

IMMUNOGENITY OF 32.2 kDa HEMAGGLUTININ BM PROTEIN OF *Escherichia coli* PILI ISOLATED FROM INFERTILE MALES' SEMEN

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ABSTRAK

Infeksi traktus genitalis pria merupakan salah satu penyebab infertilitas. Escherichia coli (E. coli) adalah penyebab utama prostatitis dan epididimitis yang dapat menurunkan kualitas spermatozoa. Salah satu faktor E. coli dapat menurunkan kualitas sperma karena kemampuan E. coli melekat ke membran plasma sperma menggunakan pili. Telah berhasil diisolasi protein hemagglutinin pili E. coli isolat semen pria infertil dengan BM 32.2 kDa. Tujuan penelitian ini adalah untuk mengetahui immunogenitas protein hemagglutinin pili E. coli BM 32.2 kDa isolat semen pria infertil. Dilakukan immunisasi pada kelinci jantan menggunakan protein hemagglutinin pili E. coli BM 32.2 kDa sebagai antigen. Titer antibodi yang terbentuk diukur menggunakan metode indirect elisa dan hasilnya dibaca menggunakan elisa reader. Reaktivitas antigen dan antibodi yang terbentuk di uji dengan metode dot blot dan western blot. Hasil penelitian ini adalah melalui uji indirect elisa, protein hemagglutinin pili E. coli BM 32.2 kDa isolat semen pria infertil dapat merangsang terbentuknya antibodi. Hasil uji dot blot dan western blot terbukti bahwa antibodi yang terbentuk bereaksi secara spesifik dengan antigen. Kesimpulan penelitian ini adalah protein hemagglutinin pili E. coli BM 32.2 kDa bersifat immunogenik dan berpotensi sebagai kandidat bahan vaksin dalam upaya pencegahan infeksi traktus genitalis pria.

ABSTRACT

Male genital tract infection is one cause of infertility. Escherichia coli (E. coli) is a major cause of prostatitis and epididymitis that can degrade the quality of spermatozoa. One factor E. coli can reduce sperm quality due to the ability of E. coli attached to the sperm plasma membrane using pili. Have successfully isolated the protein hemagglutinin pili of E. coli isolates were infertile men with BM cement 32.2 kDa. The purpose of this study was to determine the protein hemagglutinin pili immunogenitas E. BM coli isolates 32.2 kDa cement infertile men. Immunization performed on male rabbits using protein hemagglutinin pili of E. coli BM 32.2 kDa as antigens. Antibody titers formed were measured using indirect methods and the results were read using elisa reader. Antigen and antibody reactivity are formed in the test by the method of dot blot and western blot. The results of this research is through indirect elisa test, the protein hemagglutinin pili of E. BM coli isolates 32.2 kDa cement infertile men can stimulate the formation of antibodies. The results of dot blot test and western blot proved that the antibodies react specifically with antigen. The conclusion of this study is the protein hemagglutinin pili of E. coli BM 32.2 kDa is immunogenic and potentially as a candidate vaccine ingredient in preventing male genital tract infections.

Key words: protein hemagglutinin pili of *E. coli* BM 32.2 kDa, immunogenicity, indirect elisa, western blot, dot blot

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INTRODUCTION

Government program of population control through family planning (FP) has been successful. But the people who hardly have descendants (infertile) also should receive attention because infertility is a problem in a marriage. Society assumes that women as a cause can not give offspring. Yet according to Sikka (2001), 30-50% of infertility is caused by the men. Male genital tract infection is a cause of infertility for 10% (Khanna, 1992). Outbreak of infection is generally through sexual intercourse with a partner who alternated which are grouped into STD (Sexually Transmitted Disease). This STD is one of the issues of concern to experts, not only

in Indonesia but also throughout the world. STD that causes the typical symptoms such as Gonorrhoe can now be treated thoroughly. Genital tract infection and male accessory sex glands include prostatitis, epididymitis, vesiculitis, and orchitis does not cause symptoms or are asymptomatic. The results hinting (2006), found that from 1032 infertile men who visit Surabaya in infertility clinic, the incidence of accessory sex gland infections 13.4%.

E. coli is a major cause of prostatitis and epididymitis (Liu et al. 2002). *E. coli* can reduce the motility of spermatozoa (Huwe et al. 1998, Diemer 1996). Kohn (1998) showed that incubation of spermatozoa with *E.*

coli in vitro effect on the acrosome reaction. Fusion of spermatozoa with hamster oocytes was reduced after incubation of spermatozoa with *E. coli*. *E. coli* also showed close the equatorial segment of human spermatozoa membrane. According to Cummins (1995) segment equator spermatozoa membrane functions as a fusion of spermatozoa with the oocyte. Thus the closing segment of the equator spermatozoa can lower levels of fertilization.

E. coli factors can degrade the quality of spermatozoa due to the ability of *E. coli* attached to the membrane using the spermatozoa found on the tip adhesin pili. Antibiotics were not able to completely eliminate bacteria and infections that result in leukocyte infiltration in the elimination difficult. This opinion is supported by Sharma et al. (2001) which states that antibiotic therapy is often successful in reducing the white blood cells, but found 80% of semen samples containing white blood cells microbiologically negative.

Block the attachment of bacteria to the host is the most effective strategy to prevent bacterial infection. The attachment blocking used molecular adhesin (Wizemann et al. 1999). Potential immunogenicity and protectivity is an absolute requirement to be met by a protein adhesin to be able to block bacterial adhesion in vivo. Have succeeded in isolation and characterized isolates of *E. coli* proteins hemagglutinin pili cement infertile men with BM 32.2 kDa. Hemagglutinin protein has been demonstrated pili of *E. coli* is able to block adherence of *E. coli* to human spermatozoa in vitro. The purpose of this study was to determine whether the protein hemagglutinin pili of *E. coli* 32.2 kDa molecular weight is immunogenic via Indirect Elisa test, Western blot and Dot blot.

MATERIALS AND METHODS

Protein pili *E. coli* isolates were infertile men with BM cement 32.2 kDa. Local male rabbits, androgynous male, aged about 3 months with a weight about 2.5 kg.

Preparation of Antibodies

Before the rabbits were used, they were acclimatization for 2 weeks in advance. At the first place, the treated rabbits had their blood drawn as a control (pre-immune). Antigens used were hemagglutinin pili proteins of *E. coli* BM isolates 32.2 kDa cement infertile men with BM 32.2 kDa. Immunization procedure in rabbits is 800 mg antigen suspended with 800 µl PBS, plus complete Freund adjuvant 800 µL, were mixed to form a white emulsion. Then injected sub cutaneous at 5 points, where the part to be injected in

the first disinfection with 70% alcohol. Intermittent repeated one week later injected antigen mixed with incomplete Freund adjuvant on the sub-cutaneous at 5 points. Immunization was continued every week in the same way until the end of week five. Blood sampling is done every week through the lateral vein in the ear. This blood was collected in tubes every week that have been labeled and stored in cupboards ice. Blood stored in a cupboard ice will be split into sections containing blood cells and serum. Then in each tube was taken and the serum was transferred to another tube.

Purification of antibodies

SAS serum plus 50% with volume ratio of 1:1, the vortex, allowed to stand 30 minutes in a cupboard ice, then centrifuged 6000 rpm, temperature of 40C for 10 minutes. SAS pellets plus 50% by volume of 10 x the volume of pellets, in the vortex, settling in cupboards ice 30 min and then centrifuged 1000 rpm, temperature of 40C for 10 minutes. Furthermore, pellets plus 0.2 M phosphate buffer pH 8 with a ratio of 1:1, in the vortex. Performed dialysis in 0.1 phosphate buffer pH 7 in refrigerator overnight. Furthermore, coupled with cold ethanol volume ratio of 1:1 and stored in a cupboard ice 30 minutes. Then centrifuged 6000 rpm 10 minutes and put the freezer 5 minutes. Final pellet Tris HCl plus 200 mL and stored at -200C for use Elisa test, Dot blot and Western blot.

Indirect Elisa

Protein hemagglutinin pili of *E. coli* BM 32.2 kDa (as antigen) is mixed with the coating buffer with a ratio of 1:10, in the vortex. The mixture was superimposed on the Elisa plate wells, and incubated overnight at a temperature of 40C. After that in the wash with 100 mL PBS Tween 20, 3 times each wash for 3 minutes. Furthermore it was blocked using blocking buffer 1% BSA in PBS, and incubated for 2 hours at room temperature, then washed with 100 mL PBS Tween 20 for 3 minutes, washing was repeated 3 times. After that the coating with primary antibodies (antibodies derived from rabbit serum that had been purified) mixed with 1% BSA with a ratio of 1:20, and incubated for 2 hours. Further, it was washed with 100 mL PBS Tween 20 for 3 minutes 3 times. Later in the coating with secondary antibody (anti-rabbit Ig G alkaline Phosphatase Conjugate) with a 1:2500 dilution, and incubated for 1 hour at room temperature. The results in the wash with 100 mL PBS between 20, 3 times each wash for 3 minutes. Then added substrate pNPP in Diethanolamin 10%, incubated 30 min at room temperature. Later, the termination reaction was performed by adding 3 M NaOH and incubated 15 minutes at room temperature.

The result is read by Elisa Reader at a wavelength of 405 nm

"Dot Blot"

Membrane soaked Nitrocellulose PBS for 30 minutes, then assembled on the tool dot blotter. Antigen (protein pili of *E. coli* BM 32.2 kDa) that has been in dilute with Tris Buffer Saline (TBS) 1% sodium azide at a ratio 1:4 Nitrocellulose dripped on paper each 50 mL wells. Then it was carried out using a vacuum pump de gas for 3 minutes. Then do the blocking with TBS-Skim Milk 5% and incubated 1 hour. After that in the wash with 50 mL TBS Tween 20, 0.05% for 3 minutes in repeated 3 times. It was dropped with 50 mL each sinks with primary antibodies (antibodies derived from rabbit serum) diluted in TBS with a ratio of 1:20, incubated 2 hours while in the rocking. Further washing with 50 mL TBS Tween 20, 0.05% for 3 minutes in repeated 3 times. It was dropped with 50 mL of secondary antibody anti-rabbit Ig G alkaline Phosphatase Conjugate that in dilute with TBS on a comparison of 1:2500, and incubation for 1 hour while gently shaken. Then wash with 50 mL TBS Tween 20, 0.05% for 3 minutes in repeated 3 times. Furthermore, with 50 mL Western blue substrate was dropped in a dark room. Wrap the appliance with aluminum foil, incubated 30 min while shaken gently. Termination of the reaction carried out by adding 100 mL each distilled water wells, incubating 15 minutes. Then the membrane was dried nitrocellulosa. If there is a blue colored stain mean specificity of positive reactions, if not formed stain blue means negative reaction specificity.

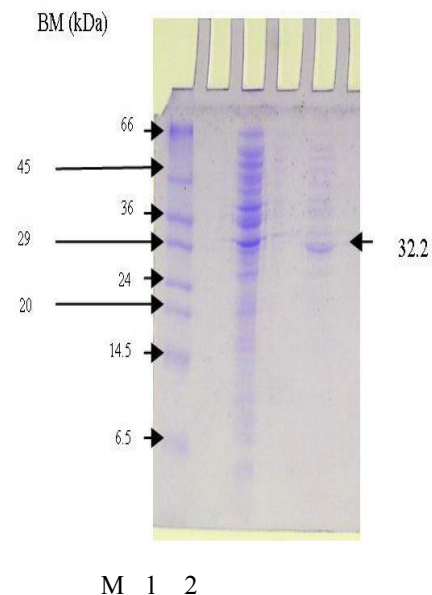
"Western Blot"

Nitrocellulose membrane and gel electrophoresis results soaked in transfer buffer for 40 minutes. Four filters and four sheets of thick filter paper moistened with the buffer used to transfer. Each arranged in sequence from top to bottom is 2 pieces of thick filter-paper filter 2 pieces - Membrane nitrocellulose - Gel electrophoresis results - 2 sheets of filter paper - 2 pieces of thick filter. Sandwiches are set on trans tools semi-dry blotting and the Bio Rad device is connected to the Power supply set 0.3 A, 20 volts for 2 hours for the transfer process. Furthermore, the membrane in washing with distilled water nitrocellulose to remove gel that is still attached. Ponco then immersed in 2% for 30 minutes, and cut markers. The membrane was washed with distilled water nitrocellulose without marker until the color disappeared. Then in blocking with 3% BSA TBS and then incubated overnight at 40C. Wash with TBS 0.05% Tween 20 for 10 minutes 2 times. The primary antibody (antibody from rabbit serum) was dropped and diluted in TBS with a ratio of 1: 20, 2-hour incubation with the

rocking slowly. Then wash with TBS 0.05% Tween 20 for 10 minutes 2 times. It was dropped with the secondary antibody anti-rabbit Ig G alkaline Phosphatase Conjugate that in dilute with TBS on a comparison of 1:2500, 1 hour incubation with the rocking slowly. Wash with TBS 0.05% Tween 20 for 10 minutes 2 times. Western blue substrate was dropped done in the darkroom. It was shaken for 30 minutes in a light-tight container. The reaction was stopped by adding distilled water. Nitrocellulose dried membrane, the results are immediately recorded.

RESULTS

The results of SDS-PAGE protein isolate pili cement infertile men



Description: M: Marker 1: whole cell *E. coli*; 2: pili *E. coli*

Indirect Elisa

Test specificity of antigen - antibody quantitatively performed by the method of Indirect Elisa. The absorbance data are seen in Table 1. These data suggest that in rabbits immunized antigens capable of inducing the formation of antibodies against these antigens. Absorbance value indicates that the results of immunization on week 5 has the highest absorbance values. It can be concluded that in week five produced the highest antibody titers compared to controls or in the previous week.

Table 1. Antibody absorbance value of immunization of rabbits with hemagglutinin protein of *E. coli* BM 32.2 kDa using the method of Indirect Elisa

No	Treatment	Absorbance
1	Control	0.394
2	Week I	0.402
3	Week II	0.422
4	Week III	0.424
5	Week IV	0.444
6	Week V	0.890

"Dot Blot"

"Dot blot" is done using a dot blotter. Purpose test "Dot Blot" is to prove the existence of the reaction of protein hemagglutinin pili of *E. coli* BM 32.2 kDa (antigen) with antibodies produced by rabbits injected with the protein hemagglutinin pili of *E. coli* BM 32.2 kDa. If no reaction means that the antigen recognized by the antibodies. The occurrence of the reaction is characterized by the formation of blue colored stain. The results of the "dot blot" is as follows:

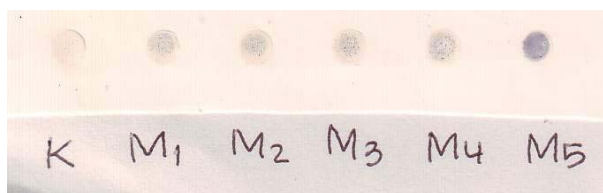


Figure 1: The "Dot Blot"

Description:

K. controls	M1. The first week
M2. Week two	M3. Week three
M4. Week 4	M5. Week 5

Shown in Figure 1 that there are shades of stain as a result of reaction of antigen with antibody. Gradation of color is influenced by variations in the concentration of antigen and antibody. At week five formed blue stain darker than the previous week. It shows in week five levels of antibodies formed larger, because the higher levels of antibodies that react with antigens produced by the dark color.

"Western Blot"

"Western Blot" is used to determine that the protein hemagglutinin pili of *E. coli* BM 32.2 kDa as antigens reacting with antibodies specific induction results. The results of "Western Blot" as shown in figure 2. Shown in the picture antigen antibody reacts specifically with

the induction by these antigens are proteins hemagglutinin pili of *E. coli* BM 32.2 kDa recognized by the antibody.

Western blot results shown in the picture below:

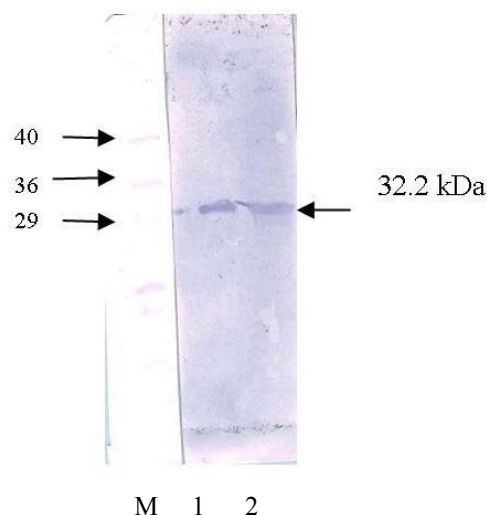


Figure 2: The "Western Blot"

Description: M: Marker. 1,2: Samples of *E. coli* proteins BM 32.2 kDa. protein hemagglutinin pili of *E. coli* BM 32.2 kDa that are recognized by antibodies induced the protein hemagglutinin pili of *E. coli* BM 32.2 kDa.

DISCUSSION

The results of the male rabbit immunization with the protein hemagglutinin pili of *E. coli* BM 32.2 kDa produce antibodies. To determine the titer of antibodies formed Indirect Elisa test done. The result is an injected antigen capable of stimulating antibody formation. Antibody titers are the highest form generated at week 5.

Reactivity of antibodies obtained from immunization results in the test method "Dot Blot". In "Dot Blot" indicates that the antibody has reactivity with an antigen. This suggests that the antigens can be recognized by the antibody. The resulting blue color is darker in week 5 than in week 1, 2, 3 or 4. These results are consistent with the Indirect Elisa that in week 5 produced the highest antibody titers. At high antibody titers are much stronger reaction between antigens and antibodies that produce a darker blue color.

The "Western Blot" was also performed to test reactivity. The result is a pili antigen hemagglutinin protein of *E. coli* BM 32.2 kDa antibody response by

antigen immunization results. It is characterized by the formation of protein bands at 32.2 kDa BM is colored blue. Conjugate anti-rabbit Ig G alkaline phosphatase labeled as secondary antibody will bind to the primary antibodies, i.e. antibodies produced by rabbit immunization with pili protein *E. coli* BM 32.2 kDa. Giving the substrate will bind to the alkaline phosphatase resulting blue color in a specific band.

The results of this study can be concluded that the protein hemagglutinin pili of *E. coli* 32.2 kDa molecular weight is immunogenic. In previous studies researchers have also proved that the 32.2 kDa protein hemagglutinin pili BM *E. coli* isolates semen of infertile men to function as adhesin (Sukarjati 2008). Thus, this protein has potential as a vaccine candidate material to prevent male genital tract infections. This is in accordance with the opinion Salyers and Whitt (1994) which states that if the adhesin protein is immunogenic, the protein is able to stimulate the formation of anti-adhesin antibodies that potentially as a vaccine, which can then be used in prevention of infection.

Adhesin responsible for the recognition and binding to specific receptor structures on the host cell. Bonding that occurs can stimulate host cell signal transduction in host defense cells that activate or inhibit cellular processes that facilitate bacterial invasion. Attachment may also stimulate the expression of new genes in microbes that are needed in the process of pathogenicity (Soto and Hultgren 1999)

The study of protein adhesin as a vaccine material to give hope in the future. Development of vaccine materials that are based on adhesin molecule gives two advantages of antibodies to the adhesin causes the attachment of bacteria to Host barriers and improve the process of elimination of bacteria (Henahan 1997).

Studies that use pili *E. coli* as a vaccine is tested in animal models to prevent urinary tract infections has been done by previous researchers. Experiments in mice immunized with the fimH antigen can reduce colonization of *E. coli* in the bladder more than 99% (Salyers and Whitt 2002). Langermann et al. (1997) reported that humoral Ig G antibodies directly against the adhesin of type 1 (Fim H) that protects mice from renal infection with *E. uropatogenik coli*. Aronson et al. (1979) reported the provision of soluble receptor analog (methyl- α -D-mannopyranoside) can block pili-mediated adherence of type 1 to the network has to prevent a UTI (Urinary Tract Infection) in the mouse. Thankavel et al. (1997) also reported an antibody that specifically binds fimH significantly reduced infection of *E. coli* kidney in experiments using mouse models. Connell et al. (1996) from the results of his research

found that the fimH *E. coli* showed reduced ability to live on with murine UTI model.

CONCLUSION

The results of this study can be concluded that the protein hemagglutinin pili of *E. coli* 32.2 kDa molecular weight is immunogenic.

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