

Characterization of *flgK* gene and FlgK protein required for *H pylori* Colonization—from cloning to clinical relevance

Jiunn-Jong Wu, Bor-Shyang Sheu, Ay-Huey Huang, Shin-Ting Lin, Hsiao-Bai Yang

Jiunn-Jong Wu, Departments of Medical Technology, National Cheng Kung University Hospital, 138 Sheng Li Road, Tainan 70428, Taiwan, China

Bor-Shyang Sheu, Department of Internal Medicine, National Cheng Kung University Hospital, 138 Sheng Li Road, Tainan 70428, Taiwan, China

Ay-Huey Huang, Hsiao-Bai Yang, Departments of Pathology, National Cheng Kung University Hospital, 138 Sheng Li Road, Tainan 70428, Taiwan, China

Shin-Ting Lin, Institutes of Microbiology and and immunity, National Cheng Kung University Hospital, 138 Sheng Li Road, Tainan 70428, Taiwan, China

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Correspondence to: Professor Bor-Shyang Sheu, Department of Internal Medicine, National Cheng Kung University Hospital, 138 Sheng Li Road, Tainan 70428, Taiwan,

China. sheubs@mail.ncku.edu.tw

Telephone: +886-6-2353535-5368 Fax: +886-6-2370941

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CONCLUSION: FlgK encoded by *flgK* is important for flagella formation and *H pylori* motility. Deficiency in FlgK or an enhanced serological response to r-FlgK can interfere with *H pylori* colonization. FlgK of *H pylori* could be a novel target for vaccination.

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Key words: *H pylori*; Colonization; Isogenic mutant; BALB/c mice; Flagella; Vaccine.

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Abstract

AIM: To characterize the role of *flgK* and its protein product in *H pylori* colonization.

METHODS: The PCR cloning method identified the *flgK* gene. An isogenic *flgK* mutant was constructed by gene replacement and confirmed by Southern blot analysis and PCR analysis. The recombinant FlgK protein (r-FlgK) was purified. Electron microscopy (EM) was applied to demonstrate the flagella of *H pylori*. An in vitro motility test was assessed in semisolid medium. The densities of *H pylori* colonization with either the wild-type strain or its *flgK* mutant were compared among BALB/c mice with or without pre-immunization with r-FlgK. The serological responses to r-FlgK were analyzed for 70 clinical patients with different densities of *H pylori* colonization.

RESULTS: From a duodenal ulcer strain, the *flgK* gene was cloned and it contained 1821 bp, with a 95.7% identity to the published sequences. No flagella were observed under EM for the mutant strain, which had a loss of motility. *H pylori* density was lower in the BALB/c mice inoculated by the mutant or with pre-immunization with r-FlgK compared to unimmunized mice or mice inoculated by the wild-type strain ($P < 0.05$). In the *H pylori*-infected patients, the serological responses to r-FlgK were uniformly low in titer.

INTRODUCTION

H pylori is now well established as a causative agent and predisposing factor for peptic ulcers and even gastric malignancy^[1-5]. However, the actual mechanism by which gastroduodenal diseases develop in response to *H pylori* infection remains unknown. The putative pathogenic factors of *H pylori* are categorized as colonization, persistence, and disease inducing factors^[4]. Colonization in the host is a prerequisite of bacterial infection and subsequent pathogenesis. The putative pathogenic factor of *H pylori* for colonization could be the possible target for preventive or even therapeutic strategy, such as vaccination.

Besides urease production and adhesin (to contact with the gastric epithelium), motility enhanced by unipolar flagella is essentially required for *H pylori* colonization^[5-7]. The motility of *H pylori* is provided by two to six polar, sheathed flagella, the filaments of which consist of two flagellin types, FlaA and FlaB^[5,6]. The *in vitro* experiments with *flaA* and *flaB* isogenic strains disclosed that both genes are required for full motility for colonization^[7]. Moreover, the establishment of persistent *H pylori* infection in a mouse model requires full motility and the presence of both flagellins^[8]. Another *H pylori* *fliD* gene, encoding a 76 kDa HAP-2 homologue, was also disclosed to be required for the assembly of flagellar filaments and motility^[9]. Infection of mice with a *fliD* mutant of *H pylori* SS1, a mouse-adapted strain, demonstrated that the FliD protein is necessary for colonization^[9]. Therefore, flagellar genes and their associated proteins should be potential tar-

gets for therapy and vaccine development against *H pylori* infection.

However, there are several genes related to the flagella in *H pylori*. Besides *flaA*, *flaB*, *flaE*, and *fliD*, the other flagellar structural proteins, such as *flgK*, are not well understood. This study thus describes the molecular cloning and characterization of the *H pylori flgK*. Applying the isogenic mutant of *flgK* and the recombinant FlgK protein of *H pylori*, this study has introduced both the *in vitro* and *in vivo* assay to define the role of *flgK* and its associated FlgK protein for *H pylori* colonization. Furthermore, this study has elucidated the clinical relevance of the anti-FlgK serological response to the colonization density of *H pylori* in humans.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *H pylori* isolate (hp250) from a Taiwanese patient with duodenal ulcer was selected for the cloning of the *flgK* gene. The *Escherichia coli* strains used were DH5 α and JM107. The *E. coli* BL21 strain was used to express the FlgK protein. Plasmid pZero-2 (Invitrogen, Carlsbad, CA) was used to clone the *flgK* gene. Plasmid pET30b (Novagen, Madison, WI) was used to express the FlgK protein. The growth and culture conditions of *H pylori* were described previously^[10].

Construction of *flgK*-mutant

Part of the *flgK* gene was amplified by PCR using *H pylori* hp250 DNA as a template with the first primer (5' CGGGATCCCGTCGCCACATCAAAATTTCCC 3') in the *flgK* gene and the second primer (5' GCTC-TAGAGCTTCACTCAACACTTCTTA CACC 3') designed to be complementary to *flgK*. PCR was performed in a DNA thermal cycler (Perkin-Elmer Corporation, Norwalk, Conn.) that had been programmed for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. Following amplification, the 1.4 kb PCR product was digested with *Bam*HI and *Xba*I (New England Biolab, Beverly, MA) and ligated to pZero-2. The 0.8 kb chloramphenicol resistance cassette (*cat*) was inserted into an *Eco*RI site to construct plasmid pMW336. Analyzing digestion patterns obtained with appropriate endonucleases and DNA sequencing confirmed the construction.

Transformation

E coli and *H pylori* was transformed based on the methods applied before^[11,12], and the selection for chloramphenicol resistance was done with 5 μ g/mL.

FlgK expression and purification

The *flaK* gene was amplified by PCR using the sense primer 5'GGGGATCCAATGGGCGGGATCTTATC3' and the antisense primer 5'CGCTCGAGTTATTGTT TAATCCCCAA3'. The PCR product was purified and cloned into the pT7 blue T vector (Novagen) and then sub-cloned to the pET30b vector digested with *Bam*HI and *Xba*I. The recombinant plasmid was transformed into the *E. coli* BL21 strain. Cells were grown at 37°C for

2-3 h in LB medium to an A_{600} up to 0.4-0.6. Isopropyl- β -D-thio-galactopyranoside (1 mmol/L) was added to the culture, and it was further incubated at 30°C for 4 h to induce protein production. Cells were harvested by centrifugation and lysed by a French press. The supernatants were collected and went through the Ni²⁺-chelate chromatography (Amersham Biosciences, Piscataway, NJ). r-FlgK was eluted using a linear gradient of 60 mM-1 M imidazole (Merck, Rahway, NJ) with a flow rate of 60 mL/h, and 1-ml fractions were collected. SDS-PAGE was performed to analyze the proteins in the soluble fractions. The desired proteins contained in the gel slices were identified by amino acid sequencing (Applied biosystems 477A autosequencer) after the proteins had been eluted. The final proteins were stored at -70°C.

Preparation of anti-r-FlgK antibody

Rabbits were injected intramuscularly with 1 mg of r-FlgK mixed with complete Freund's adjuvant; four subsequent immunizations with 500 μ g of r-FlgK mixed with incomplete Freund's adjuvant were given at 2-wk intervals. The serum anti-r-FlgK titers were determined by enzyme-linked assay seven days after the final boost.

ELISA for mice and human serum

Wells of microtiter plates (Dynatech) were coated with r-FlgK and blocked with bovine serum albumin. Serially diluted patient's serum was then added to the wells and incubated at room temperature for 2 h. Goat-anti-rabbit-IgG-HRP as secondary antibody was added and incubated at room temperature for 2 h. After the wells were washed, a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine was added and incubated at room temperature for 1 h. The reaction was stopped with 2N H₂SO₄, and the results were read with a microtiter reader (V-max; Molecular Devices Corporation, Menlo Park, Calif.) at a wavelength of 450 nm.

Electron microscopy for the bacterial morphology

Both the wild-type strain and its isogenic mutant were harvested from blood agar plates and gently suspended in phosphate-buffered saline. The negative stain was based on the method of Josenhans *et al*^[7]. The morphology, especially the flagella filaments, was compared between these two strains under the transmission electron microscope (Hitachi, H7000, Tokyo, Japan).

In vitro motility testing and in vivo animal experiments

Both the wild type strain and mutant were used in *in vitro* motility tests based on the method of Kim *et al*^[9]. Besides the *in vitro* motility test, *in vivo* animal experiments were conducted. A total of 90 six- to eight-wk-old male BALB/c mice (Iffa, L'Arbresle, France) were allocated into three study groups, challenged with wild-type *H pylori* (W group, *n* = 30), the *flgK* mutant (M group, *n* = 30), and wild-type strain plus pre-immunization with r-FlgK (I group, *n* = 30). In the I group, mice were immunized ip With 100 μ g of r-FlgK mixed with complete Freund's adjuvant, followed by one further immunization with 50 μ g of r-FlgK with incomplete Freund's adjuvant. These mice were inoculated through gastric gavage with

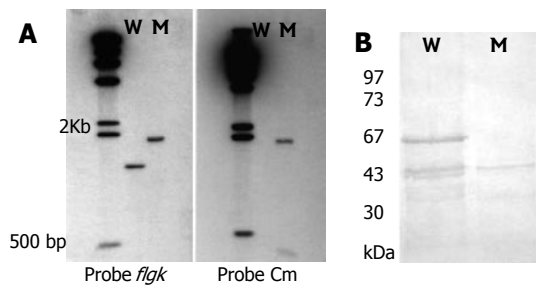


Figure 1 A: Southern blotting of genomic DNA from *H pylori* 250 and its *flgK* mutant digested with *Hind*III. The probes used were specific for the *flgK* gene, chloramphenicol resistance (Cm) gene and λ DNA. Lane W, wild-type strain; lane M, mutant; λ *Hind*III marker used as a molecular size standard; B: Western blotting of anti-r-FlgK polyclonal antibodies to confirm the phenotype of *flgK* isogenic mutant (absence of 67 kDa band). Lane W, wild-type strain. Lane M, mutant. Low molecular weight protein markers.

5×10^8 CFU of wild-type or mutant strains for consecutive 3 d periods^[13]. One week after inoculation, half of the surviving mice were sacrificed by spinal dislocation in each study group. The remaining mice were all sacrificed on the end of 4th wk. Gastrectomy was then performed in each mouse, to be stained with hematoxylin-eosin (HE) for severity of gastric inflammation and Warthin-Starry silver stain for colonization of *H pylori*. These slides were evaluated on a blind-coded basis to assess the total density of *H pylori* colonization (HPD) of each mouse ranging from 0-15^[13]. The serum of each sacrificed mouse was also collected for the serological assay.

Serological response of antibody to FlaA and r-FlgK

This study has prospectively enrolled 70 dyspeptic patients, who have no known history of anti-*H pylori* therapy, to receive panendoscopy and serum samplings. During panendoscopy, the endoscopic diagnosis and the gastric biopsy were taken for the checkup of *H pylori* density (HPD, range 0-15)^[14]. The serological responses of these clinical patients to r-FlgK, and also to the other *H pylori* flagella related proteins like FlaA^[15] were analyzed to test their correlation to the colonization density of *H pylori* in the human stomach. The sera of 70 dyspeptic patients were re-sorted for determination of the antibody titer to r-FlgK and FlaA (HPA 5040, Ibt-Immunological & biochemical test system GmbH, Reutlingen, Germany) by the ELISA method. In addition, the sera of the mice in 3 different study groups were also re-sorted to check the antibody titer to r-FlgK and FlaA.

Statistical analysis

The Student's *t*-test was applied as appropriate for the parametric difference of HPD between mice sacrificed on the 1st wk and on the 4th wk within the same group. The one-way-ANOVA test with Bonferroni's method was used for multiple testing of data, including HPD, titer of anti-r-FlgK, and titer of anti-FlaA in both human and mice settings. All tests of significance were two-tailed with a *P* value < 0.05 taken as significant.

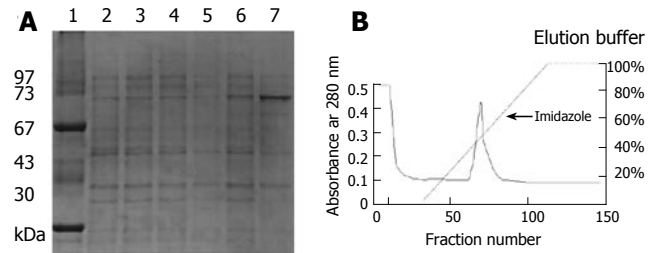


Figure 2 The r-FlgK (73 kDa) can be expressed under IPTG induction by SDS-PAGE. A: Lane 1: Low molecular protein marker, Lane 2: *E. coli* BL21 strain, Lane 3: *E. coli* BL21 strain, IPTG induction, Lane 4: pET-30b/*E. coli* BL21 strain, Lane 5: pET-30b/*E. coli* BL21 strain, IPTG induction, Lane 6: *flgK*/*E. coli* BL21 strain, Lane 7: *flgK*/*E. coli* BL21 strain, IPTG induction; B: The purification of r-FlgK protein by Ni²⁺-chelating chromatography column.

RESULTS

Disruption of the *flgK* gene and cloning of the r-FlgK protein

A pair of primers was used to amplify the *flgK* gene. The PCR product was confirmed by sequencing analysis. The *flaK* gene of *H pylori* hp250 contained 1821 bp, coding for a predicted 606 amino acids, and had 95.7% identity with published sequences^[16]. It has been deposited in GenBank under the accession No. AF333079. Gene replacement was used to disrupt the *flgK* gene. The successful creation of the *flgK* mutant was confirmed with southern blotting (Figure 1A), and western blotting analysis was used to detect the protein phenotype (Figure 1B). The r-FlgK protein was present in a 73 kDa band on the SDS-PAGE gels (Figure 2A). The r-FlgK protein was efficiently eluted from the Ni²⁺-chelating chromatography column by 500 mmol/L imidazole (Figure 2B). Identification of the purified protein of FlgK was confirmed via N-terminal sequence analysis of the protein; the first 10 amino acids (Gly-Gly-Ile-Leu-Ser-Ser-Leu-Asn-Ala-Ser) were confirmed exactly as predicted from the sequence.

Morphological difference under electron microscopy and in vitro motility assay

Under EM, no flagellar filament was observed in the mutant strain (Figure 3B), whereas the polar flagella were observed in wild-type strain (Figure 3A). No motility (indicated by the absence of a halo) was observed in the mutant strain (Figure 3D), whereas the halo was observed in wild-type strain (Figure 3C). Adding a high titer (as high as 1:50) of anti-r-FlgK polyclonal antibody (Figure 3E) had a more evident inhibition of the motility of wild-type *H pylori* than adding a lower titer (1:200) of antibody (Figure 3F).

Colonization assay and serological responses of the in vivo mouse model

All the mice in the three study groups, sacrificed on either the 1st wk or the 4th wk, had *H pylori* colonization. In Table 1, on the 1st wk of the study, the mean HPD of the mice in M group infected by the *flgK* mutant was significantly lower than that of I group, receiving pre-immunization by r-FlgK, and that of W group, infected by the wild-type strain (*P* < 0.05). On the 4th wk, the mean HPD of the mice in the M group remained significantly lower than that

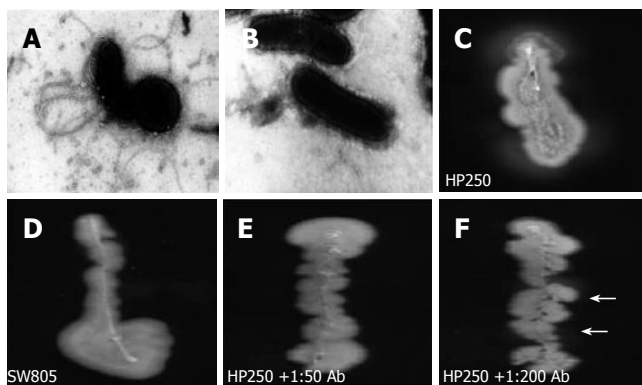


Figure 3 The electron microscopy disclosed wild-type *H. pylori* had full-length sheathed flagella (A), which was not observed in the *flagK* mutant (B). *In vitro* motility testing disclosed halos of wild-type strain (C), whereas the mutant was without halo (D). An evident halo (arrows) indicated a poorer inhibition of the motility of wild-type *H. pylori* by adding 1:200 anti-r-FlgK polyclonal antibody (F) than by adding 1:50 of the antibody (E).

of the W group. The mean HPD of mice in the I group had a significant decrease in those sacrificed on the 4th wk, when compared to those on the 1st wk (3.89 vs 5.24, $P < 0.005$). Moreover, the mean HPD of mice sacrificed on the 4th wk became significantly lower in the I group than in the W group (3.89 vs 6.59, $P < 0.05$).

The serological titers to FlaA, in either 1-wk or 4-wk experiments, were similar among the three mice groups (Table 1). In contrast, the serological titer to r-FlgK of both 1-wk and 4-wk serum samples, were (ranked in an downward order): I group, W group, and then M group ($P < 0.05$).

Serological response to flagella protein in clinical patients

In Table 2, the serological response to FlaA was significantly higher for patients with high *H. pylori* colonization than for patients with low or no *H. pylori* colonization ($P < 0.05$). However, the serologic response to r-FlgK were all less than 0.6 (in titer level) for the three patient groups, and thus were not different between any two patient groups (Table 2).

DISCUSSION

Besides urease production and adhesin, motility enhanced by unipolar flagella is required for *H. pylori* colonization^[5,6]. The motility of *H. pylori* is provided by sheathed flagella, consisting of two flagellins (FlaA and FlaB) encoded by *flaA* and *flaB*^[6,7]. *In vitro* experiments with isogenic *H. pylori* strains of mutated *flaA* and *flaB* confirmed both flagellins are required for motility^[8]. In our study, the isogenic *flagK* mutant of *H. pylori* was disclosed to be without flagella under EM (Figure 3B). *In vitro* experiments revealed the mutant (Figure 3D) had a worse motility than the wild-typed strain (Figure 3C). Our findings confirmed that loss of flagella in *H. pylori* could occur by the disruption of its *flagK*, as well as by other previously reported flagellar genes.

Linkages between flagella and virulence have been observed previously in bacteria other than *H. pylori* isolates. In bacterial species other than *H. pylori*, loss of the ability to produce flagella generally has resulted in a less virulent

Table 1 *H. pylori* gastric colonization and the corresponding serologic responses to FlaA and r-FlgK of *H. pylori* in different mouse groups

Mean (SD)	W group	M group	I group
1-wk mouse groups (n = 15)	(n = 15)	(n = 15)	(n = 15)
Anti-FlaA (A)	0.312 (0.141)	0.301 (0.115)	0.289 (0.128)
Anti-r-FlgK (A) ^{a,b,c}	0.459 (0.066)	0.329 (0.085)	1.258 (0.692)
HPD (0-15) ^{a,c}	5.35 (2.08)	3.65 (2.87)	5.24 (2.15)
4-week mouse groups (n = 13)	(n = 14)	(n = 14)	(n = 14)
Anti-FlaA (A)	0.345 (0.105)	0.324 (0.115)	0.298 (0.101)
Anti-r-FlgK (A) ^{a,b,c}	0.682 (0.395) ¹	0.351 (0.052)	1.325 (0.171)
HPD (0-15) ^{a,b}	6.59 (2.78) ¹	4.25 (2.15)	3.89 (1.85) ¹

W group: mice challenged with wild-type *H. pylori*; M group: mice challenged with isogenic *flagK*-mutant; I group: mice received pre-immunization of r-FlgK before challenge of wild-type *H. pylori*; HPD: total *H. pylori* density of the mice's stomachs by histology. The value of anti-FlaA and anti-r-FlgK was indicated as optical density (A) checked by ELISA with a 1:1000 dilution of serum. ^aindicated significant difference between W and M mice groups; ^bbetween W and I mice groups; ^cbetween I and M mice groups (One way ANOVA test with Bonferroni's method, $P < 0.05$); ¹ indicated significant difference of this parameter between the 1-wk and the 4-wk samples within the same mouse group (Student's *t* test, $P < 0.05$).

Table 2 The serologic responses against FlaA and r-FlgK of *H. pylori* among patients with different gastric colonization

Serological A titer	<i>H. pylori</i> -negative	Low colonization	High colonization
Mean (SD)	(n = 20)	(n = 20)	(n = 30)
Anti-FlaA ^{a,b}	0.268 (0.152)	0.298 (0.118)	0.398 (0.159)
Anti-r-FlgK	0.468 (0.154)	0.501 (0.258)	0.521 (0.233)

Low colonization and high colonization were defined by the total *H. pylori* density in the human stomach scored as less or equal to 5 and more than 5, respectively. A: indicates the optical density of titer checked by ELISA with a 1:1000 dilution of serum. ^aindicated significant difference between patients without *H. pylori* infection and with high colonization; ^bindicated significant difference between patients with low and with high colonization (One way ANOVA test with Bonferroni's method, $P < 0.05$).

organism^[17-19]. With respect to *H. pylori*, nonmotile mutants of *flaA*, *flaB*, or *fljD* genes could decrease motility and thus decrease the ability to establish persistent colonization of *H. pylori* in an *in vivo* mouse model^[6-9]. Such data demonstrated that full motility, mediated by these three flagellar associated genes or others, should be an essential virulence factor of *H. pylori*. These flagellar associated genes are thus possible targets for novel therapeutic or preventive vaccination for *H. pylori* infection. Besides *flaA*, *flaB*, and *fljD*, our study should be the first study to reveal another novel target, *flagK*, as able to regulate the presence of flagella on *H. pylori*.

In Figure 2, the r-FlgK protein of *H. pylori* was purified in this study. The anti-r-FlgK polyclonal antibody was obtained from a rabbit challenged with such purified r-FlgK. The study thus further tested whether anti-r-FlgK polyclonal antibody could inhibit the motility of the wild-type *H. pylori* isolates. *In vitro* motility testing of wild-type *H. pylori* was more inhibited by a 1:50 titer (to give no halo) (Figure 3E) than by a 1:200 titer of anti-r-FlgK polyclonal antibody (which resulted in a faint halo) (Figure 3F).

These findings supported the hypothesis that the immune response to r-FlgK of *H pylori* could possibly interfere with *H pylori* colonization of the infected host.

In Table 1, on the 1st-week study, the mice inoculated with the mutant were found with significantly lower HPD than those inoculated with the wild-type strain (3.65 vs 5.35, $P < 0.05$). The titer of anti-r-FlgK was also lower in the M group than in W group (0.329 vs 0.459, $P < 0.05$). The poor serological response to r-FlgK in the M group indirectly indicated that deficient production of FlgK occurred after destruction of the *flgK* gene. Moreover, deficiency of FlgK in the mutant strain decreased its colonization ability in mice. Such above-mentioned difference was consistently found on the 4th wk (Table 1). On the 4th wk study, the titer of anti-r-FlgK of the immunized group was significantly higher than those of the other two groups (1.325 vs 0.682 and 0.351, $P < 0.05$). On account of this higher titer of anti-r-FlgK antibody (which has the ability to inhibit motility) (Figure 3E and 3F), mice receiving pre-immunization had lower HPD than those mice in W group (3.89 vs 6.59, $P < 0.05$). This evidence was also supported by the limited (< 1.0) titers to r-FlgK of the clinical *H pylori*-infected patients (Table 2). These interesting findings supported the ability of an enhanced immune response to r-FlgK to inhibit the motility of *H pylori*.

The mean titers of anti-FlaA in these three mouse groups were not different on either the 1st or 4th wk study (Table 1). This data suggested the interference with *H pylori* colonization in mice was mainly accounted for by the response to the *flgK* gene product rather than the *flaA* gene.

In contrast to the poor serological responses to anti-r-FlgK, these clinical patients were disclosed to have significant differences in the titer of anti-FlaA Ab among patients with different *H pylori* colonization density (Table 2). This implied the serological response to FlaA could be more sensitive than anti-r-FlgK to predict the presence of *H pylori* infection. However, the titer of anti-FlaA was increased with the increment of *H pylori* colonization (Table 2). The function of anti-FlaA antibody may just serve as a serological marker rather than a promising immune protection against *H pylori* infection.

In summary, FlgK encoded by the *flgK* gene plays an important role in flagella formation and motility of *H pylori*. The deficient FlgK of *flgK*-mutant and the pre-immunization with r-FlgK can effectively decrease *H. pylori* colonization. Therefore, FlgK of *H pylori* may serve as a potential target to inhibit the motility and colonization of *H pylori* in the infected host.

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