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The use of a 49.6 kDa pili protein of *Helicobacter pylori* for serological diagnosis in mice

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ABSTRACT

Bacterial culture is currently not recommended for routine evaluation of *H. pylori* infection in humans. For this reason, serological techniques play a critical role in diagnosing *H. pylori* infection in humans, particularly for initial pre-endoscopy or pre-treatment screening in dyspeptic patients. However, several current "in-office" tests appear to be less accurate or would need further validation before being recommended for use in a primary clinical care level. Preparation of specific and immunogenic antigens is an important step to improve the sensitivity and specificity of serologic assays, particularly for confirming the peptic ulcer status. The purpose of this study was to use a sub-unit protein of *H. pylori* with a molecular mass of about 49.6 kDa for the evaluation of *H. pylori* infection in mice. Fifty mice were orally infected with live *H. pylori,* and a similar number of mice were only orally given sterile phosphate-buffered saline (PBS). This process was repeated three times with a three-day interval between each administration, and blood samples were collected before and after each infection. The sera were tested using dot blot and ELISA. Seroconversion was detected two weeks after infection, and ELISA showed 98% for both its sensitivity and specificity. This study has indicated that the 49.6 kDa subunit pili proteins recognized homologous antibodies against the microorganism. Further studies are required to confirm the reaction of this protein against serum originated from *H. pylori*-infected human.

Keywords: Pili 49.6 kDa, *H. pylori*, cholera toxin, *in vivo*
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INTRODUCTION

Since the original description of *H. pylori* by Marshall and Warrant in 1982,[1] these bacteria play a pivotal role in the pathogenesis of several gastro-duodenal pathologies. Infection of the bacteria triggers both local and systemic antibody response (Youri Glupczynski 1998). It has been shown that antibody response to *H. pylori* infection increased in atrophic gastritis without histologic evidence,[2,3] suggesting that the accuracy of gastric-biopsy-based tests might be limited if the number of *H. pylori* is very low. Many serological tests are made to detect the presence of CagA and VacA antigens,[4] but antibody response against these antigens will appear late after infection.[5,6] This may suggest that the antigen used in serological tests should be antigenic in nature to recognize specific early immune responses. Preparation of immunogenic and immunodominant antigens is important to step to improve sensitivity and specificity of serologic assays for *H. pylori*. It has been established that a sub-unit protein of *H. pylori* with a molecular mass of about 49.6 kDa has a pathogenic effect in causing peptic ulcer. A very recent study has demonstrated that this protein was found to be adherence to mice gastric epithelial cells *in vitro*. Moreover, the attachment of intact *H. pylori* cells on the purified mice gastric epithelial cells could be protected by the presence of polyclonal antibodies produced against the homologous protein, indicating the protein was dominant and immunogenic.[7] The purpose of this study was to use a sub-unit protein of *H. pylori* with a molecular mass of about 49.6 kDa in serological tests to detect the immune response of *H. pylori* infection in mice.

MATERIALS AND METHODS

H. pylori isolate and cultivation

The technique used to prepare *H. pylori* isolates was based on previously published paper.[7] Briefly, a sample of *H. pylori* strain was originally isolated from patient with gastritis and duodenum ulcer, and then re-cultured using media Trypticase Soy Agar (TSA) and Trypticase Soy Broth (TSB) supplemented with 10% sheep blood, Dent supplement, and Isovitalex. The culture is then incubated at 37°C in a microaerophilic atmosphere. Subsequently, the bacteria were transferred into a 10 ml sterile tubes containing about 10^6 cells/ ml and kept for not more than1 hr at 5°C until used.

Isolation of 49.6 kDa subunit pili protein of *H. pylori*

The supernatant containing pili was done electrophoretically by SDS-PAGE based on the method.[8] The product of electrophoresis in the form of gel was cut straight at a molecular weight of about 49.6 kDa. The gel pieces were then sliced and inserted into

the dialysis membrane by using electrophoresis running buffer fluid. Subsequently, the desired protein was electroeluted by placing the membrane horizontally in the negative electrode with 20 mA current for 15 minutes. The dialysis was performed on the product of electroelution with 2 liters of pH 7.4 PBS buffer fluid for 2 × 24 hours. Dialysis fluid was changed three times, and the dialysis fluid in membrane dialysis as a result of electroelution of SDS-PAGE band was collected. Total protein was measured using a method derived from DC Protein Assay (Bio-rad), suspended to a concentration of about 10 ng per ml and kept at -20°C until used.

Dot blot assay

Gradual sero-conversion of H. *Pylori* infection was evaluated using dot blot assay, based on the modified technique of IW Tenaya 1998. Briefly, a total of 5 uL protein 49.6 kDa was placed on the nitrocellulose membrane *(Invitrogen)* with the size of about 15 mm^2 using Airjet Dispenser (*Biodot*), blocked with 5% *reagen blocking* for 1 hr. The membrane was subsequently soaked with mice serum diluted 1:200 in PBS and incubated for 1 hr at RT. After washing with PBS-Tween, the membrane was reacted with *conjugate* (*Goat anti-mouse IgG-HRP*) for 1 hr, rewashed, and finally reacted with the substrate (*4 Chloro napthol*). When color development occurred, the reaction was stopped by washing with water and photographed.

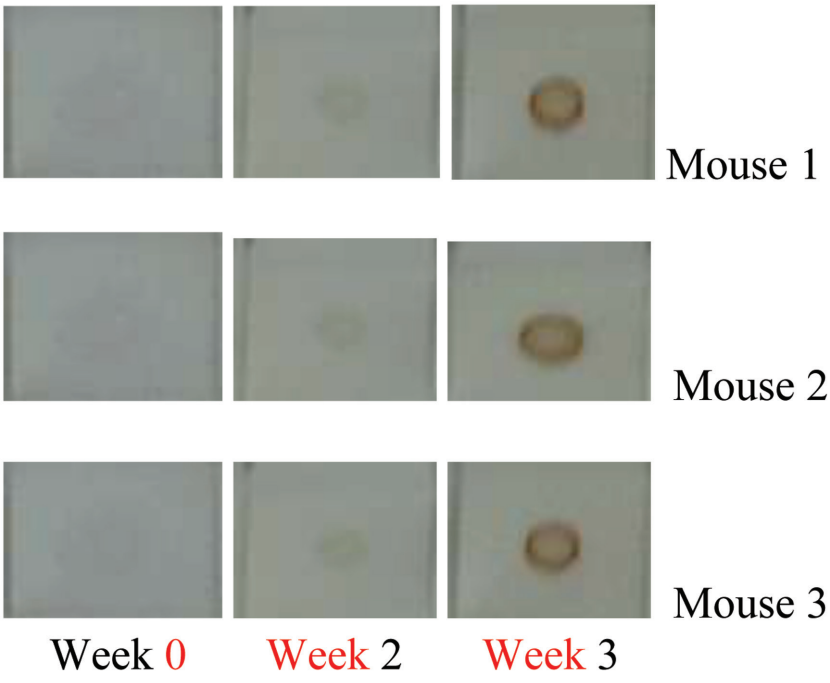

Figure 1 Dot blot analysis of 49.6 kDa protein reacted with mice sera infected with *H. pylori*. Note: all the three mice produced antibody response gradually from the 2nd weeks after the initial infection

ELISA tests

An Enzyme linked-immunosorbent assay (ELISA) was used to detect antibody response to *H. Pylori* infection according to the method of Graham et al. (1996). Firstly, a flat-bottom microplate (Maxisorp, Nunc) was coated with 50 uL 49.6 kDa protein per well. After blocking with PBS containing 5% BSA, mice serum diluted 1/400 in PBS was added into the well and incubated for 2 hr at 37°C. The plate was washed with washing buffer (PBS pH 7.0 containing 0.1 % (V/V Tween-20)). Subsequently, conjugate anti-mouse IgG-HRP diluted 1/1000 in PBS was added to the plate and incubated for 1 hr(?) at 37°C. After a further washing as before, conjugate OPD(?) was added and incubated for 30 min. at room temperature (RT) and the reaction was stopped by adding a stop buffer (3M NaOH). Optical density(OD) was read using a microplate reader with a wavelength of 450 nm (Thermo, Multiskan EX). The cut-off value was calculated by calculating the difference between positive reaction and negative control divided by 2.

RESULTS

The dot blot assay showed that immune responses against *in vivo* infection of *H. pylori* increased gradually 2 weeks after the initial infection and were clearly detected at 4 weeks; no antibody was observed before the infection (Figure. 1). This reaction confirmed that *H. pylori* isolates used to infect mice orally in this study was immunogenic and released specific antibody that reacted with homologous 49.6 kDa protein in vitro. The absent of antibody response in mice before the initial infection suggesting that all animals used in the study were free of being infected with *H. pylori*.

ELISA test confirmed the dot blot assay by testing 100 mice (50 infected and 50 non-infected mice) with life *H. pylori* isolates. Sera obtained from infected mice gave higher OD value compared with those of the control group. From a total of 50 infected mice, 48 of them (96%) gave positive ELISA results of ≥ 0.535, with a cut of value (COV) =0.535. In contrast, 48 out of 50 mice (96%) of the control group showed negative ELISA results of ≤ 0.535. Based on the formula of Sugiono (2006) (Figure.2), the sensitivity of the ELISA in this study was 48/48+2×100% (96%). Meanwhile, the specificity of the test was 48/2+48x100% (96%). This results suggest that ELISA test with the application of the isolated 49.6 kDa protein had high diagnostic value for detecting immune responses to *H. pylori* infection. Compared to the dot blot test above, the ELISA test was considered to be more practical and could be applied to test massive samples.

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H. pylori Positive Serum				
		Positive	Negative	Total
ELISA of *H. pylori*	Positive	48	2	50
	Negative	2	48	50
	Total	50	50	100

Figure 2 The formula of Sugiono (2006) to demonstrate the specificity and sensitivity of ELISA test. Note that explanation of the Fig. is presented in the text

DISCUSSION

The ability of *H. pylori* to adhere to the mucosal constituents in the human stomach is a predisposition site for the attachment of this unique niche, which has become the major habitat of the microorganism.[14] It has been recently published that a subunit pili 49.6 kDa protein of this bacteria was found to be immunogenic, immunodominant, and considered to have adhesion properties.[11] *In vivo* study has shown that the application of this protein for a vaccine in an animal model, provided a protective immune response. Similarly, a subunit pili protein of Shigella *dysentriae* and *Salmonella typhi* with a molecular weight of 49,8 kDa and 48 kDa respectively were also a hemagglutinin with adhesion properties.[15] For this, we reasoned that the 49.6 kDa protein of *H. pylori* reported in the previous study may be a potential antigen for developing serological assays to detect immune responses against *H. pylori* infections in mice.

The serological diagnostic assays developed in this study were considered specific and sensitive. The dot blot test confirmed that the 49.6 kDa protein of *H. pylori* specifically recognized homologous antibodies against infection of this bacteria. Although detectable antibody using this technique could be demonstrated as early as 2 weeks after infection, it was considered specific as no antibodies were not detected before the initial infection. This data demonstrated that all animals used in the study were free of being infected with *H. pylori* in nature. Two weeks after initial infection, the stronger antibody response was detected, suggesting that the released antibody titer was associated with the normal immune mechanism, although antibody response after this point was not collected and tested.

The 49.6 kDa subunit pili of *H. pylori* coated in the ELISA was associated with its potential to cause peptic ulcer.[11] In the previous study, it was proven that the use of this protein in a vaccine trial in mice, produced protective antibodies. For this reason, it was assumed that the developed ELISA in the current study could detect the presence of protective IgG antibodies which correlate with the development of a peptic ulcer. Lee, C.K, *et al.* (1999) reported that the use of urease as a vaccine candidate that triggered the release of IgG and IgA on the saliva of the rhesus macaque, but had no protective properties to challenge. Moreover, application of inactivated whole-cell vaccines in human could also release IgA and IgG in serum but was not able to eradicate *H. pylori* (Kotloff, K.L, *et al.* 2001). These findings have indicated that both the urease and inactivated whole-cell vaccines antigens may not be able to protect the presence of peptic ulcer, and the secreted antibodies may have less value if used in serological assays. The *in vivo* study reported here has confirmed that the 49.6 kDa subunit pili protein of *H. pylori* could be used in a serological assay to test the immune responses against natural infection which may correlate well with peptic ulcer. However, preparing this antigen was considered technically difficult. Therefore, it may be necessary to provide a recombinant protein using gene encoded this antigen for further study. The most important work is to provide a recombinant protein which is able to test antibodies originated from H. pylori-infected human for the confirmation of peptic ulcer status.

CONCLUSION

The serological studies reported here confirmed the biological properties of the 49.6 kDa subunit pili protein of the *H. pylori* which seemed to be useful for clinical application. Although this was the first report on using this protein for serological tests, it was found to be sensitive and specific. Further study is required to provide recombinant protein using gen encoded this antigen, in order to facilitate serological diagnosis for evaluating peptic ulcer status in human infected with *H. Pylori*.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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