Original Article

Study of Biofilm Formation in C57BL/6J Mice by Clinical Isolates of *Helicobacter pylori*

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ABSTRACT

Background/Aim: Despite the significant number of studies on *H. pylori* pathogenesis, not much data has been published concerning its ability to form biofilm in the host stomach. This study aims to evaluate the potential of clinical isolates of *H. pylori* to form biofilm in C57BL/6J mice model. **Materials and Methods:** Two strains of *H. pylori* were selected from a collection of clinical isolates; one (19B), an efficient biofilm producer and the other (4B), with weak biofilm-forming ability. Mice infected through gastric gavages were examined after one and two weeks. Colonization was determined by CFU and urease activity; the anti-*H. pylori* IgA was measured by ELISA, and chronic infections were evaluated by histopathology. Bacterial communities within mucosal sections were studied by immunofluorescence and scanning electron microscopy (SEM). **Results:** Successful infection was obtained by both test strains. Strain 19B with higher ability to form biofilm *in vitro* also showed a higher colonization rate in the mice stomach one week after infection. Difference ($P < 0.05$) in IgA titers was observed between the infected mice and the controls as well as between 19B and 4B infected mice, two weeks after the last challenge. Immunofluorescence and SEM results showed tightly colonizing *H. pylori* in stomach mucosal sections and in squamous and glandular epithelium. **Conclusion:** *H. pylori* is able to form biofilm in the mouse stomach and induce IgA production, reflecting the same potential as in humans. Firm attachment of coccoid form bacteria to host cells suggests the importance of this state in biofilm formation by *H. pylori*. Occurrence of biofilm in squamous and glandular epithelium of the mouse stomach proposes that *H. pylori* can colonize all parts of the upper gastrointestinal tract.

Key Words: Biofilm, C57BL/6J mice, chronic infection, coccoid form, *Helicobacter pylori*

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Helicobacter pylori colonize human stomach in approximately half of the world's population. Many of the pathogenic effects of *H. pylori* infection are related to chronic active inflammation, which persist lifelong and cause peptic ulcer disease (PUD) or gastric cancer in the absence of appropriate treatment.[1,2]

The relationship between persistence of pathogenic bacteria in host and the ability of biofilm formation, has been studied for numerous bacteria. The National Institute of Health and Center for Disease Control and Prevention has estimated that over 80% of all bacterial infections involve a biofilm stage in their disease period.[3-5]

The common characteristics of the biofilm formation ability are the capability to attach to a substratum by embedding into a matrix of extracellular polymeric substances.[3-5] This property allows more resistance to host defense and antibiotics by reducing their access to the bacterial population residing within the biofilms. Furthermore, microbial populations within the biofilm can better tolerate nutrient starvation, pH changes, and toxic compounds such as oxygen radicals.[6] Resistance of biofilms to multiple

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factors may be due to specific characteristics such as slower growth rate and physiological heterogeneity of the inhabitants, as well as its matrix, which is predominantly composed of exopolysaccharides.[7] Furthermore, bacteria growing within a biofilm can secrete substances that may play a role in signaling of gene expression, resulting in phenotypic heterogeneity within the biofilm.[7] The features of biofilm formation have been studied for several pathogenic bacteria.[8] However, despite the significant number of studies published on the pathogenesis of *H. pylori*, not much data is available concerning its biofilm-formation behavior in the host stomach. The investigators have proposed that *H. pylori* has the ability to form biofilms on various surfaces in aquatic environments.[9] Carron *et al.* and Coticchia *et al.* proposed that urease-positive organisms formed thick biofilms in the host stomach compared with urease-negative isolates.[10,11] Yonezawa *et al.* isolated a strong biofilm-forming strain of *H. pylori* and suggested that the growth rate of biofilm-forming populations is a principal factor in the biofilm development *in vitro*.[12] However, the effects of these complex phenomena and their consequence in the pathogenesis of *H. pylori* infection *in vivo* remain to be determined.

Establishing *in vivo* models of biofilm formation may enable us to understand the conditions, which play important roles in *H. pylori* pathogenesis.

This study aims to evaluate the biofilm formation ability of *H. pylori* clinical isolates associated with chronic infection in a C57BL/6J mouse model.

MATERIALS AND METHODS

Bacterial strains and growth condition

A collection of 30 clinical *H. pylori* isolates from children and adult patients with chronic gastritis were employed for this study. The isolates were grown on modified Campy blood agar plates containing brucella agar base (Merck, Germany) with defibrinated sheep blood (5%), and antibiotics. The plates were incubated at 37°C under microaerophilic atmosphere for 3 days. The grown colonies were identified by Gram staining, standard biochemical tests, and polymerase chain reaction using *H. pylori*-specific primers for *16SrRNA* and *ureC*.[13]

In vitro screening of the isolates for biofilm formation ability

The isolates were screened for biofilm formation as previously described with some modifications.[14] Colonies from culture plates were inoculated into brucella broth (Biolife, Italy) supplemented with fetal calf serum (2%) and glucose (0.3%) (Merck, Germany). Bacterial suspensions were incubated at 37°C under microaerophilic atmosphere, centrifuged at 100 rpm to an optical density of 0.2 at 600 nm (A600) equivalent to 5-8 x 10^3 CFU/mL approximately, at the beginning of the exponential phase. Portions (250 μL) of the cultures were inoculated into the wells of 96-well flat-bottomed tissue culture plates (BIOFIL, Jet Bio-Filtration Products Co., China) and were incubated at 37°C under microaerophilic conditions for 6 days. Three independent experiments with eight replicates for each strain were performed and in each test the brucella broth without bacteria was used as the negative control. The wells were vigorously washed three times with sterile phosphate-buffered saline (PBS), in order to remove nonadherent bacteria. The bacteria attached tightly to the wells were fixed with 99% ethanol (200 μL per well) for 20 min, and air-dried. Plates were then stained with 1% crystal violet (200 μL per well) for 5 min and the excess of stain was rinsed by running tap water. To dissolve the dye attached to the adherent cells, the dried plates were treated with 33% glacial acetic acid (160 μL per well) and the optical density (OD) of the wells was measured at 505 nm using an ELISA reader (SCO GmbH, Thu, Dingelstadt, Germany). Culture medium without bacterial cells was used as negative control. Quantitative evaluation of the biofilms was reported as the mean ± standard deviation of three independent experiments.

Animals

All experiments with animals were in accordance with the Institutional Animal Ethical Committee guidelines. Five groups of 10 mice (C57BL/6J, Razi (Razi Institute, Iran), aging between 6 and 8 weeks were employed. Mice (two groups for each strains) were infected (3×) through gastric gavage with 10^8 of 19B and 4B strains, respectively. Negative controls (also 10 mice) received PBS. One week and two weeks after the last challenge, blood samples were collected and the mice were humanely killed. The stomachs were then removed and divided into four portions for determining the CFU, measurement of urease activity, histopathologic and microscopic examination.

Determination of urease activity and CFU

Urease activity in the homogenized gastric tissue was performed according to the previously described method.[15] For enumeration of colonized bacteria, stomach samples were homogenized with sterile PBS; cultured on the brucella agar plates and incubated at 37°C under microaerobic conditions. Colony counts were performed after 3–5 days of incubation.

Histopathology

Portions of mice stomach were processed for histologic examination according to the standard protocols.[16] Briefly, the segments containing all parts of the stomach including the antrum and corpus were fixed at 10% neutral buffered

formalin, embedded in paraffin, sectioned by standard methods, and stained with hematoxylin and eosin (H and E) as well as Giemsa stain.[16] Density of polymorphonuclear leukocytes, and the degree of lymphocytes and plasma cell infiltration in the gastric mucosa were evaluated on the sections stained by H and E. Colonization and bacterial density were evaluated by Geimsa staining. The degree of cell infiltrations was graded according to the protocol previously adopted by Chen *et al*.[17] The stained biopsies were scored by observing chronic inflammatory cell densities (lymphocytes, plasma cells) as follows: 0, none; 1: Less than 10 in each high power field; 2: >10 cells/high power field; 3: Some areas with heavy inflammatory cell infiltration; 4: Diffuse and dense cell infiltration; 5: Presence of dense chronic inflammatory cells nearly in all parts of the whole mucous such that they separate the gastric glands; 6: Entire mucosa contains a dense chronic inflammatory cell infiltrate.

Measurement of the anti-*H. pylori* IgA

To measure the amount of IgAs in mouse serum, a method described by O'Riordan *et al*. and Akhiani *et al*.[15,18] was employed. Their method is based on comparison of optical density (OD) obtained for increasing dilution of mice antiserums. As increasing dilution of the antiserums was made until reaching to the background level (the level of OD for negative control), statistical analysis of OD values may be of a high-quality criteria. Therefore, the titers of IgAs for each mouse would be the reciprocal value of the dilution without unite.

Ninety-six-well microtiter plates (Nunc, Kamstrup, Denmark) were coated with 10^8 CFU/mL of the *H. pylori* in carbonate buffer (0.1 M sodium carbonate, pH 9.6). The plates were washed with Bovine serum albumin (BSA) (1% in PBS) and incubated for 1 h at room temperature (RT), after which time they were washed with PBS containing 0.05%Tween-20 (PBST). The wells were then inoculated with 100 µL of serially diluted mouse serum (1:100 to 1:12000 in PBST) and incubated at 37°C for 1 h. The plates were washed with PBS (6×) and diluted (5000×) goat peroxidase-coated anti-mouse-IgA (Sigma, St. Louis, MO, USA) was added to each well before incubation at RT for 1 h. The wells were then washed (6×), TMB peroxidase substrate (Sigma, Deisenhofer, Germany) was added and incubated for 10 min. The reaction was stopped by hydrochloric acid (1N) and absorbance was measured at 405 nm using an ELISA reader (SCO GmbH, Germany).

Immunofluorescence

Specimens were fixed in 4% paraformaldehyde at 4°C overnight, washed (30 min) with PBS (3×), dehydrated using 50% and 70% ethanol (ETOH), respectively. The fixed tissues were embedded in paraffin, placed in xylene for 5 min (3×), followed by ETOH (100%) for 5 min (2×) and air-dried. Slides were washed in PBST for 5 min, blocked with BSA–Tween-20 (3% in PBS) for 1 h and washed in PBST for 5 min. The slides were then incubated with a rabbit polyclonal anti-*H. pylori* antibody (1:1000 in PBST containing 1% BSA) raised against *H. pylori* outer membrane proteins.[19] After overnight incubation, the slides were washed with PBST (3×) for 15 min. For immunoflourescence examination, the slides were treated with the goat antirabbit IgG (0.01 µg/mL in PBST with 1% BSA) conjugated to Rhodamine (Sigma, SAB3700877), incubated in the dark for 1.5 h and washed with PBST (3×) for 15 min. Nuclear staining of the cells was performed according to a previously described method.[20] Briefly, the slides were placed in DAPI (4', 6-diamidino-2-phenylindole, Roche, Mannheim, Germany) or PI (propidium iodide, Molecular Probes) diluted with PBST (1000×) for 5 min, rinsed once with PBS for 5 min and air dried.

Scanning electron microscopy

All histological samples were fixed in 2.5% glutaraldehyde for 3 h followed by dehydration with increasing concentrations of ETOH: 25% (15 min), 30% (15 min), 50% (15 min), 70% (15 min), 90% (15 min), and 100% (15 min, 2×). Samples were stored in desiccators until coated with gold–palladium sputter for two 200-s intervals (Nano Structured Coating Co. Iran). Scanning electron Microscopy (SEM) was performed using TESCAN VEGA3S electron microscope, at 30 KV.[11]

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) one-way test with Minitab 16 statistical software, and probability levels of $<0{\cdot}05$ were considered as statistically significant.

Significant difference between the numbers of bacteria colonizing the mice stomachs (counted by CFU method), was assessed by ANOVA one-way, Dunnett's simultaneous tests. Also, significant differences between the IgA titers, (comparing their ODs) was assessed by ANOVA one-way, Dunnett's simultaneous tests.

RESULTS

Screening of clinical strains for biofilm formation

Two biofilm-forming strains were selected: A strong biofilm producer (strain 19B) and a weak biofilm producer (strain 4B). Figure 1 displays the results of *in vitro* biofilm formation by 19B and 4B strains studied by SEM. *H. pylori* strain 19B showed the highest levels of biofilm formation and strain 4B the lowest amount of biofilm. The OD at 505 nm was 0.136 ± 0.0152 for strain 19B, 0.0692 ± 0.0084 for strain 4B and 0.0306 ± 0.00517 for control.

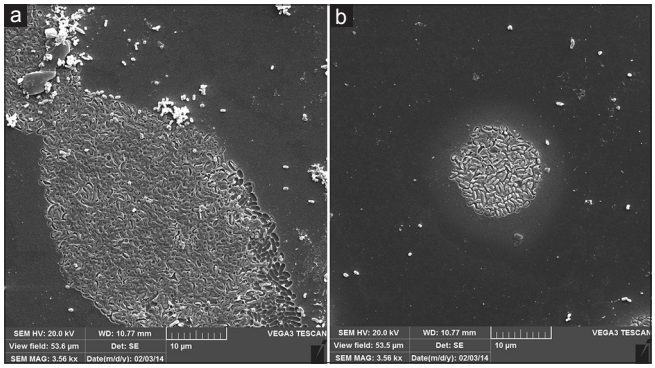

Figure 1: Representative scanning electron microscope images of mature *H. pylori* biofilms on cover slip. (a) *H. pylori* strain 19B (b) *H. pylori* strain 4B

Colonization of the mice stomach

Colonization of the gastric mucosa by 19B and 4B *H. pylori* strains was evaluated by bacterial enumeration and urease test. Both *H. pylori* strains were able to colonize the mouse stomach and produce a positive urease reaction. Bacterial enumeration, revealed a higher number for 19B strain colonization, one week after infection. However, no significant difference was observed between the two strains two weeks after infection [Table 1].

Serum IgA

There was no difference in serum IgA titers between the two mice groups (19B and 4B) as well as the control mice, one week after infection [Table 1]. Both strains were able to induce humoral immune response two weeks after infection. However, the higher biofilm-producing strain, 19B induced a significantly higher IgA response compared with 4B strain, two weeks after infection ($P < 0.05$).

Histopathology

One week after challenge, no significant cell infiltration was seen in the infected mice stomachs by any of the strains. However, after two weeks, a significant cell infiltration was observed by both strains in comparison with the controls. However, no significant difference was observed between two strains (Score of chronic inflammatory infiltrates in the gastric mucosa were 2.66 ± 0.33 and 2.33 ± 0.33 for 19B and 4B strain, respectively). Microscopic examination of the stained stomach sections by H and E revealed significant cell infiltration and tightly attached organisms with the coccoid *H. pylori* morphology to the gastric glands compared with the control group [Figure 2].

No bacterial cell was observed in the gastric glands of the controls sections stained either by H and E or by Giemsa. In addition, the bacteria embedded in the amorphous matrix, were also observed in the squamous and glandular epithelium of the stomach in the infected mice groups, two weeks after challenge [Figures 3 and 4].

Table 1: Results of bacterial enumeration, urease, and production of anti-*H. pylori* IgA

Mouse group	CFU/g ± SD	Urease	IgA titers
Strain 19B after one week	$39±8.26×10^3$	+	500±0.025
	*P value (0.000)		P value (0.999)
	t-value (17.90)		t-value (0.20)
Strain 19B after two weeks	$55±7.66×10^3$	+	8000±0.068
	P value (0.000)		P value (0.000)
	t-value (25.13)		t-value (32.21)
Strain 4B after one week	$7±1.92×10^3$	+	500±0.058
	P value (0.010)		P value (0.998)
	t-value (3.18)		t-value (0.23)
Strain 4B after two weeks	$41±7.58×10^3$	+	5000±0.043
	P value (0.000)		P value (0.000)
	t-value (19.01)		t-value (20.26)
Control	0	−	500±0.026

*The significance between the numbers of bacteria colonized the mice stomachs (counted by CFU method), was assessed by Dunnett's simultaneous tests. Significance between the IgA titers, using the optical density of titer taking into account the dilution factor was assessed by ANOVA one-way, Dunnett's simultaneous tests

Visualization of biofilm by immunofluorescence and SEM

Evaluation of the sections by immunofluorescence using anti-*H. pylori*-specific antibodies demonstrated the dense *H. pylori* communities in the glandular epithelium and squamous of the stomach [Figures 5 and 6]. Similarly, bacterial presence was visualized by SEM images in both mice groups [Figure 7]. SEM images showed higher densities for strain 19B compared with 4B to some extent [Figure 7].

DISCUSSION

In this study, the higher ability of 19B strain to form biofilm *in vitro* and higher colonization rate in the mouse stomach only one week after infection is consistent with the conclusion of Yang *et al*., that formation of *H. pylori* biofilm is time dependent *in vitro* and may also be time dependent *in vivo*.[21] However, no difference was observed between the ability of the two strains to colonize or form biofilm in the stomach of the experimental mice after two weeks. Furthermore, induction of mucosal immunity in mice occurred by both strains of *H. pylori* two weeks after challenge [Table 1], a significantly higher titer of IgA was observed in response to 19B strain, which could be related to its rapid ability to produce a successful infection/biofilm *in vivo*. In contrast, absence of significant IgA titers one week after challenge suggests that mucosal immune response initiates at least two weeks after colonization. Overall, enumeration of bacterial cells in the infected mice stomach, measurement of IgA titer, and images of light microscopy

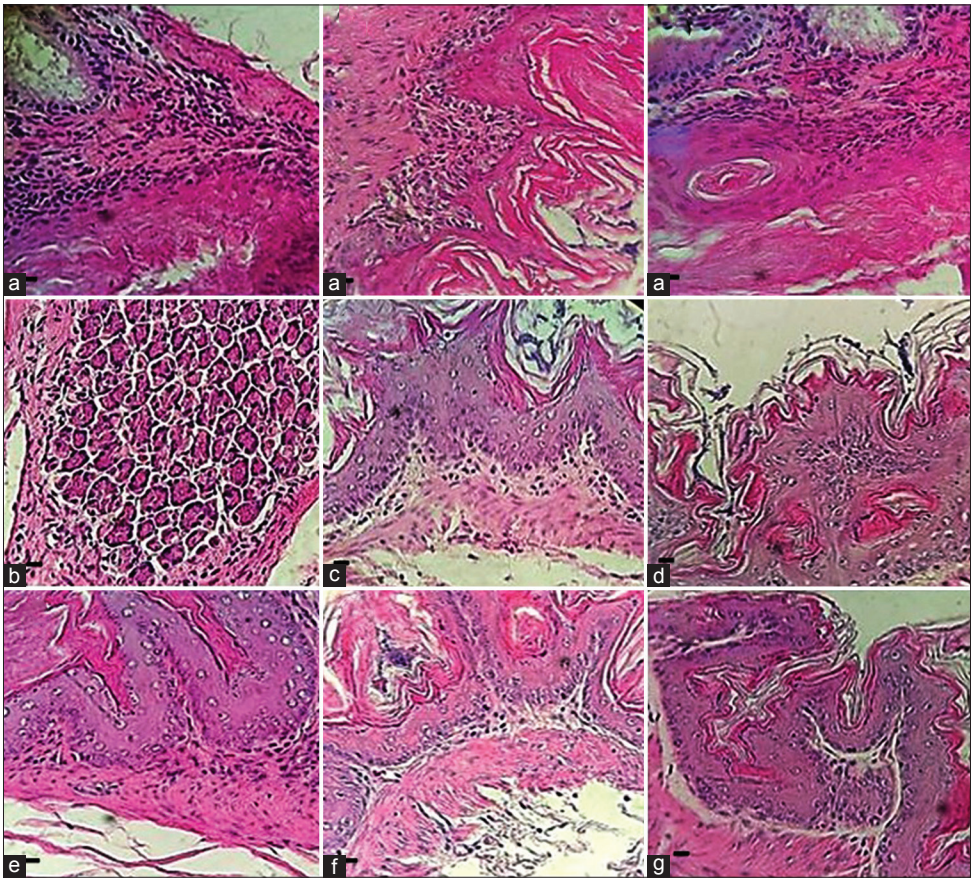

Figure 2: Mice stomach sections, stained by H and E. (a) mice challenged by *H. pylori* strain 19B displaying moderate cell infiltration after two weeks; (b and c) mice challenged by *H. pylori* strain 19B after one week where no cell infiltration was observed; (d and e) mice challenged by *H. pylori* strain 4B after two weeks where no cell infiltration was seen; (f) mice challenged by *H. pylori* strain 4B after one week where no cell infiltration was seen; and (g) mice control group (Scale bar, 10 μm)

confirmed that the rate of colonization and and subsequent biofilm formation, was not similar for the two selected strains. The formation of less strain 4B biofilm *in vitro* may be due to its slower growth rate. However, *in vitro* it is not practically possible to find a strain that produces no biomass (null) since the biofilms formed by various strains may only be quantitatively different in a shorter time. Costerton *et al*. have previously suggested that growth rates of the isolates growing in rich media in batch cultures may differ profoundly for the same species.[22] They also showed that the organization of fine structures named biofilms *in vivo* are different from those formed by pure cultures growing in nutrient-rich media *in vitro*.

The presence of inflammation in infected mice characterized by observation of chronic inflammatory cell infiltration [Figure 2] confirmed occurrence of chronic infection in the mouse model infected with *H. pylori*, somehow mimicking the situation observed in humans.

As described by O'Toole *et al*., biofilms are communities of microorganisms, surrounded by amorphous matrices.[23] Images of light microscopy [Figures 3 and 4], SEM, and immunofluorescence [Figures 5–7] obtained for two *H. pylori* strains exhibited thick communities of coccoid form bacteria in gastric mucosa suggesting formation of typical biofilms in our mouse model.

In the present work, the coccoid form of *H. pylori* was dominant in gastric mucosa; these bacterial cells were not only embedded in amorphous matrix, but were also seen in squamous and glandular epithelium suggesting that *H. pylori* is able to form a biofilm in all parts of the stomach in C57BL/6J mice [Figures 6 and 7]. Presence of *H. pylori* strains in the coccoid form might play an important role in resistance and transmission of infection.[24] Studies have shown that *H. pylori* in its coccoid form viable and can decrease its DNA and RNA levels.[25] In humans, *H. pylori* is commonly colonizes the antrum but colonization in the esophagus has not been proved yet. This may be due to the difference between the gastric anatomy of mice and humans. The stomachs of rodents are divided into glandular and nonglandular portions. The nonglandular part is generally thin walled and lined by keratinized stratified

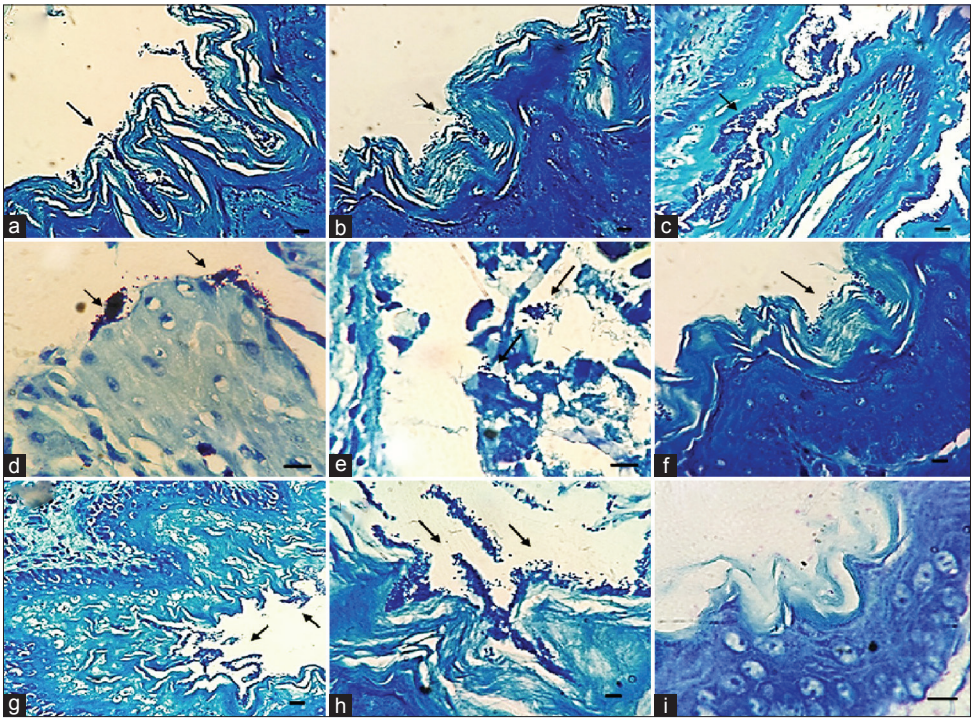

Figure 3: Representative light microscopic image demonstrating biofilm formation by *H. pylori* in sections stained by Geimsa. (a and b) biofilm formation by 19B strain after one week in the squamous area of the mice stomach; (c and d) biofilm formation by 19B strain after two weeks in the squamous and antral area of the mice stomach respectively; (e and f) biofilm formation by 4B strain after one week in the antral and squamous area of the mice stomach respectively; (g and h) biofilm formation by 4B strain after two weeks in the squamous area of the mice stomach; and (i) squamous area of the mice stomach in control group. (Scale bar, 10 μm)

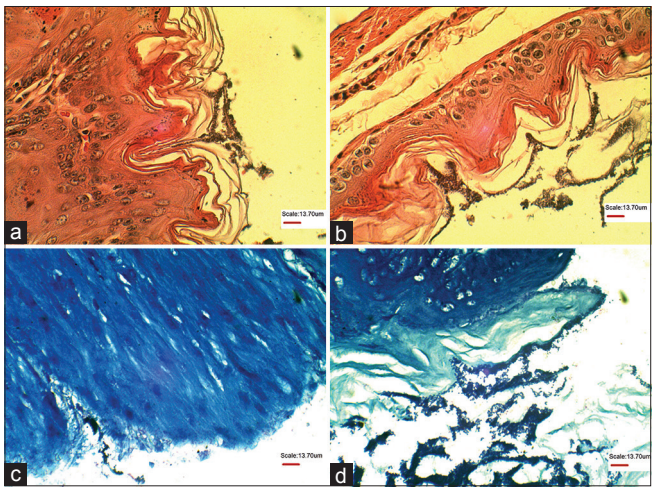

Figure 4: Representative light microscopic image demonstrating biofilm formation by 19B strain in the squamous area of the mice stomach after two weeks in sections stained by Hematoxylin and Eosin (a and b), and Geimsa (c and d)

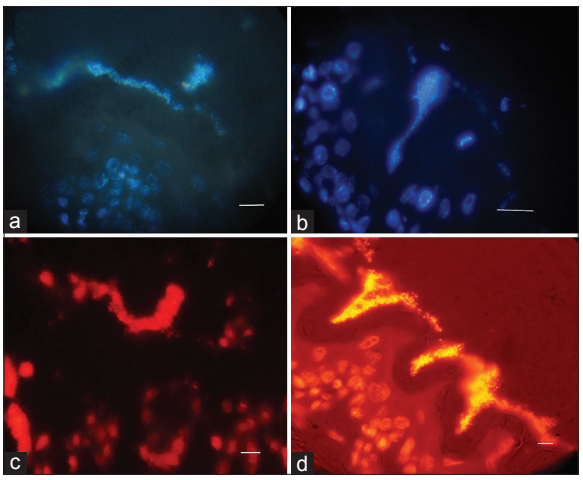

Figure 5: Representative immunofluorescence image of a mature biofilm formed by 19B formed in the antral and squamous areas of mice stomach respectively after two weeks. Fluorescent bacteria are caught near the gastric mucosa. (a and b) Nuclear staining with DAPI visualized by UV filter (c and d) Nuclear staining with PI visualized by red filter. (Scale bar, 10 μm)

squamous epithelium used for food storage and digestion. The glandular part is thick walled and covered by columnar epithelia. Furthermore, the squamocolumnar junction in mice is not entirely near the gastroesophageal junction as seen in normal human anatomy.[26,27] Thus, *H. pylori* may be able to form biofilm in all parts of the mouse stomach. This would be interesting since there are a few reports indicating that *H. pylori* can also colonize other parts of the gastrointestinal tract in humans.[28] For example, Jabbari Moghadam *et al.* observed that *H. pylori* can colonize children's tonsillar tissues and may have a possible role as

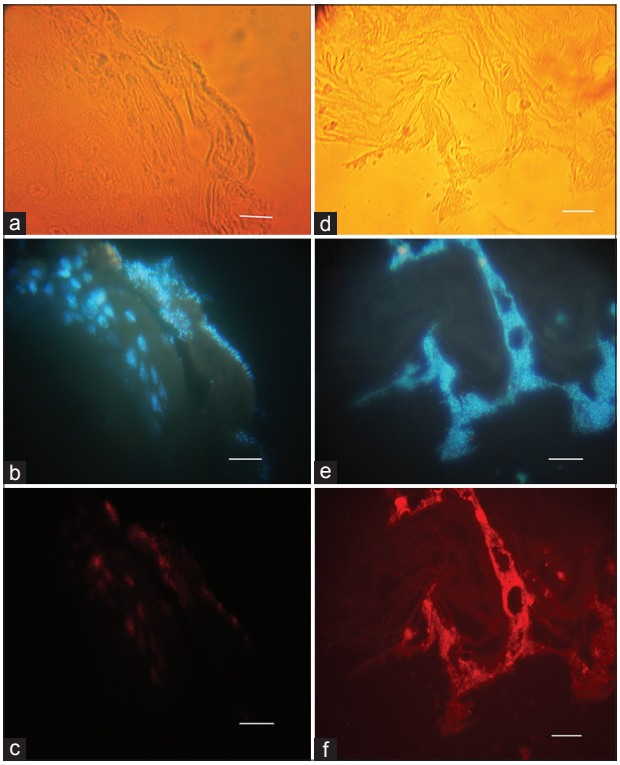

Figure 6: The representative immunofluorescence image of a mature *H. pylori* biofilm corresponding to 19B and 4B strains, respectively, after two weeks, respectively, formed in the antral and squamous areas of mice stomach. (a and d) visualized by visible light; (b and e) Nuclear staining with DAPI visualized by UV filter; (c and f) Immunofluorescence staining with rhodamin conjugated to antirabbit antibody visualized by red filter. Fluorescent bacteria are seen near the gastric mucosa (Scale bar, 10 μm)

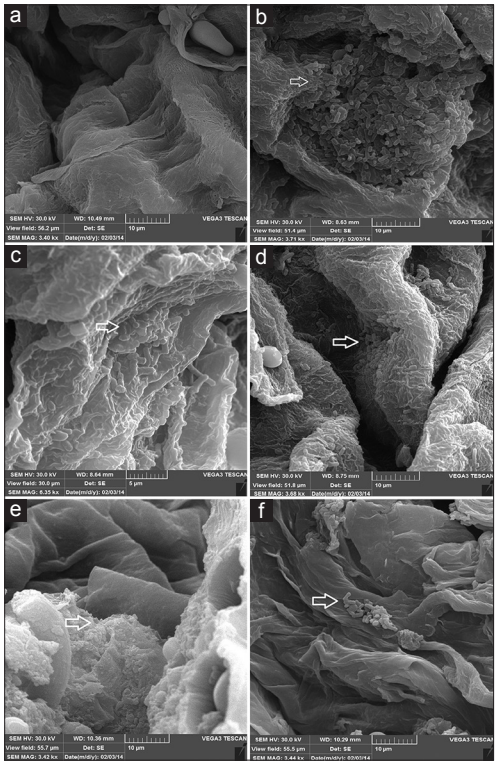

Figure 7: Representative scanning electron microscope images of a mature *H. pylori* biofilm formed in mice stomach after two weeks. (a) mice control group; (b and c) mice challenged by *H. pylori* strain 19B where the microcolonies of bacteria are seen near the gastric mucosa; and (d–f) mice challenged by *H. pylori* strain 4B

reservoir for *H. pylori* in children. In addition, Aslan *et al*. observed that despite no visible colonization, *H. pylori* may contribute to chronic tonsillitis, especially at the mucosal layers.[29] Furthermore, Gutierrez *et al*. showed that *H. pylori* can colonize heterotypic gastric mucosa in the upper esophagus (inlet patch) and provide a reservoir for oral–oral transmission or a niche for *H. pylori* to avoid antibiotics.[30] Biofilm formation on the medical implants have also been considered as real problem with serious consequences in recent years.[31] Although the significance of biofilm formation has not been well evaluated in pathogenesis of *H. pylori* infection, many researchers have suggested that it may contribute to the failure in eradication of *H. pylori* infection. One reason may be survival of microorganisms in the focal sites and further possibility of re-infection, which can help to increase resistance of *H. pylori* to currently used antimicrobials.[1,32] Consistent with this, Yonezawa *et al*. demonstrated that expression of efflux pump genes and resistance to clarithromycin significantly increased in the biofilm-forming cells compared with the planktonic populations.[33] Cammarota *et al*. reported that targeting *H. pylori* biofilm infections with molecules such as N-acetylcysteine prior to antibiotic treatment, increased the eradication rate compared with that of nontreated group.[34]

CONCLUSION

An important finding of the present study was showing the ability of *H. pylori* to form typical biofilms in our mouse model during chronic infection, which may reflect the same potential in the human stomach. Furthermore, biofilm formation in squamous and glandular epithelium of the mouse stomach suggests that *H. pylori* can colonize the upper gastrointestinal tract, such as esophagus, which can also act as a reservoir for *H. pylori*. Although this study was performed in a mouse model, presence of firmly attached coccoid cells, suggests that this state may also occur in humans. This may be shown by microbiological examinations of biopsies obtained from chronically infected human subjects.

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Conflicts of interest
There are no conflicts of interest.

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