./T.)	+++% (No./T.)
1 day					
	Treated	50% (5/10)	30% (3/10)	20% (2/10)	0 (0/10)
	Control	22% (2/9)	22% (2/9)	44% (4/9)	12% (1/9)
1 week					
	Treated	70%** (7/10)	0 (0/10)	30% (3/10)	0 (0/10)
	Control	20% (2/10)	0 (0/10)	50% (5/10)	30% (3/10)
4 weeks					
	Treated	60%** (6/10)	10% (1/10)	20% (2/10)	10% (1/10)
	Control	0 (0/10)	60% (6/10)	20% (2/10)	20% (2/10)
12 weeks					
	Treated	40%** (4/10)	20% (2/10)	10% (1/10)	30% (3/10)
	Control	0 (0/10)	0 (0/10)	20% (2/10)	80% (8/10)

*Treated: mice passively immunized with IgY-Hp58 1 day, 1 week, 4 weeks and 12 weeks after infection with 3×10^8 CFU/mL; Control: mice passively immunized with IgY-N at 1 day, 1 week, 4 weeks and 12 weeks after infection with 3×10^8 CFU/mL. All mice were killed 8 weeks after passive immunization.

**Significantly different from the control mice ($p < 0.001$).

Histopathological Findings

The untreated *H. pylori* infected mice revealed severe inflammatory reaction due to long period of infection. However, the severity of inflammation was lower in mice passively immunized with the IgY-Hp58 after one day of *H. pylori* infection. Moreover, the degree of gastritis in mice passively immunized after 1 week, 4 weeks, or 12 weeks from infection was significantly ($p < 0.001$) lower than the control, (Table 4). On the other hand, there was no significant difference in the severity of gastritis between infected mice passively immunized with IgY-N and untreated infected mice as controls (data not shown).

DISCUSSION

Tremendous efforts have been made to identify alternatives to antibiotic-based therapies in the form of more broadly based means of suppressing *H. pylori* infection.[23] In this regard, oral administration of active antibodies specific to *H. pylori* may be advantageous in that recognition of *H. pylori* by the antibody, which would efficiently inhibit adhesion of the bacterium to human epithelial cells.[24] However, a stable and reliable *H. pylori* infection animal model would be necessary for evaluating vaccine efficacy and helpful for understanding the pathological mechanism of the organism.[11] Experimental animals used for studying *H. pylori* infection have included monkeys, dogs, piglets, domestic cats, and rodents.[25,26] However, these models are not optimal, because it cannot be handled with ease or in large numbers, or infection rates are rather low. Alternatively, animal models using small rodents such as mouse that mimic human disease have developed with promising success.[27,28] Fox et al.[29] demonstrated that the stomach of rat at 8 weeks post infection had the most severe inflammation than after 2 and 4 weeks. Wang et al.[30] followed *H. pylori* infection by 3 different isolates in C57BL/6 and BALB/c mice for 23 months. Marchetti et al.[31] reported that bacteria passages in vivo are more efficient than clinical isolates in establishing a detectable infection, possibly because selection of good colonizers occurs in vivo. Moreover, the ability of *H. pylori* to colonize was enhanced by serial dosage of *H. pylori* in mice.[32–34] On the other hand, fasting of animals was recommended before infection by several investigators.[31,33,35] In the present study, BALB/c mice infected with a human pathogenic strain of *H. pylori* as experimental model was established when animals were challenged on three alternative days. Microbiological culture and immunohistochemical staining showed that high colonization density could be due to multiple time of infection, passage of bacteria from mice to another mice and fasting of animals before each inoculation.

In addition, it has been found that the severity of gastritis was much higher using 3×10^9 CFU/mL doses than other low doses ($p < 0.05$) and increases with time. These results are consistent with the work of Lee et al.[27] who showed that severity of gastritis in mice was increased gradually from two weeks post-infection until reach maximum at 8 weeks after infection. Our results bring the direct evidence that BALB/c mice infected by 3×10^9 CFU/mL for multiple times represent a good model for *H. pylori* infection and induction of gastritis. So, the human strain-infected BALB/c mouse seems to be a suitable animal model for *H. pylori*-related research. The usefulness of specific egg yolk immunoglobulin Y (IgY) has been assessed for therapeutic application by passive immunization therapy through oral ingestion of IgY for prevention of *H. pylori* infection.[35,36] Furthermore, fortification of food products with this immunoglobulin would significantly decrease the *H. pylori* infection.[13] In the present study, we preliminary investigated the efficacy of egg yolk antibodies from hens immunized by pathogenic *H. pylori* whole cell lysate (IgY-HpLysate) after one day of infection of adapted BALB/c mice model. Our data based on *H. pylori* culture and immunostaining suggested that IgY-HpLysat antibodies could inhibit *H. pylori* infection in ~67% infected BALB/c mice one day after infection. The severity of inflammation was lower in mice passively immunized with IgY-Hp58 after one day of *H. pylori* infection. Moreover, the degree of gastritis in mice passively immunized after 1 day from infection was significantly lower than the control. Although anti-*H. pylori* whole-cell lysate IgY was strongly reactive to *H. pylori* proteins, a cross-reactivity exists between anti-*H. pylori* whole-cell lysate IgY and other bacteria in the human gastrointestinal tract which could decrease the efficacy of IgY-Hp[36] Therefore, selective antigens with high immunocompetence from *H. pylori* proteins are needed to produce a more specific IgY and also reduce side-effects.[37] Targeting of proteins in the gastric lumen using oral antibody requires that the target antigen of *H. pylori* be located on the surface of the bacterial body. The immunodominant proteins recognized by IgY against *H. pylori* whole-cell lysate were reportedly related to the urease, heat shock protein 60, peroxiredoxin and thiol peroxidase of *H. pylori*.[23,36,38] Recently, we have identified a 58-kDa *H. pylori* antigen in sera of infected patients,[19] a protein that probably represent a fragment of the cytotoxin domain of VacA protein.[39] Fortunately, the developed IgY-HpLysate identified the 58-kDa antigen in whole cell lysate of pathogenic *H. pylori* using the western blot. Consequently, it was applied as an antigen for hen immunization and the developed IgY antibodies (IgY-Hp58) applied as an oral passive immunization for *H. pylori*. Based on our preliminary encouraging data using IgY-HpLysate, several passive immunization experiments were done to investigate the efficacy of isolated egg yolk antibodies to a purified

58-kDa *H. pylori* antigen (IgY-Hp58) against challenge exposure of the adopted BALB/c mice to *H. pylori* infection. Our results suggested that egg yolk antibodies from hens immunized by the 58-kDa *H. pylori* antigen inhibited *H. pylori* infection in mice. *H. pylori* culture demonstrated that the percent of reduction in mice passively immunized after 1 day, 1 week, 4 weeks, or 12 weeks with IgY-HpLysate was much higher than infected untreated control group. Specific IgY-Hp58 antibodies eradicated *H. pylori* infection in 70% of infected mice in comparison with infected untreated control groups ($p < 0.05$) as assayed by culture technique and the immunohistochemical staining. The highest passive immunization was achieved in *H. pylori* infected mice that orally administered IgY-Hp58 after 1 week of infection. The difference between culture and immunostaining results may be attributed to the difference in specificity and sensitivity of both techniques.[40,41] In addition, a very significant difference, ($p < 0.05$) was found in the degree of gastritis in mice passively immunized with IgY-Hp58 after one week of infection and the infected untreated control group. Wang et al.[42] reported that the degree of gastritis could be used as an indicator of *H. pylori* density. In addition, the severity of gastritis in therapeutically immunized mice was lower than control in spite of presence of *H. pylori*. These results are most due to efficacy of these developed antibodies against virulence factors of *H. pylori*, which cause severe inflammatory reaction in the stomach. *H. pylori* could burrow into the intracellular spaces that could explain the difficulty in complete eradication of this organism with active surface agents.[43] Moreover, this bacterium has many effects on the host immune system, and that the net result is an ineffective immune response that fails to eradicate the organism.[44] However, our results confirmed many studies have shown that egg yolk from an immunized hen has an antibody capable of specific recognition in an abundant quantity, provide a novel alternative approach to treatment of *H. pylori* infection and is therefore economical.[35,45,46] In conclusion, the generated egg yolk antibodies from hens immunized by the 58-kDa *H. pylori* antigen inhibited *H. pylori* infection in mice and may advantageous to the management of *H. pylori* infection. Further studies to evaluate the effect of anti-Hp58 IgY on *H. pylori* infection in *H. pylori* positive volunteers as well as in gastritis patients will be performed.

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