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Combinations of Protein Sub-Unit PILI 37.8 KDA *V. Cholerae* with Cholera Toxin Sub-Unit B *V. Cholerae Can* Protect Come Out of the Solution in the Intestinal Mice

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ABSTRACT

Pili of *Vibrio cholerae (V. cholerae)* have a role in the pathogenesis of cholera. Previous our study had proved that protein 37,8 kDa pili *V. cholera*e was a molecule adhesin. Molecule adhesion of *V. cholerae* and cholera toxin subunit B (CTB) appears important factor in pathogenesis of cholera. The aim of this study is to verify that the protein adhesion 37.8 kDa combined with the CTB can induce s-IgA and therefore have a protective function toward the diarrhea caused by *V cholerae*. This study used a balb/c. The balb/c is vaccinated by CTB and protein 37.8 kDa sub unit pili *V. cholerae* combined with oral CTB. The production of s-IgA was checked by the ELISA method. A protectivity test was used by efflux water into small intestine. Analysis of the study utilize of the ANOVA and Tukey's test. The results showed s-IgA had a lot of divergence between treatment and statistically no different, mean while for protectivity test, it also shows significant difference between treatment (p=0.05). In conclusion, protein adhesin pili *V. cholerae* 37.8 kDa combined with CTB is able to induce an immune response with increased of content of s-IgA and protection toward liquid secretion in the small intestine.
Keywords: Pili V. *Cholera* 37.8 kDa Cholerae Toxin subunit B, vaccine

INTRODUCTION

The first step in the pathogenesis of cholera is the attachement of a *V. cholerae* to the surface of an enterocyte in the intestinal tract. This is followed by a second step where the bacteria *V. cholerae* is colonized as well. During colonization the bacteria will produce a cholerae toxin (CT). CT has a role in the secretion of water in the intestinal tract. A sub unit B protein is part of the cholerae toxin protein which attaches to GM1 on a mucous of the intestinal surface. The attachement of sub unit B is followed by internalization of sub unit A1 cholerae toxin protein into the enterocyte which can activate the adenylate cyclase enzyme. This enzyme causes ATP to become c-AMP, which is produced to come out of sodium chloride and water in the enterocyte to the lumen of intestine and to become diarrhae [1][2][3][4].

Some vaccines have already been developed to prevent diarrhae caused by *V. cholerae*. Killed whole-cell *V. cholerae* O1 with purified recombinant B-subunit of cholera toxoid (WC/rBS), variant of the WC/rBS and an attunuated live oral VCD 103 HgR are a recent recombinant vaccine which has been developed to prevent cholera. Efficacy trials have shown that the vaccines are safe and confer 60-95% protection. The result of the study of the VCD 103 HgR vaccine in Jakarta Indonesia is not satisfactory [5].

Our recent study found that the 37.8 kDa protein at the tip of pili *V. cholerae* has a role in molecule adhesion. This study wanted to investigate whether the 37.8 kDa protein as a molecule adhesion at the tip of pili *V. cholerae* combined with sub unit B cholera toxin can provide protection against diarrhea. This the molecule adhesion 37.8 kDa combined with CT as might become a new candidate vaccine for cholera.

METHODS

Bacteria *V. cholerae* (El Tor Ogawa) comes from a patient suffering from diarrhae in the General Hospital, Faculty of Medical, University of Brawijaya, Malang, East Java of Indonesia.

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Bacterial culture

The TCG media was used to enhance pili production of *V. cholera* according to the Ehara method. The content of TCG media was 0.02% thioproline, 0.3% $NaHCO_3$, 0.1% mono sodium l- glutamat, 1% bactotryptone, 0.2% yeast extract, 0,5% NaCl, 2% bacto agar and 1 mM β-amino ethyl ether –N,N,N,"n",-tetra acetic acid (EGTA) [6].

Isolation protein pili

Bacteria cultur was collected from the TCG media in 20 roux bottles. The pili bacteria was isolated according to our method by using a pili bacteria cutter. The pili bacteria cutter was developed from a modification of an omni mixer. The differences between the tool and omni mixer are their naif, speed and time adjustment. The pili bacteria cutter does't have a naif. The naif has been replaced by a metal cylinder which can rotate. The tip of metal cylinder has a rough, sharp surface. The metal cylinder is rotated and adjusted by time and speed. Therefore the pili bacteria is cut in this way.

The collection of bacteria from 20 roux botles media were re-suspended by tri chlor acetic acid (TCA) until the concentration reached 3%. The suspension was shaken thoroughly for 30 seconds and stood at room temperature for 1 hour. A pellet was collected by centrifugation of the suspense at a speed 6.000 rpm for 30 minutes and at 4^o C temperature. Three grams of the pellet was re-suspended using 6 ml of PBS pH 7.4. The suspended bacteria were placed into the tube of the pili bacteria cutter.

Following this, the pili bacteria cutter was adjusted of the speed at 5000 rpm, for 30 second and at 4^o C temperature. The isolation of the pili bacteria continued by centrifugation of a suspense of the cutting pili at speed of 12,000 rpm, for 30 minutes and at 4^o C temperature. The supernatant contained rich pili protein of bacteria separated from the pellet and stored at 4^o C. The collection of supernatant of the second to third cutting was isolated from the pellet using same procedure of the first cutting. The collection of pili continued from the forth to seventh cutting. The only difference between the first and the forth to seventh cutting was an adjustment to speed 10000 rpm, within 60 seconds of pili bacteria cutter.

SDS-PAGE

SDS-PAGE was performed as described by Laemli [7]. The samples were boiled for 5 min in a final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue with 5% (v/v) 3 mercaptoethanol prior to electrophoresis through 5% stacking and 15% separating gels. The gels were then stained in Coomassie brilliant blue [7]

Coupling protein 37.8 kDa and CTB

The procedure of coupling sub unit pili 37.8 kDa with CTB was used by the following method. Protein sub unit pili 8 mg in PBS 1.5 ml was mixed with CTB 0.23 mg in 1.5 ml PBS and 2% glutaraldehyd 3 cc. The mixture was stirred at temperature of 4^o C for 1 hour. After that glycine 2.24 ml pH7,2 was added with and dialyzed at night.

Sample large

A large sample of 18 Balb/c mice and were divided into 3 groups, so each group had 6 Balb/c mice. The groups were provided with namely PBS is three kind of treatments were PBS, sub unit B cholera toxin and protein 37.8 kDa coupled with sub unit B cholera toxin.

Immunization

Every Balb/c mause was taken a natrium bicarbonat 0.2 M, 0.3 ml before treatment. Dose of treament for every mice was 250 µg of protein. Immunization was given every week 4 times.

Examination of s-IgA

Pooled mucous was taken from the illeum of the mice. Illium was cut at a lengthof 10 cm and mucous come out from the cut illeum where the entrails were removed. The rich mucous of s-IgA suspensed with an equal volume PBS. Collection of s-IgA in the mucous used centrifugation of suspense with a speed of 6.000 rpm, for 30 minutes and at a temperature 4^o C. Supernatant rich s-IgA was stored in 4^o C.

The content of s-IgA in the mucous was measured by ELISA using standart procedure.

Protectivity test

The researchers developed a new methode for measuring excretion of water from the enterocyte into the illeal lumen. The weight of the section of the of illeal loop was measured and after that was placed in a 250 cc bottle with 200 ml of Hank's balance salt, NaHCO3 and HEPES solution. The bottles were placed in shaker at a temperature of 37^o C for 1 hour with agitation at a rate of 60 X per minute. After 1 hour the agitation weight of the illeal loop was measured.

Data analysis

Data analysis used *SPSS for Windows* 13.

RESULTS

Profile protein pili *V. cholerae* is cut by pili cutter found in Figure 1.

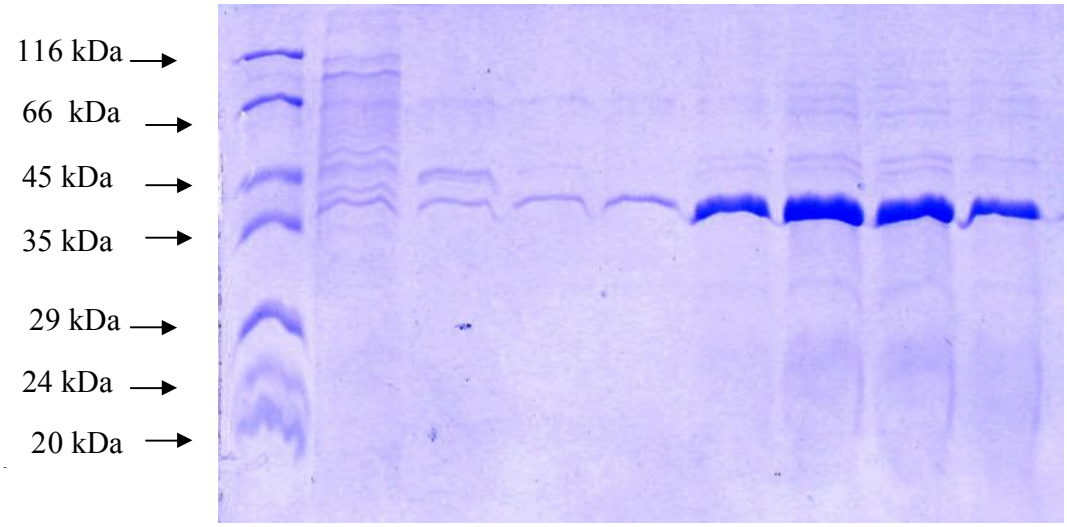

Figure 1 Profile protein pili Vcholerae by using pili cutter

Lane 1: Marker (Sigma) *)
2: Whole cell
3: 1st cutting
4: 2nd cutting
5. 3rd cutting
6. 4th cutting
7. 5th cutting
8. 6th cutting
9. 7th cutting

The result demonstrate that the protein 37.8 kDa appear consisten from the fourth until the seventh cutting. If it is compared with the protein in the first untill third cutting, there is marked difference.

The result an examination of a s-IgA depigted in Table 1

Table1 Result of the examination of a s-IgA among difference treatment

Macam Perlakuan	Mean	SE	SD	Sig p<0.05
Protein 37.8 kD + CTB	31.5	14.4	5.9	a
Protein 37.8 kDa	37.5	11.2	4.6	a
CTB	49.8	20.4	8.3	a
PBS	57.6	25.4	10.4	a

Although mean of the s-IgA control compared with the s-IgA CTB statically not different, if the mean of s-IgA control or s-IgA CTB compared with s-IgA protein 37,8 kDa the result is significantly different.

Calculations of the s-IgA in the treatments is found in bar digrams Figure 2.

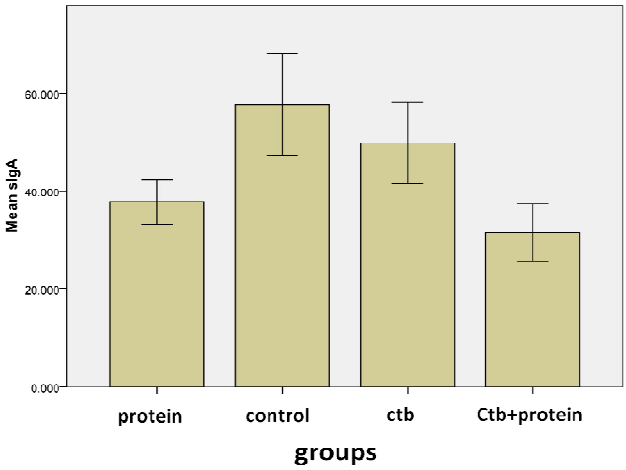

Figure 2 Results of the experiments with various treatments. Bar diagrams in Figure 2 show the difference among the three types of treatments.

The protectivity of various treatments by using ileal loop tie test was depicted in Figure 3.

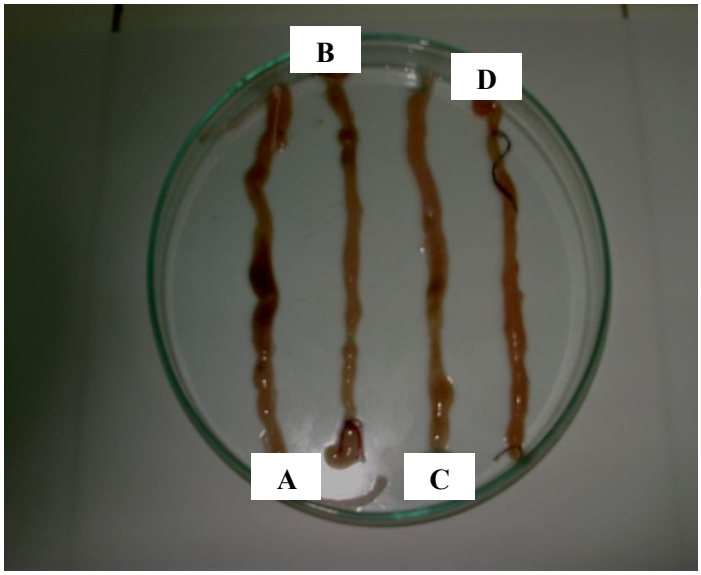

Figure 3 The result of the protectivity of various treatments using mice ligated ileal loop tie test on mice.

A. Not immunized + *V.cholerae*
B. Immunized with protein 37,8 kDa + CTB + *V. cholerae*
C. Immunized with CTB + *V. cholerae*
D. Immunized with 37.8 kDa + PBS

Result: the difference of measurement of the weight in various treatments with mice ileal loop tie model found in Table 2.

Table 2 Results of various treatments by weight in the mice ileal loop test.

Macam Perlakuan	Mean	SE	SD	Sig p<0.05
Protein 37.8 kD + CTB	477.00	3.251	3.521	a
Protein 37.8 kDa	491.33	37.399	37.399	a
CTB	647.00	1.506	3.688	b
PBS	701.17	0.509	2.317	c

A comparison of the results show that non immunized + *V cholerae* between immunized protein 37,8 kDa + CTB + *V. cholerae,* immunized protein 37,8 kDa + *V. cholera,* and immunized CTB + *V cholerae* is significally different. Immunized protein 37.8 kDa + CTB + *V. cholerae* between immunized protein 37.8 kDa + *V. cholerae* is not significally different. Figure 4 shows the difference among treatments

The amount of bacteria *V. cholerae* that attach to the surface of intestine cell is depicted Figure 5.

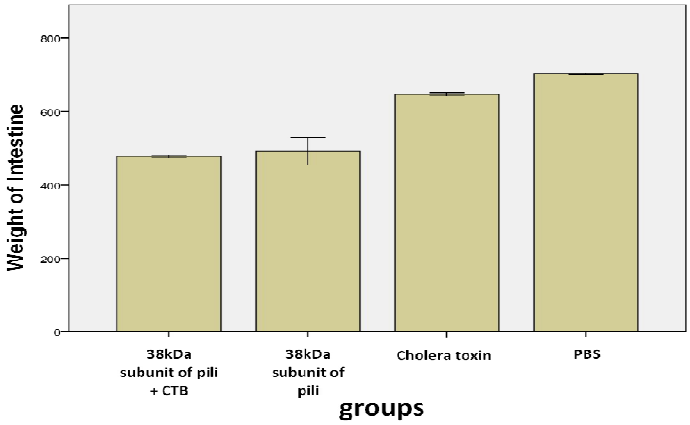

Figure 4. Results of the experiments of protectivity with various treatments

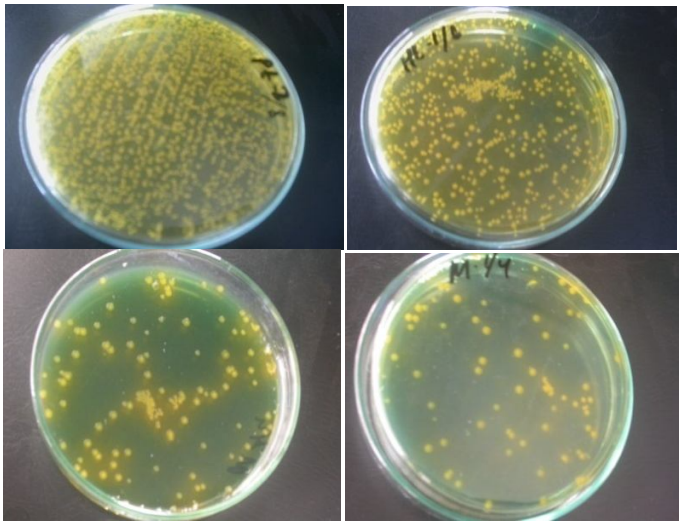

Figure 5 Colony forming unit in every sample treatment

DISCUSSION

The profile of the protein a sub unit pili *V. cholerae* in Figure 1 is consistent. The results of the first cutting to the third cutting of the profile protein is identical and is not found to be protein dominant. In fourth to seventh cutting the profile of the protein sub unit pili is also identical. The results of the cutting show that any dominant protein has a molecular weight 37.8 kDa. May be the localization of the 37,8 kDa is at the tip of the pili *V. cholerae* and has a function of molecular adhesion. Naturally in the outer membrane bacteria *V. cholerae* have a protein dimer molecular form weight of 76 kDa. Protein weight 37.8 kDa is as monomer form of outer membrane protein weight 76 kDa. The protein weight 37.8 kDa at the tip of the pili *V. cholerae* is identical with monomer form of weigh 37.8 kDa outer membrane proteins *V. cholerae.* Sperandio [8] also found the protein of

37.8 kDa in the outer membrane of *V. cholerae* (Manuscript in preparation).

We have already designed pili bacteria cutter which was used for the tool isolation of pili bacteria. The principle of difference our tool between omni mixer was usually used is in the knife. Omni mixer was usually used for isolation pili of *V. cholerae* [6] has four knives which make a cross each other. The cutter of pili made from stainless steel stick which has 11 cm long and diameter 18 mm. The tip of stick has a long 3.5 cm and diameter 22 mm and the surface was cartel led 1 mm deep. So the surface of this part has the smooth and sharp protrudes and has a function as knife. Opposite of the tip one, the tip was fixed to the motor apparatus so the stick can rotates. The tube of pili bacteria cutter for collecting the sample is smaller than the omni mixer. Three grams of the pellet was re-suspended using 6 ml of PBS enough and can put it in. The pili bacteria cutter is reproducible and maybe is accepted as a tool for bacterial pili collection beside omni mixer.

An examination of a s-IgA is presented in Table 1 and Figure 2. The result show the mean of s-IgA control compared with s-IgA CTB, protein 37.8 kDa and protein 37.8 kDa *V.cholera* combines with CTB are statistically not different. This study may be used anti human s-IgA.

Figure 3 presented the results of experimental protectivity using the mice ligated ileal loop model (MLIL). The model has some advantages if compared with a rabbit ileal loop. In the mice ileal loops tie model is easier than the previous model. The last model is friendly to animals and does not cause pain. The rabbit animal model was pain full in the second step of the ileal loop. The second step was a replacement of abdomen content into the abdominal cavity. After that an abdominal skin incision was closed by a suture. Before it was killed the animal still needed alive for 8 to 10 hours so it was pain full. May be in the future MLIL can replace rabbit ileal loop model [9].

Table 2 and Figure 4 shows the result of the difference of measurements of a weight ileal loop tie in various animal model treatments. Comparison of the result of immunized CTB + *V. cholerae* between non immunized + *V. cholerae,* immunized CTB + *V. cholerae* between immunized protein 37.8 kDa + CTB + *V. cholerae* and immunized CTB + *V. cholerae* between non immunized +*PBS* are different but not statistically. May be the results are due to the other toxin of *V. cholerae* still being active. The toxin is a zonulla occludence toxin (zot). In the experiment the zonulla occludence toxin may be still active. Zot can damage zonulla occludence of the enterocyte. Zonulla occludence damage can leak a sodium chloride ion and water to discharge into the lumen gastro intestinal. A comparison of the results of experiments in immunized protein 37.8 kDa + CTB + *V. cholerae* between none immunized + *V. cholerae* and non immunized + PBS between non immunized + *V. cholerae* are statistically different [10][11][12].

The antibody of anti adhesion molecule protein was 37.8 kDa s-IgA give protections to colonization of the bacteria *V. cholerae*. Without colonization of bacterial *V. cholerae* zot is not produced. The production of antibody CTB s-IgA can protect attachment of CTB receptor ganglionic monoxide (GM1) in a mucous surface of yeyunum/ileum. A failure of attachment CTB to GM1 cause c-AMP cyclase is inactive, because sub unit A1 can not to be introduced into the enterocyte. The sodium chloride ion and a water pump in the enterocyte is not a disturbance [13][14][15].

To date three oral cholera vaccines are available. All have been shown to the safe, immunogenic and effective. One vaccine consists of killed whole-cell *V. cholerae* O1 with purified recombinant subunit of cholera toxoid (WC/rCB). Efficacy trials have shown that this vaccine is safe and convers 85-90% protection. The protection declined rapidly after six months in young children, but was still about 60% in older children and adults after two years. A variant of the WC/rCB vaccine containing no recombinant B-subunit has been produce and tested in Vietnam. A field trial conducted in 1992-1993 in Vietnam showed a protective efficacy of 66% at eight months in all groups. CVD 103-HgR, a vaccine consist of an attenuated live oral genetically modified *V. cholerae* O1 strain has been trialed in United State of America, Micronesia and Indonesia. A large field trial CVD 103-HgR vaccine performed in Indonesia has not shown convincing protection in a population exposed to cholera for an extended period after immunization (WHO 2009).

Finkelstein [16] proposed that the ideal cholera vaccine should have an adhesive molecule, a CTB, taken orally, not produce diarrhea and with no obvious there are no side effects. This studied huffily can fulfill the ideal cholera vaccine.

CONCLUSION

The goal of these experiments was to verify that the protein adhesion 37,8 kDa combined with the CTB can induce s-IgA and therefore have a protective function toward the diarrhea caused by *V. cholerae*. The results of these research had seen that protein adhesin pili *V. cholerae* 37,8 kDa combined with CTB was able to induce an immune response with increased of content of s-IgA and protection toward liquid secretion in the small intestine.

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