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IN - VIVO CONFIRMATION OF A 49.6 KDA PROTEIN PILI OF HELICOBACTER PYLORI TO PREVENT DESTRUCTION OF GASTRIC CELLS AGAINST LIVE HOMOLOGOUS BACTERIA IN MICE

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ABSTRACT: Peptic ulcers is one of the major gastrointestinal disorder in human being that generally associated with the infection of *Helicobacter pylori*, a gram-negative microaerophilic bacterium in stomach. It is also linked to the development of the stomach cancer. The aim of this study was to investigate the *in vivo* properties of the sub-unit pili proteins with molecular weight of about 49,6 kDa in mice. The bacterium was firstly cultured on the plate of TSA-B (Trypticase Soy Agar with 5% Sheep Blood) to prepare the protein of interes using bacterial cutter and SDS-PAGE. The purified protein was used for a vaccine emulsified with commercial cholera toxin and give orally. The immunized mice showed a significant protection against challenge with live *H. pylori* cells. In contrast, animals that received the 49, 6 kDa protein without adjuvant as well as the negative control with PBS failed to inhibit adherence of the bacteria, as indicated by a severe damages of gastric tissues. This study has indicated that the sub-unit pili proteins trigered the release of protective antibodies againts the microorganism if combined with cholere toxin adjuvant. Further study is required to investigate the biological functions of this protein as a vaccine candidate for protecting the infection by this microorganism in causing gastric ulcers.

INTRODUCTION: During the last decade it has been established that the presence of microbes in the stomach has been associated with gradual increase of gastric cancer. The perception of the bactericidal activity of stomach acid to hamper the ability of bacteria to cause this condition has been arguable. A number of investigators have demonstrated that *Helicobacter pylori*was considered as a major cause of gastric cancer [1, 2]. The presence of the bacteria in the human stomach coincided with a variety of gastric disorders such as peptic ulcer, gastritis chronic and gastric carcinoma even gastric lymphoma [3].

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However, morbidity may varied in different some individuals associated with acquired influences that stimulate defenses of host against the infection[4].

The ideal regimen to treat this infection has been difficult primarily due to human habits including smoking, overweigh, poor compliance [5], and re-infections associated with antibiotic resistance [6]. However, eradication of the bacteria using various therapeutic schemes could potentially prevent gastric cancer [7]. It has been demonstrated that H. pylori colonizes the epithelial surface of the gastric mucosa by forming a specific adhesion mechanism [8], with the involvement of immunogenic proteins such as outer membrane proteins and sub-unit proteins: Cag A, Vac A, adhesion A and Oip A [9]. With this process, the microorganism must leave the mucus membrane and adhere to the underlying epithelium [10]. However, the precise mechanisms

underlying H. pylori adhesion have yet massively to be identified.

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 on mice gastric epithelia 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 [11].

However, the *in vivo* studies to demonstrate the biological activities of the protein in causing pathological consequences in the stomach are very limited. The purpose of this study was to confirm the in vivo pathogenesis of the 49.6 kDa protein of *H. Pylori* in causing gastric epithelial cells damages in mice. Furthermore the in vivo protection of the epithelial cells by providing local vaccine contained the pili protein against intact *Helicobacter pylori* was also evaluated.

MATERIALSAND METHODS:

H. pylori isolate and cultivation:

A stock sample of *H. pylori* strain was kindly provided by Biomedical Research Unit West Nusa Tenggara Provincial Hospital. The bacterium 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 and incubated at 37^{o}C on microaerophilic atmosphere. Subsequently, the bacteria were transferred into 10 ml sterile tubes containing about 10^6 cells/ ml and kept for not more than1 hr at 5^{o}C until used.

Isolation of *H. pylori* pili:

Isolation of *H. pylori* pili was performed by the method [12] with a slight modification. Bacteria pili was cut by using pili bacterial cutter which was carried out for 30 sec at speed 5000 rpm while the second to five cutting used the same speed. Subsequently, the isolation of pili fraction by centrifugation of cutting result was done at 12000 rpm at 4^{o}C. The supernatant containing the bacterial pili were stored at 4^{o}C, a sample of it was checked under an electron-microscope for confirmation.

Isolation of *H. pylori* sub unit pili 49, 6 kDa protein:

Research methods [12] with slight modification. The supernatant containing pili was done electrophoretically by SDS-PAGE based on the method [13]. 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 current 20 mA for 15 minutes.

The dialysis was performed on the product of electroellusion with PBS pH 7.4 buffer fluide as much as 2 liters during 2 x 24 hours. Dialysis fluid was changed three times, and dialysis fluid in membrane dialysis as a result of electrophoellusion of SDS-PAGE band was collected. Total protein was measured using a method derived DC Protein Assay (Bio-rad), suspended to a concentration of about 10 ng per ml and kept at -20^{o}C until used.

Confirmation of the sub unit pili 49, 6 kDa protein that inhibits colonization of *H. pylori* in gastic mucosa of mice:

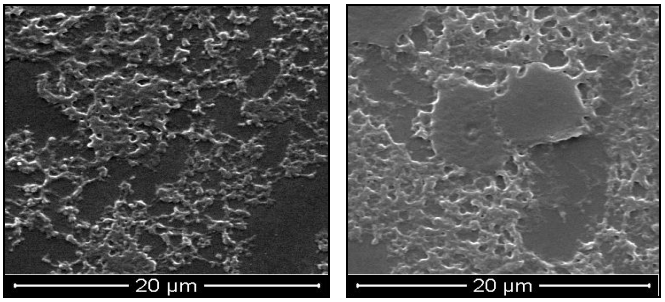
Three groups of experimental mice consisting 4 mice in each group designed as group A, B and C were used in this study. Mice in group A were orally immunized with 0.5 ml of immunogen contained 200 µg of sub unit pili 49,6 kDa protein emulsified with 10 µg cholera toxin sub unit B, as recommended by the factory (Sigma, USA). This was repeated 3 times in 1 week intervals.

In contrast, animals in group B and C were only orally given cholera toxin and PBS respectively. At fourth week of experiment, all animals in the three groups were challenged orally with 0.5 ml of live *H. pylori* at a concentration of about 10^6 cells/ml, the clinical signs were observed daily. The animals were kept for one week before being killed for gastro-intestinal sample collections. The samples were then process using H&E staining as published previously.

RESULTS:

Confirmation that *H. pylori* pili was isolated:

To confirm if pili of *H. pylori* could be isolated as desired was checked by observing a sample of it under electron-microscope, before being used for further study. This study found that the pili was isolated as expected, indicated by a uniform white sediments without any cellular morphology **(Fig. 1A),** compared to the whole cells indicated by intact cells connected with pili **(Fig. B)**



A **B**

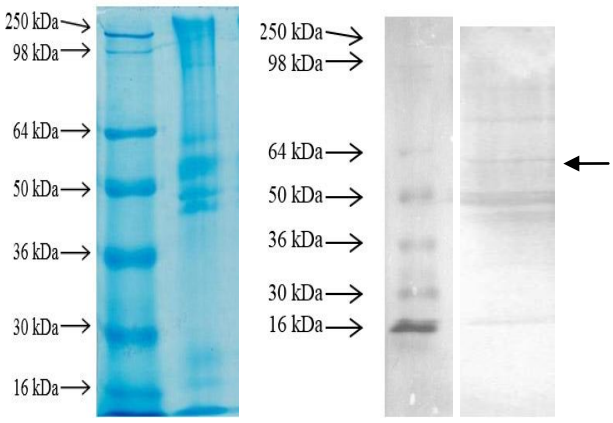
FIGURE 1: MORPHOLOGICAL FEATURE OF ISOLATED PILI (A) AND WHOLE CELLS (B) OF *H. PYLORI* UNDER ELECTRON MICROSCOPE EXAMINATIONS, THE MAGNIFICATIONS ARE NOTED.

Isolation of *H. pylori* subunit pili 49, 6 kDa protein:

A sample of the isolated pili was subjected into SDS-PAGE to localized the position of the sub unit pili 49, 6 kDa protein, before being cut and purified. As it was published previously, the position the protein of interest was quite clear with molecular weight of about 49, 6 kDa, suggesting the desired protein could precisely be localized **(Fig.2 A).** Furthermore, this protein was assumed to be immune-dominant and immunogenic, indicating by a strongest protein band compared to other proteins in Western blot analysis **(Fig. 2 B)**. This protein band was then purified for the immunization of mice, although product of the purified protein is not shown.

In Vivo confirmation of the sub unit pili 49, 6 kDa protein to prevent colonization of *H. pylori* in gastic mucosa of mice:

The immunized mice with the sub unit pili 49, 6 kDa *H. pylori* demonstrated a protective reaction against live infection of *H. pylori.* There were no significant damages of gastrical tissues found after one week incubation *in vivo* **(Fig. 3a).** In contrast, a severe lost of epithelial cells was observed in animal without the presence of cholera toxin adjuvant in the inoculums given **(Fig. 3b)** and in negative control with PBS **(Fig. 3c).** No lesions were observed in normal tissues (3d).



A **B**

FIGURE 2: THE PRECISE LOCATION OF THE SUB UNIT PILI 49,6 kDa PROTEIN OF *H. PYLORI* WAS CONFIRMED BY USING SDS-PAGE (A) AND WESTERN BLOT ANALYSIS (B). THE POSITION OF THE PROTEIN IS INDICATED (ARROWED).

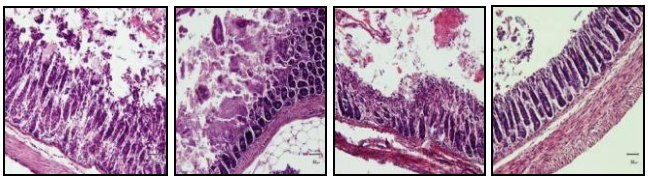


FIG. 3A **FIG.3B** **FIG. 3C** **FIG. 3D**

FIGURE 3: DEMONSTRATION INFECTION OF INTACT CELL OF *H. PYLORI* IN VARIOUS ANIMAL TREATMENTS.

A significant protection of tissue in animals immunized with sub unit pili 49,6 kDa (3a); a serious tissue damages observed in non-immunized animals (3b and 3c) after challanged with live bacterial cells; and a normal feature of gastrical tissues (3d).

DISCUSSION: The initial step of colonization process by *H. pylori* is their ability to adhere the mucosal surface of gastric epithelial cells. The adhesion of *H. pylori* to mucus 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]. In other bacteria, it was reported that a sub unit pili protein of *Shigella dysentriae* and *Salmonella typhi* with molecular weight of 49, 8 kDA and 48 kDa

respectively were hemaglutinin with adhesion properties [15]. With *H. pylori* it selves, it has just recently published that a sub unit pili 49, 6 kDa protein of this microorganism was found to be immunogenic and immune dominant *in vitro* **[11]**. For this, we reasoned that this protein may be a potential candidate for protection against natural *H. pylori* infections.

In this study, the *in vivo* biological properties of the sub unit pili 49,6 kDa protein of *H. pylori* were investigated. It was previously published that this protein could be purified for the *in vitro* studies which showed its immunogenicity [11]. In animal models, application of this protein mixed with a commercial cholera toxin adjuvant, revealed a significant inhibition against live *H. pylori* challenge in the mucosal gastric. It was reported that *H. pylori* possess a a type IV secretion system encoded by the *cag* pathogenicity island, the effector protein CagA, the vacuolating cytoxin (VacA) and others that responsible in causing pathological effect in *H. pylori* infections [16].

This feature was not experienced in this study, suggesting that the sub unit pili 49, 6 kDa protein may has a crossed-protected reactivity to the toxin *in vivo*. However, compared to the normal samples prepared from normal animals, the degree of protection was less complete.

This may be due to vaccination regime used, in which the dose of antigen and adjuvant need to be further optimized. In contrast, the introduction of the protein alone, without the adjuvant failed to protect gastric mucosa, suggesting that protein was immunogenic as reported previously and the adjuvant play important role in providing a good protection. With this regard, inhibition of *H. pylori* adhesion to human gastric mucus was proved by giving a high-molecular weight constituent of cranberry juice **[10]**. However, it seemed likely that the inhibition by using the juice may be due to physical protection, rather than biological reactions as it was reported in the current study.

In line with our previous data that, application of the isolated IgG on the pre-treated mice gastric epithelial cells with the purified sub unit pili 49, 6 kDa proteins, repealed an inhibition of *H. pylori* cells to adhere the cells. This further suggested that the sub unit pili 49, 6 kDa proteins had a specific adhesion molecules to bind the target gastric cells of mice. The inability of the bacteria to attach the gastric epithelial cells particularly when a high titer of IgG was added, confirming the sub unit pili 49,6 kDa protein was a functional protein that may associated with the pathogenesis of the bacteria. The *in vivo* study reported here has confirmed that the protein could provide a protection against natural infection as demonstrated by histological observations.

However, the protection was specific only against the *H. pylori* cells, but not against cholera toxin (data not shown).

This result suggested that the both the sub unit pili 49,6 kDa protein of the bacteria and cholera toxin as an adjuvant had synergetic actions in protecting the gastric tissues in mice against live challenge of *H. pylori*, and therefore it may be necessary to use this protein as a vaccine candidate for the prevention against *H. pylori* infections.

CONCLUSION: The *in vivo* study reported here has further confirmed the *in vitro* characteristic of the 49, 6 kDa sub unit pili protein the *H. pylori* that was previously published. The current result has suggested that the protein was also found immunogenic, causing significant damages to gastric tissues of infected mice. However, the corporation this protein with a commercial cholera toxin adjuvant and given orally, indicated that the 49, 6 kDa sub unit pili protein the *H. pylori* was an useful antigen associated with protection against this microorganism when emulsified with the of cholera toxin. Further study is required to investigate the biological functions of this protein as a vaccine candidate for protecting the infection by this microorganism in causing gastric ulcers.

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CONFLICT OF INTEREST: The authors report no conflict of interest.

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