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A 15.13 kDa*Enterobacter gergoviae* Biofilm Predictive Protein is Recognized bysIgA in Bronchial Aspirate from Patients with VAP

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***Abstract*:** *Ventilator-associated pneumonia (VAP) is difficult to diagnose, expensive to treat, and is associated with significant morbidity and mortality. The major part of VAP pathogenesis is the formation of a biofilm structure which also plays role in its resistance to treatment. A strategy to preventVAP isby removing the biofilm structure. The aim of this study is looking up the biofilm predictive protein of Enterobacter gergoviae (E. gergoviae) which is recognized by Secretory Immunoglobulin A (sIgA) as a mucosal immune response in VAP patient. This study used a tube adherence method for the detection of biofilms, protein profiling techniques with SDS PAGE and blotting for the evaluation of the antigen antibody response. The sIgAwere isolated from bronchial aspirate of VAP patient at ICU RSSA Malang. From two E. gergoviae samples, one sample biofilm positive (sample 1), and theother was biofilm negative (sample 2). The biofilm predictive protein was detected using SDS PAGE, and is discovered to be 15.13 kDa. The sIgA from sample 1 (p 1) reacted with protein band 15.13 kDa, 38.02 kDa, 45.71 kDa and 47.21 kDa whilesIgA from sample 2 (p 2) reacted with 38.02 kDa, 45.71 kDa, 47.21 kDa, and 50 kDa. This finding was predicted that protein MW 15.13 kDa played role in biofilm formation.*

Keywords:*VAP,biofilm, Enterobactergergoviae, sIgA.*

I. Introduction

Ventilator-associated pneumonia (VAP) is a nosocomial pneumonia which happens after 48 hours of ventilator use through the trachea or also tracheostomy[1,2]. Although major advances in techniques in patient management has established and effective procedures, VAP as a complication of ventilator-use still occurs in about 8-28 % of cases[2,3,4]. Statistically, VAP risk in ventilator use-patient is about 1-3 % per day[5]. The mortality rate of VAP patients after 48 hours is about 24- 76% [3].

An important step in the development of VAP requires bacterial colonization in the oropharyng[2]. The aspirate pools around the cuff of endotracheal tube and is easily to contaminated[6]. Thus, this bacterial colonization results in theformation of biofilm structure, which is a main part of VAP pathogenesis[2,4].

Biofilm is defined as a complex structure of bacterial community on a surface (biotic or abiotic) and is responsible in the pathogenesis of infection associated with the use of medical devices (internal or external)[7]. This structure increases the resistance of the bacterial community to treatment[7,8].One prevention strategy of VAP isby removing the biofilm structure [9].

Commonly, a response to mucosal infection is shown as the increase of locally secreted antibody, mainly composed of Secretory Immunoglobulin A (sIgA).This acts as a protective antibody which increases host defenses to biofilm structure [10]. It is the first line of defense to the pathogenic microorganisms. It has a multi functional, in which can act as a protection (high affinity) to foreign substances and pathogenicmicrobes, a comensal microbes protection, and a protection of mucosal inflammation [10,11].

The bacteria which is dominant in VAP varies depending on the case, institution, first antibiotic response, local resistance patterns, and ventilator use-patterns[2,5]. Many studies show more than 60 % of VAP cases is caused by Gram negative rod *Enterobacteriaceae*in which the prognosis would be worst than Gram positive bacteria(in antibiotic sensitive bacteria) [3]. In last decade, many cases of*Enterobacter*bacteremia, especially in ICUis reported. This bacteremia occurs1.3 – 2.5 more often in men, neonates and old age with a mortality rate of 20-35 %. Otherwise, this species also shows the resistance of antimicrobial drugs [12].

An annual report from the Microbiology Department of Saiful Anwar Malang Hospital (RSSA) in 2010 shows that the majority of the bacteria found in the sputum of ICU patients is *Enterobacter gergoviae* (43%)[13].The aim of this study is looking up the biofilm predictive protein of *Enterobacter gergoviae* (*E. gergoviae*) which is recognized by Secretory Immunoglobulin A (sIgA) as a mucosal immune response in VAP patient.

II. Material and Methods

2.1 Collecting the bronchial aspirate.

Bronchial aspirate was collected from the patientstreated in the ICU which shows aClinical Pulmonary Infection(CPIS) score ≥ 6 with the patients consent[5]. This research is also approvedby the ethical committee in RSSA Malang. The aspirate is collected after 3 days of ventilator[14].Bacterial identification usesMcConkey Agar, Gram staining and *Microbact System* 24E. The *Microbact*24E was used because it is considered accurate for the *Enterobacteriaceae*family[15].

2.2 The detection of Biofilm formation

The detection method of biofilm formation wasby a tube adherencemethod described by Christensen *etal*, 1982. The suspensions of the tested strains were incubated in glasstubes which contained Tryptic Soy Broth (TSB) with glucose supplementation 0.25 % aerobicallyat 37°C for a period of 2 days.Then, thesupernatant was decanted and the tubes stainedwith a 1 % crystal violet solution. Lastly, the tube is washed with distilled water 3 timesand dried. A positive result was defined as the presence of a layerof the stained material which adheres to the inner wall of the tubes.A stained ring at the air-fluid levelwas considered as negative [16].

2.3 Ekstracellular Protein Isolation

The bacteria was inoculated in 10 mL nutrient broth, and incubated at 37º C. After 20 hours (18-24 hours),the broth wassentrifugated at 6000 rpm, for 15 minutes, at 4ºC to separate the extracellular proteins and the bacteria.[17,18,19].

2.4 Secretory Immunoglobulin A preparation

Secretory Immunoglobulin A(sIgA) preparation was obtained from the bronchial aspirate of VAP patient using precipitation methods with an extracting buffer. The bronchial aspirate was added with extract buffer, sentrifuged at 4000 rpm for 30 minutes, and supernatanwas decanted for next centrifugation. The final pellet was added with 50µL TrisHCl 6.8 0.5 M [20]. The extract buffer was added by Triton X-100 and a cocktail of proteinase inhibitor (Sigma) to prevent protein degradation. The concentration of sIgA was measured by *spectrophotometer nano drop*.

2.5 SDS Page Electrophoresis

Determining weight molecular protein was done by SDS-PAGE using theLaemli method.In this study, we compare the extracellular protein of biofilm positive and negative bacteria. Sample was heated for 5 minutes, 100ºC in a buffer which contains TrisHCl 5 mM pH 6.8, 2-*mercapto ethanol* 5%, *Sodium Dodecyl Sulfate* 2.5% w/v, glycerol10% v/v with *Bromophenolblue*. The separating gel concentration was a mini slab gel 12.5% and stacking gel 3%. The sample was run at 120 mV, 400mA for 90 minutes. Then, the protein was stained with C*ommassie Brilliant Blue R-250*[21].After profiling, the spesific protein was cut and inserted to dialyses tube for electroelution and dialysis for further protein purification [22].

2.6 Dot Blotting and Western Blotting

Dot blotting was done for a semiquantitative examination of the immune reactionfor the protein. Proteinswere diluted in a Tris-buffered saline (TBS)and 50 µL were inserted into each wellcovered by nitrocellulose paper. The protein was carried out using a vacuum pump de gas for about 3 minutes. After blocking with TBS-milk (TBS containing 3% nonfat dry milk powder)for 1 hour and washing with TBS tween for 3 times, sIgA as a primary antibody was added in each well and incubated at room temperature for 2 hours on shaker.After washing with TBS-tween, IgA antihuman alkaline fosfatase conjugate was added in each well with a dilution 1:2500. Chromogenic substrate (BCIP-NBT) was added after the final wash with TBS Tween[23]. The dot was measured by *Corel Photo Paint X6*.Western blotting was done for a qualitative examination of antigen-antibody reaction. After electrophoresis, the transfer to nitrocellulose membrane was done in about 120 minutes. Membranes were blocked overnight at room temperature usingTBS-milk. The following stepswere the same with dot blotting[24].Spectra multicolor broad range protein ladder were used.

2.7 Statistical Analysis

The mean value of *Corel Photo Paint X6* was analysed by Kolmogorov Smirnov test to determine the data distribution. The data distribution was normal ($p > 0,05$), so the Pearson correlation was used to correlate the dilution of antibody and the mean ($p<0,05$)[25].

III. Result

This study was done at RSSA Malangand Faculty of Medicineof Brawijaya University Malang. The total VAP cases at ICU RSSA Malang since February until Juny 2013 were 22 cases in which 3 cases were infected with *E. gergoviae*.One sample was accidentally broken. The subjects used in this study was a man, age 30 (patient 1) and a woman, age 21(patient 2). Both isolate showed a resultof 93.93% of Microbact system.

The detection for biofilm formation wasaccomplished usinga tube adherence method. From two isolate, 1 sample showed positive biofilm (Figure 1).Figure 1 showed that isolate *E.gergoviae*1 (tube BF) was positive for biofilm production because it presented a purple stained layer which adhered to the tube.

After the tube adherence method, the scrapping of the stained layer was done to look at the microscopic structure. There was a thick layer in sample 1 (Figure 2A), which was different from sample 2 (Figure 2B).

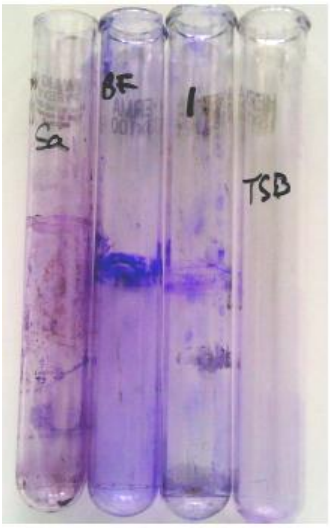

Figure 1. The detection of Biofilm with Adherence Tube Method
Sa: *Staphylococcus aureus*biofilm positive (Positive Control) showed a purple layer which adhered to the tube; BF: *E.gergoviae*1 showed a purple layer which adhered to the tube; 1: *E.gergoviae*2 showed no purple layer ; TSB: *Tryptic Soy Broth* (Negative Control).

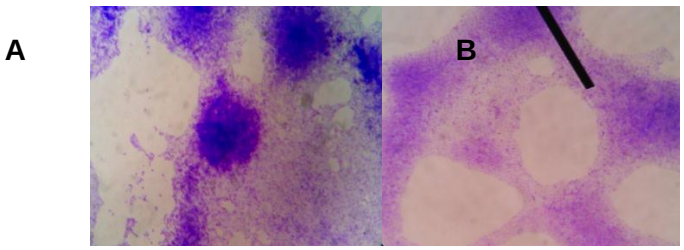

Figure 2.Microscopic Examination Directly from Tube Adherence Method (1000x)
A: *E.gergoviae* 1 showed a thick layer which covers the bacterial colonies and B: *E. gergoviae* 2 showed bacterial colonies.

The next step of this study was isolation of the extracellular protein from *E.gergoviae*. All isolates were grow on a nutrient broth (NB) to compare the protein bands.Besides the extracellular protein profile of both isolates, the profiling of *whole cell* (pellet)from all isolates was done to ensure the biofilm predictive protein that shown.

Figure 3 showed the differences between ekstracellular protein of *E. gergoviae* 1 and 2 when grown on NB (lane 3 and 4). The protein profile was compared with *whole cell E. gergoviae* 1 and2 (lane 1 and 2). A protein band with a size of 15.13 kDa observed in *E.gergoviae* 1 and not in *E.gergoviae* 2.

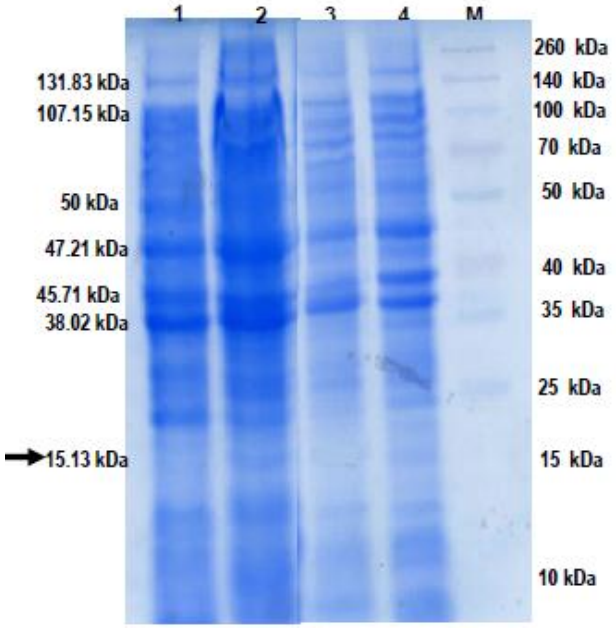

Figure 3. SDS PAGE of Protein *E.gergoviae*

1: *Whole cell* (pellet) *E.gergoviae* 2 (dilution 20x); 2 : *Whole cell* (pellet) *E.gergoviae* 1 (dilution 20x) ; 3: Supernatan *E.gergoviae* 2 in NB; 4: Supernatan *E.gergoviae* 1 in NB; M: Marker

The bronchial aspirate was collected from VAP patient and brought to the Biomedic Laboratory of Medical Faculty of Brawijaya University for sIgA isolation. It was processedby precipitation with extract buffer and sentrifugation, and measured by*nanodropspectrophotometer* (BioRad). It showed the concentration of p 1 (sIg A from sample 1) and p2 (sIg A from sample 2) were13.76 mg/mL and 1.33 mg/mL, respectively.

For dot blotting, each wells were added the antigen and antibody in many dilution. The best result was the dilution which shows the darkest spot by Corel Photo Paint X6. The antigen was a 15.13 kDa protein, and the primary antibody was p1 and p2. The secondary antibody was human anti Ig A with *alkaline fosfatase* enzyme.

Figure 4 showed the best reaction in p1 group was 1/1000 of antigen and 1/10 of antibody. In other group, the concentration of antigen protein and p 2 was $1/10^6$ and 1/100, respectively. These concentrations were used for the following western blotting methods.

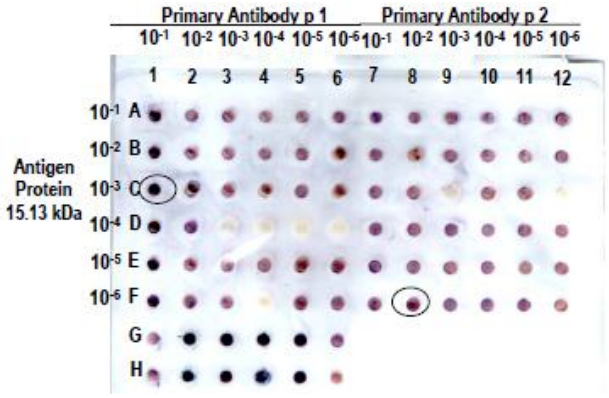

Figure 4. Dot Blotting between Antigen Protein 15.13 kDa with p 1 and p 2

G1 and H1 : antigen protein without dilution ; G2 anf H2: p 1without dilution; G3 dan H3 : p 2without dilution; G4 and H4 : antigen protein and p 1without dilution; G5 and H5: Antigen protein and p 2without dilution; G6 and H6: TBS without dilution. Black circles showed the strongest titer from each group of primary antibody.

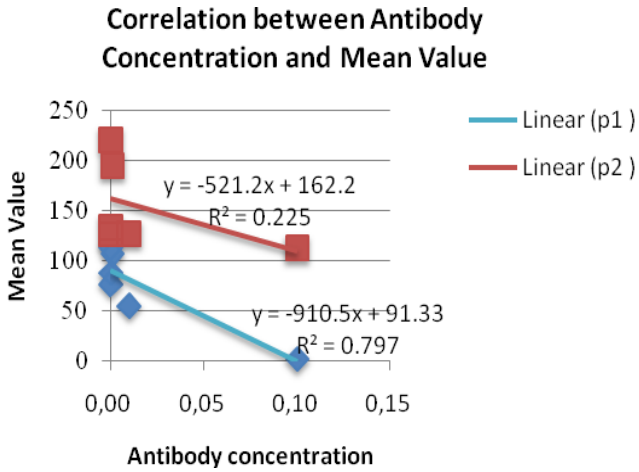

Figure 5. Correlation between Antibody Concentrationand Mean Value from Dot Blotting.
Blue line showed the reaction between antigen and p1 with R^2 =0.797and p=0.017 (p<0.05). Red line showed the reaction between antigen and p2 with R^2 = 0.225 and p=0.341 (p>0.05)

Figure 5 showed the correlation between antibody concentration and mean value from Dot Blotting. With the same antigen, the dilution of antibody concentration will affect the mean value of Corel Photo Paint X6 as a marker of antigen-antibody reaction. The Pearson correlation of p1 was -0.893 and p2 was -0.475. The correlation between the dilution of p1 concentration and the reaction of antigen antibody was significant (p<0.05) while the correlation between the dilution of p2 concentration and the reaction of antigen antibody was not significant (p>0.05).

Western blotting showed the differences of protein bands which reacted with p1 and p2. Primary antibody p 1 reacted with protein band 15.13 kDa, 38.02 kDa, 45.71 kDa and 47.21 kDa whilep2reacted with 38.02 kDa, 45.71 kDa, 47.21 kDa and 50 kDa.Protein 15.13 kDa just reacted with p1.

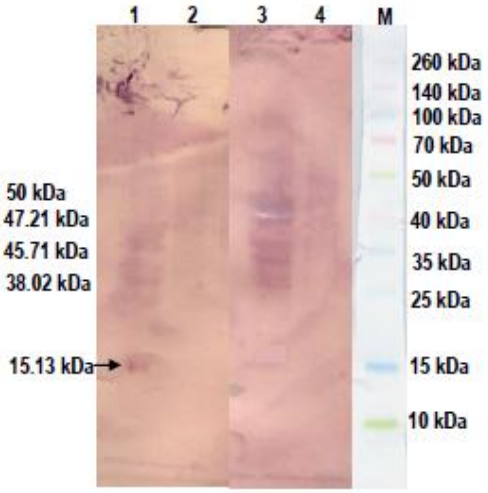

Figure 6. Western Blotting of Antigen Protein with p 1 (lane 1 and 2) and p 2 (lane 3 and 4) with marker (M).
Lane 1: supernatant *E.gergoviae* 1 with p1; 2: supernatant *E.gergoviae* 2 with p2; 3: supernatant *E.gergoviae* 1 with p2; 4: supernatant *E.gergoviae* 2 with p2.
Black arrow showed the biofilm predictive protein 15.13 kDa

IV. Discussion

VAP patients which were infected with *E. gergoviae* in this study was 13.6 %. Enterobacteriaceae was showed by many studies as a cause of VAP, with a worse prognosis compare to Gram positive bacteria. It caused morbidity and mortality and may also be multiresistant[5].

The biofilm formation played a main role of this infection pathogenesis. It was formed by bacterial colonization in oropharyng, and continued by the formation [2,4,6]. This formation may increase the drug

resistance and a phagocytosis response [7,18,26]. This biofilm could be difficult for the therapy, so a diagnostic tool was needed.

This study showed that *E.gergoviae* 1 was biofilm positive (Figure 1 and 2) using a tube adherence method. Niveditha *et al*, 2012 showed the sensitivity of the tube method and Congo Red Agar (CRA) method to be much the same about 44% and 56%, respectively[27]. Oliveira and Cunha, 2010 compared both methods to the polystirene plate methods and the best method was the tube adherence with a sensitivity and spesificity 100%. The polystyrene plate had a sensitivity of 97.6 %, and a spesificity of 94.4 % whilst CRA had a sensitivity of 89 % and a spesificity of 100 %[28]. From the microscopic examination, the thick layer with violet staining surrounding the bacteria (Figure 2A) was compatible with a polysaccharide extracellular matrix described generally as main constituent of bacterial biofilms[29].

The SDS PAGE was done to compare protein band from two isolates (the supernatant and the whole cell-*pellet*) which was grown on NB. The biofilm predictive protein was important because it played a main role in the secondary adhesion of biofilm formation. Secondary adhesion (locking)was mediated by a spesific adhesin protein on an abiotic surface and was followed by the formation of irreversible polysaccharide complex[8]. Flagella, pili, conditional adhesin or surface adhesin can be an early mediator for biofilm formation [7,30]. The different band was protein 15.13 kDa (figure 3). This band appeared only in *E. gergoviae* 1. A study of *Staphylococcus aureus* showed the result of SDS-PAGE from positive biofilm bacteria in an enrichment medium and in nutrient broth had a lower molecular weight protein[31]. Contrary to Loehfelm *et* al, 2008, the study showed a high molecular weight antigen (> 460 kDa) that reacted with monoclonal antibody of a biofilm associated protein from *Acinetobacter baumanii* [32].

The primary antibody of this study was asIgA from the bronchial aspirates of VAP treated patients with *E.*gergoviae (p 1 and p 2). We used sIgA because the biofilm was formed in mucosa, so the mucosal immune response was primarily by sIgA [10]. The concentration of p1 (13.76 mg/mL) was higher than p2 (1.33 mg/mL). It showed that sIgA from bronchial aspirate of VAP treated patients with biofilm positive *E.gergoviae* was higher than biofilm negative *E.gergoviae*.

Dot Blotting methods (Figure 4) showed the reaction of antigen antibody showed that the 15.13 kDa can bind with sIg A from VAP patient with biofilm positive (p1) and biofilm negative (p2). But, Figure 5 showed the difference correlation between the dilution of antibody concentration (p1 and p2) and the reaction of antigen-antibody.The correlation between the dilution of p1 concentration and the reaction of antigen antibody showed the medium significance correlation (R^2 =0.797; Pearson correlation=-0.893; p=0.017) while the others not (R^2 = 0.225; Pearson correlation=-0.475; p=0.341). It means there was a significant correlation between the dilution of sIgA from bronchial aspirate of VAP treated patients biofilm positive *E.gergoviae* concentration and the reaction of biofilm predictive protein-sIgA.

In western blotting method, the 15.13 kDa reacted with p1, and not reacted with p2. It showed that the protein with MW 15.13 kDa is a predictive biofilm protein which was isolated from bronchial aspirate of VAP patient.Therefore, this is expected to be a diagnostic tool of *E.gergoviae* biofilm positive.

The many bands which is showed on western blotting can be explained by various reasons, such as the use of polyclonal sIgA. The polyclonal antibody was not specific and could respond to many antigens (pili, whole cell, and other proteins). Polyclonal antibody could bind with many epitopes and therefore would be non spesific, but sensitive. Cross reaction would often happened[33].

V. Conclusion

The biofilm predictive protein 15.13 kDa could bind the polyclonal sIgA with biofilm positive. This study still needs further research with other strain of *E. gergoviae*, using an invivo polyclonal sIgA or purified sIgA.

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