ChemTech

International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.12 pp 445-453, 2016

The Identification of Bacillus Endospore Strain 6114-RSMD Based on 16SRDNA and Anti-Nosocomial Bacteria Activity

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Abstract : Nosocomial is the harmful bacterial infection caused by Multi-Drug Resistance (MDR) that able to produce extended-spectrum beta-lactamase (ESBL) or methicillin-resistant *Staphylococcus aureus (*MRSA) that able to infect a human. One of the strain *Bacillus* endospores was discovered among others nosocomial bacteria community at the Mitra Delima Hospital, Malang, East Java. The previous study based on phenotypic identification concludes that the strain was scientifically identified as *Bacillus* endospore strain 6114-RMSD. The aims of this study were to further identify 6114-RMSD strain, discovered in Mitra Delima Hospital, using 16SrDNA and to determine its anti-nosocomial bacteria activity. The methods in this study were DNA isolation and amplification; 6114-RMSD growth curve assay, and 6114-RMSD cell-free supernatant (CFS) extraction; CFS anti-nosocomial activity test using diffusion and broth dilution method to nosocomial pathogens, MRSA, *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*; and CFS protein profiling by SDS-PAGE. Based on 16SrDNA identified that 6114-RMSD was *Bacillus amyloliquefacien*. The CFS of 6114-RMSD protein has anti-nosocomial activity potential like amoxicillin. The protein profile contained 14-71.90 kDa proteins which best growth in TBS medium and best harvested after 96 h. CFS 14 kDa protein had significant potential to inhibit nosocomial bacteria growth compared with crude CFS and amoxicillin.
Keywords : 16SrDNA identification, anti-nosocomial activity, *Bacillus* endospore, nosocomial bacteria.

Introduction:

Nosocomial is the harmful bacterial infection caused by MDR bacteria such as *Escherichia coli* and *Klebsiella pneumonia,* which able to produce ESBL or MRSA that able to infect a human. Nosocomial patients are serious medical attention in the hospital[1]. Mortality increased by 6% in the hospital due to the bacterial infection. This infection may induce uncomfortable stay-treatment in the hospital, increase the severity of the disease, increase the length of treatment in the hospital, and even patient mortality[2].

The increase of immunocompromised diseases and the occurrence of the bacterial resistance to antibiotics make this infection more severe[3]. A new breakthrough is needed to obtain bacterial agent that selective and produces antibacterial compounds[4]. *Bacillus* endospore is one of the proposed solutions to obtain the anti-nosocomial bacteria compounds. These *Bacillus* are able to adapt and survive to the hospital and its

surround extreme condition. Some of the *Bacillus* such as *Bacillus polymyxa* and *Bacillus subtilis,* are well-known as antibacterial compound producer[5].

One of the strain *Bacillus* endospores was discovered among others nosocomial bacteria community at the Mitra Delima Hospital, Malang, East Java. The previous study based on phenotypic identification concludes that the strain was scientifically identified as *Bacillus* endospore strain 6114-RMSD[6]. Therefore, this study is to further identify 6114-RMSD strain genus and species based on 16SrDNA and to determine its anti-nosocomial bacteria activity.

Method:

Phenotype Characteristic Analysis

The phenotype and biochemical metabolism of Strain 6114-RSMD's characteristics were analysed with various methods: (1) Gram staining; (2) endospore staining; (3) oxygen requirement analysis; (4) heat-resistance test; (5) pH-resistance test; (6) motility test; (7) proteolytic test; (8) catalase test; (9) oxidase test; (10) lipolytic test; (11) carbohydrate fermentation test; (12) Voges-Proskauer test; (13) Simmon-Citrate test; (14) nitrate reduction test; (15) Indol test; and (16) urea hydrolysis test. The protocols for tests no. 12, 13, and 15 were based on Brown[7], while the rest were based on Reller[8].

DNA Isolation

Strain 6114-RSMD's DNA was isolated based on the modified protocol by Natarajan et al.[9]. An overnight culture of 6114-RSMD in LB medium was centrifuged (40 °C, 4250 rpm, 15 min). The pellet was resuspended with 567 µL TE buffer pH 7.6 and 10µL lysozyme and then incubated at 37 ^{o}C for 1 h. The suspension was added with 30 µL SDS 10% and 3 µL pro-K 10mg/mL, incubated at 37 ^{o}C for 1 h. Then, 100 µL NaCl 5 M and 80 µL CTAB/NaCl was added to the suspension, homogenized, and incubated at 65 ^{o}C for 1 h. The PCI (phenol: chloramphenicol: isoamyl alcohol) solution (25:24:1) of 750 µL was added to the suspension, homogenized, and centrifuged (4 ^{o}C; 12000 rpm, 10 min). The supernatant was added with 500 µL PCI, gently shaken, and centrifuged (4 ^{o}C; 12000 rpm, 10 min). Supernatant was collected, gently shaken, and further centrifuged (4 ^{o}C; 12000 rpm, 10 min). The pellet was added with 500 µL 70% ethanol and centrifuged (4 ^{o}C; 12000 rpm, 5 min). After that, the pellet was air-dried in 55 ^{o}C and resuspended with 40 µL TE buffer pH 7,6 and stored in −20 °C. Furthermore, DNA sample was qualitatively tested by agarose electrophoresis and quantitatively tested by spectrophotometry.

PCR Amplification

The DNA sample was amplified using polymerase chain reaction (PCR) (thermocycler Amplitron®). The amplification was using universal primers: 16S rDNA 27F 5' GAG AGT TTG ATC CTG GCT CAG 3' as the forward primer and 1495R 5' CAT CGG CTA CCT TGT TAC GA 3' as reverse primer[10]. Amplified DNA was determined using electrophoresis in 1.5% agarose at 100 V for 30 min. The gel was visualized and documented using UV transilluminator[11]. GeneRuler™ DNA Ladder Mix 100 bp was used as DNA marker in the electrophoresis.

DNA sequencing and phylogenetic reconstruction

Amplified DNA was sequenced and used to reconstruct the phylogenetic tree to determine the species. Sequenced DNA samples were aligned by Bioedit software. The phylogenetic tree was reconstructed by Maximum Likelihood algorithm (Tamura-Nei method, 1000 bootstraps) using MEGA 5 software based on the aligned sequence. BLAST was also used to confirm the isolates identity based on NCBI database. The phylogenetic tree and the phenotypic data were used to confirm the species of the isolates.

6114-RSMD Growth curve

A pure colony of 6114-RSMD was inoculated into 50 mL TSB medium and incubated in rotary shaker 37 ^{o}C, 120 rpm, for 24 h. The optical density (OD) was determined using spectrophotometer (λ= 625nm). This

stock culture was diluted in the series of concentration (1:0, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7) using TSB medium to make inoculum stock. Each concentration series was added with 3 drops formaldehyde before

determining the OD (λ= 625nm). The bacterial cell of each concentration was counted using hemocytometer. The standard curve is the linear regression (r) and equation (y = ax+b) between bacterial cell and OD.

Twenty milliliters of the stock culture (OD = 0.1) was inoculated in 180 mL TSB medium and incubated at rotary shaker 120 rpm, for 1-4 days. Cell growth was observed based on the OD (λ = 625 nm) every 4 h for 4 days. The bacterial cell was determined with the standard curve.

Cell-Free Supernatant (CFS) Isolation

A single colony of 6114-RMSD was inoculated in 5 mL TSB and incubated on rotary shaker (150 rpm) at 37 ^{o}C, 24 h, with 0.5 of standard turbidity McFarland. The cell suspension was transferred to 1 L TSB in 1 % (v/v) as a subculture and then incubated in a rotary shaker (120 rpm, 37 ^{o}C). After 4 days, the cultures were centrifuged (4 ^{o}C, 12000 rpm, 30 min). The supernatant was precipitated with saturated ammonium sulfate 50 % at 4 ^{o}C for 24 h. The bioactive compound was filtered by 0.02 μm millipore and further used in this study[12].

Anti-Nosocomial Bacteria Activity

Staphylococcus aureus strain MRSA, *E. coli*, *K. pneumoniae* strain ESBL, and *P. aeruginosa* as pathogens were retrieved from Saiful Anwar State Hospital, Malang. The pathogens were inoculated in Nutrient Broth (NB) at 37 ^{o}C for 24 h while observing their OD gained 10^{8} CFU/mL (OD = 0.1). One mL of each pathogen inoculum was transferred to an individual petri dish and added with Muller Hinton Agar (MH) medium. The well holes 4-6 mm were made on the medium and injected with the CFS (100%, 50%, and 25% concentration) and antibiotic (amoxicillin) as control and then incubated at 37 ^{o}C for 24 h. The inhibition zone was calculated with calipers[13].

SDS-PAGE Electrophoresis

The protein profile of cell-free supernatant (CFS) was determined using electrophoresis. The sample was denaturated by RSB (1:1) and heated to 100 ^{o}C for 5 min. The separation of proteins in the cell-free supernatant was using 12.5% polyacrylamide gel, 200 mA, 120 V constantly until the tracking dye reached the bottom of the gel. The gel was stained and each protein band had molecular weight depended by the proteins mobility in the gel (Rf)[14].

AMP activity analysis

The gel from SDS-PAGE that contained 14 kDa protein was submerged in 15ml Muller Hilton Agar with 100 microliter nosocomial bacteria culture (OD 0.1 or 10^{8} CFU/ml). After the gel solidified, the agar was incubated in 37 ^{o}C for 24 h.

Data Analysis

The bioactive compound activity was descriptively described and statistically analyzed by ANOVA (α = 0.05) using SPSS ver. 22.

Results and Discussion:

Phenotype Analysis

The phenotypic characteristic of 6114-RMSD by microscopic observation was oval and rigged edge bacterial colony. The colony was white, with convex elevation, and semi-mucoid consistency. This strain belonged to Gram-positive bacteria. Based on the biochemical metabolism test, 6114-RMSD was capable of synthesising endospore, catalase enzyme, cytochrome oxidase enzyme, 2,3-butanadiol and acetone. This bacteria also synthesize exoenzymes, gelatinase and caseinase that allowed the bacteria to digest gelatin medium and hydrolyze casein. Based on Indol test, 6114-RMSD cannot synthesize tryptophanase, which responsible to produce indole from tryptophan. The bacteria was immobile since it did not have flagella. However, it was able to neutralize the acid environment during fermentation. This bacteria did not use citrate as their sole carbon and energy source.

Isolate 6114-RMSD was considered as excellent bacteria because of the ability to grow under uncondusive environment. In this case, the hospital environment had been always kept clean by antiseptics, antibiotics, UV rays, and disinfectants. It was known from the analysis that strain 6114-RMSD was capable to produce endospore in order to survive in their dormancy. The phenotypic analysis showed that the 6114-RMSD is related to *B. laterosporus*. The manual identification (Bergey's manual) is less accurate for species determination[15]. Environmental factor may alter the phenotypic character of the bacteria and biased the identification. Therefore, molecular identification used 16sRNA as gene marker is promising for species identification.

6114-RMSD Identification using 16S rRNA

Identification based on 16S rRNA was specific and able to distinguish up to genus and species due to its conservative nitrogen bases, which serve as annealing site for PCR primers. The 16s rRNA identification is well established and reliable in order to identify new bacteria species.

The amplification process obtained the thick DNA band at $\pm$1200 bp (Figure 1). A phylogenetic tree that reconstructed from the 16s rRNA of 6114-RMSD compared with other bacterias from the database shows that 6114-RMSD is closely related to *Bacillus amyloliquefaciens* (confidence level 99.69%) (Figure 2). The phenotypic analysis based on Bergey's Manual showed that 6114-RMSD was similar to *Bacillus* endospore. Furthermore, 16s rDNA data suggested that 6114-RMSD was *Bacillus amyloliquefaciens.*

PCR result visualization of 6114-RMSD strain with 16S rDNA

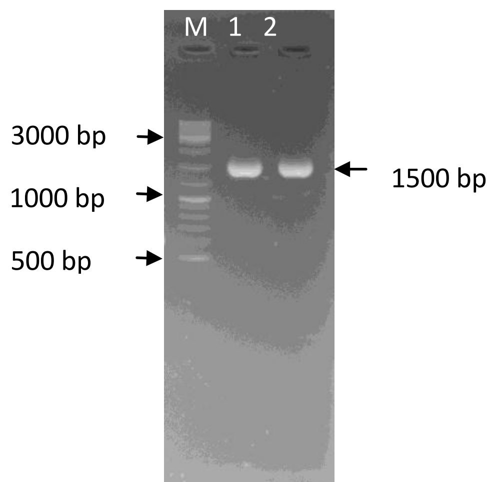

Figure 1 PCR result visualization of 6114-RMSD strain with 16S rDNA (27F and 1495R) primers in 1.5 % agarose (M: Marker)

Maximum Likelihood Phylogenetic Tree

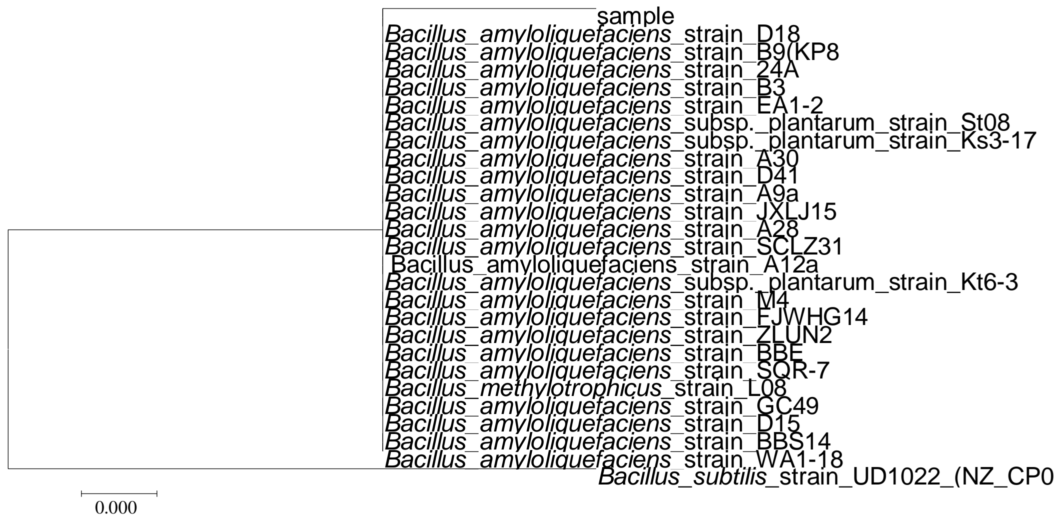

Figure 2 Maximum Likelihood Phylogenetic tree based on 16S rRNA of 6114-RSMD and other bacteria. (1000 bootstraps)

6114-RMSD Growth Curve

Based on the growth rate in the TSB media, 6114-RSMD showed logarithmic phase after 20 h incubation until 24 h from initial inoculation. *Bacillus* strain 6114-RMSD was grew rapidly until the highest bacterial density obtained in this study had reached (OD = 2.549) at 24 h. Furthermore, 6114-RMSD reached stationary phase after 44 h incubation with OD remained at 2.084. Waste product accumulation and food resource depletion occurred in this phase that could decrease the growth rate of 6114-RMSD. The bacteria lifespan count remained constant and eventually decline at some point (fluctuation). Goldstein et al. mentioned that antibiotics compounds are mainly produced in the stationary phase [16]. The secondary metabolites, such as cell-free supernatant, which have anti-nosocomial activity are also produced in stationary phase.

Anti-Nosocomial Bacteria Activity

Diffusion test

Amoxicillin was used as a control in this study because amoxicillin is standard antibiotic (wide-spectrum beta-lactam), readily available, and widely prescribed as general medicine. Amoxicillin is well known as its ability to overcome bacterial infection such as tonsillitis, pneumonia, and food poisoning, and it is safe for pregnant women. Otherwise, amoxicillin is unstable against bacteria produced β-lactamase thus some bacteria are resistant to this antibiotic[17].

From the diffusion test obtained that CFS from 6114-RMSD induces inhibition zone to pathogen nosocomial bacteria. The biggest inhibition zone was obtained against *P. aeruginosa* (17.91 mm), followed by *K. pneumoniae* (16.63 mm), MRSA (15.37 mm), and *E. coli* (15.11 mm). The inhibition zone was increasing steadily the longer the incubation period (Figure 3). The result suggested that the anti-nosocomial substance contained in the crude CFS may have the same potential compared with amoxicillin. Otherwise, it also suggested that the crude CFS contained many proteins that were not synergically working to inhibit the nosocomial bacteria since the nosocomial bacteria were still able to survive in diffusion test medium.

6114-RMSD CFS Inhibition Zone to Nosocomial Bacteria

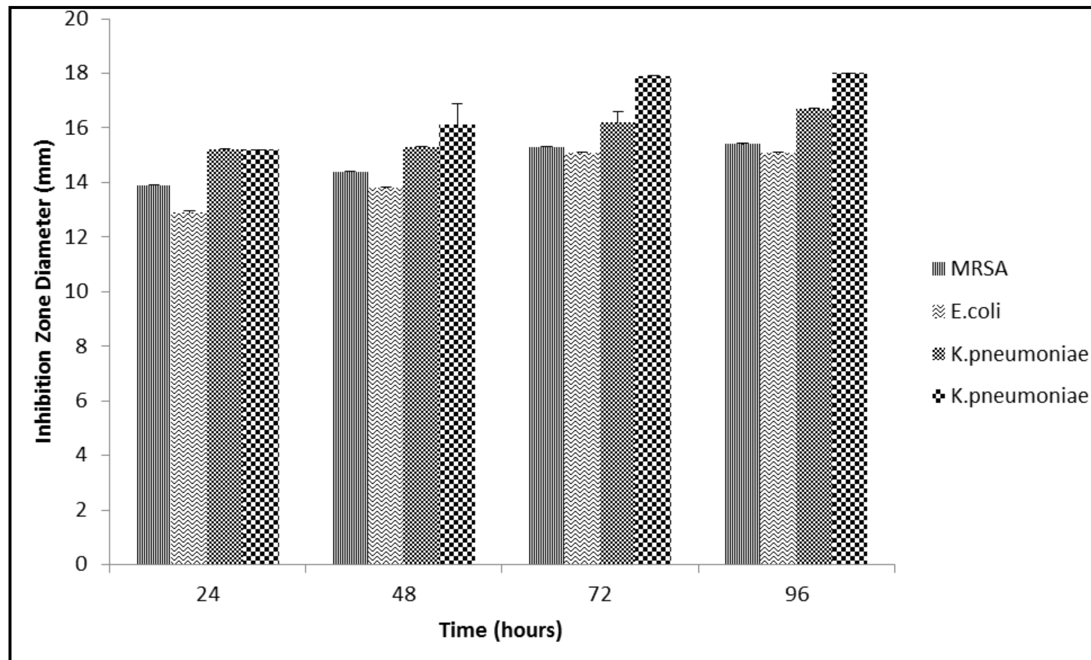

Figure 3. 6114-RMSD CFS inhibition zone to nosocomial bacteria in diffusion agar method

Broth Dilution Test

Broth dilution test was used to determine the lowest concentration of the sample to inhibit the bacterial growth. The result was based on the turbidity of the sample compared to bacterial control and medium control. The lowest concentration to inhibit bacterial growth is recorded as MIC[16].

The broth dilution test result obtained that both Gram positive bacteria and Gram negative bacteria were affected by the CFS. There was significant OD reduction in all concentration of MRSA. *P. aeuriginosa* had no significant OD reduction in 25% and 50% concentration. *E. coli* and *K. pneumonia* OD was decreasing, but not significantly decreases in any concentration. The antibacterial effect was increasing as CFS concentration increased. It suggests that 6114-RMSD CFS have bactericidal effect by binding the phosphatidylethanolamine in the cell membrane, obstruct the active transport activity, and alter the cell membrane permeability[16].

CFS Protein Profile and Antibacterial Activity

In order to determine the optimal CFS production, time variation of extraction and medium variation were conducted. CFS was extracted from 24, 48, 72, and 96 h from 6114-RMSD culture. The CFS production was used four different growth mediums: Luria Bertani (LB), NB, trypticase soya broth (TSB), brain hearth infusion (BHI). From the SDS-PAGE result obtained that the CFS protein extracted from TSB and BHI were thicker than the others. Furthermore, TBS was chosen to be a growth medium for protein profile and antibacterial activity. Based on the time variation, we predicted that CFS extracted from 6114-RMSD culture after 96 h is the best in inhibiting nosocomial bacteria growth.

Based on SDS-PAGE result, CFS contained the protein that ranged from 14 to 71.90 kDa (Figure 4). Broin *et al.* mention that those active components which serve as water soluble cationic peptide have molecular weight around 6-16 kDa[17]. The 14 kDa band in the result was wide, suggesting in the mixture of light molecular weight proteins. This result concluded that the candidate for the antimicrobial peptide (AMP) is the 14 kDa protein.

6114-RMSD CFS Protein Profile

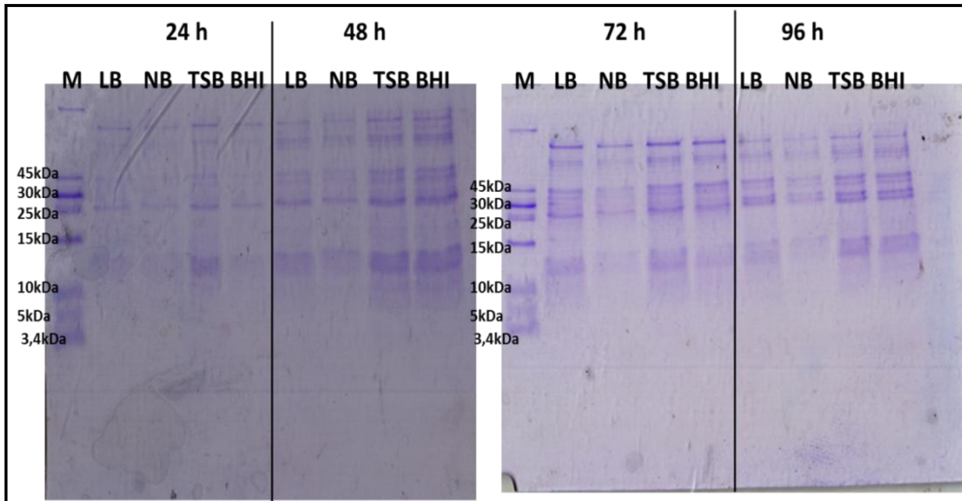

Figure 4. 6114-RMSD CFS protein profile 24, 48, 72, and 96 h culture with different medium. M: Marker; LB: Luria Bertany; NB: Nutrient Broth; TSB: Tripticase Soya Broth; BHI: Brain Hearth Infussin

The AMP activity analysis obtained such interesting results. The average of inhibition zone for nosocomial bacteria by 14 kDa against MRSA, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* was 24.73 mm, 19.96 mm, 30.37 mm, and 27.77 mm, respectively (Figure 5). The inhibition zone for the 14 kDa protein was significantly wider than the crude CFS and amoxicillin in MRSA, *K. pneumonia*, and *P. aeruginosa*. The inhibition zone by 14 kDa against *E. coli* was wider but not statistically significant. This data suggests that the 14 kDa has more potential as antibacterial medicine than the crude CFS and amoxicillin. Thus, it provided vital information to bacteria candidate exploration to the new antibacterial compound.

Comparison of Inhibition Zone for Nosocomial Bacteria

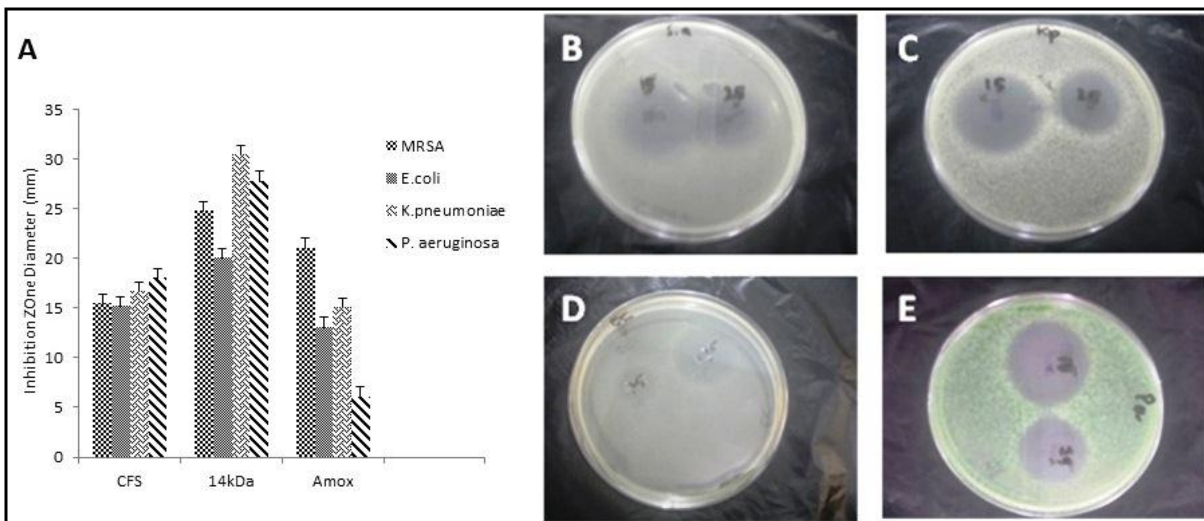

Figure 5. Comparison of inhibition zone for nosocomial bacteria by crude CFS, 14 kDa, and amoxicillin (A); inhibition zone of MRSA (B); inhibition zone of *E. coli* (C); inhibition zone of *K. pneumoniae* (D); inhibition zone of *P. aeruginosa* (E)

Conclusion:

Bacillus strain 6114-RMSD was identified as *Bacillus amyloliquefacien* based on the 16S rRNA and the phenotypical analysis. The 14 kDa proteins isolated from 6114-RMSD CFS during the stationary phase were had more potential to inhibit the nosocomial bacteria growth compared to crude CFS and amoxicillin.

Recommendation:

We recommend conducting the further exploration for the microorganism that have antibacterial activity in the numerous hospitals. It is needed to analyze based on different extraction method, such as HPLC, to obtain the pure antibacterial protein from our strain. In order to determine its application, the AMP from our strain CFS is needed to be analyzed to discover its toxicity and distribution.

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