Isolation and identification of *Staphylococcus aureus* from Raw Milk Resistant Against Non β-Lactam Antibiotics

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ABSTRACT

The aim of this research was to study the resistance of *Staphylococcus aureus* isolated from raw milk in 5 dairy farms in Surabaya against non β-lactam antibiotics. Fourthy samples were culture on Mannitol Salt Agar from which 23 samples were identified there were *Staphylococcus sp* and from it were identified 7 (30,44%) samples were *Staphylococcus aureus* by showed characteristic such as mannitol fermented, grape/cluster shape, Gram positive, catalase positive, coagulate positive and had β haemolysin on Blood Agar. Antibiotic disk that used for sensitivity test were Tetracycline, Erythromycin, and Gentamicin using Kirby-Bauer Method. The results showed that the precence of Tetracycline resistance in a sample from K and P dairy farm and the presence of Erythromycin resistance in a sample from K and W dairy farm. The existence of this resistance causes milk dangerous to be consumed by public health because the resistant bacteria, or genetic determinants of resistance, can be transmitted from animals to humans via foodstuffs and direct contact with animals.

**Key words**: *Staphylococcus aureus*, raw milk, antibiotic sensitivity test, non β-lactam antibiotics
Introduction

Based on SNI 3141.1:2011, the meaning of raw milk is fluid from healthy and clean udder, correct milking ways, the content not less or additional with anything, not have treatment except cooling process. Milk is a highly nutritious food because it contains nutrients that a complete and balanced diet such as proteins, fats, carbohydrates, minerals, and vitamins that are needed by humans. The content of high nutritional value causes the milk to a medium that is preferred by microbes for growth and development (Miskiyah, 2011). Some pathogenic bacteria are likely to exist in milk among other Bacillus spp, Brucella spp, Campylobacter spp, Enterobacter sakazakii, Escherichia coli, Listeria monocytogenes, Mycobacterium spp, Salmonella spp, Streptococcus spp, Yersinia enterocolitica and Staphylococcus aureus (Chotiah, 2010).

Non β-lactam antibiotics can be classified into 5 categories, there are class Aminoglycosides, Quinolones, Sulfonamides, Macrolides, Tetracyclines. Each antibiotics had a different structure and activity faced of pathogenic bacteria (Pieshesa, 2011). Non β-lactam antibiotics used in this research were the class of Tetracycline (Tetracycline), Aminoglycoside (Gentamicin) and Macrolide (Erythromycin). But many report from worldwide that the present of Staphylococcus aureus resistant antibiotic. Resistance to antibacterial drugs is an increasingly important problem in both humans and animals (Quinn et al, 2002). The widespread, sometimes indiscriminate, use of these drugs results in the selection of bacteria which are inherently resistant. Not only may these resistant bacteria become the predominant species in a population but also they may transfer genetic material to susceptible bacteria which then acquire resistance. Antibacterial drug resistance can been coded either in the bacterial chromosome or in plasmids. Resistance genes can be transferred between bacteria through transduction, conjugation, transposable
elements or transformation. Resistance to an antibacterial agent often results in cross resistance to other agents in the same class. This form of resistance is encountered with the Sulphonamides, Tetracyclines, Aminoglycosides and Macrolides (Quinn et al., 2002).

The purpose of this research was to determine the presence of *Staphylococcus aureus* in raw milk that is resistant against non β-lactam antibiotics. Resistant bacteria, or genetic determinants of resistance, can be transmitted from animals to humans via foodstuffs and direct contact with animals are recognised to be the main routes of resistance transfer from animals to humans (Schlegelova et al., 2008; FVE, 2010). Antibiotic resistance in humans resulting in the ineffectiveness of antibiotics by doctors in case of infection. Therefore, it is necessary to test level of sensitivity some antibiotics as one of the aspects that affect the veterinary public health (Suwito and Setyadji, 2008).

**Material and Method**

The research was conducted on February until April 2013. The raw milk sampling were taking place on five dairy farm in Surabaya such as B, K, P, T, dan W dairy farm. Isolation, identification and sensitivity test of *Staphylococcus aureus* have been done at the Laboratory of Veterinary Public Health and the Laboratory of Veterinary Bacteriology and Micology, Faculty of Veterinary Medicine, Airlangga University. Type of research design used explorative research laboratories. The material used in this research were raw milk samples were sold in 5 dairy farm location in Surabaya, Gram staining materials: Aquades, Crystal Violet, Safranin, Lugol, Alcohol 96%, Mannitol Salt Agar (MSA), Blood Agar (BA), H2O2, rabbit blood plasm, Buffer Pepton Water (BPW 1%), Muller Hinton Agar (MHA) and non β-lactams antibiotic: Tetracycline, Eritromycin and Gentamicin are antibiotic that ready to used in the discs form. The tools used in this research were ose/ loop, bunsen
burners, lighters, ruler, test tubes, centrifuge tube, rack for test tube, steril cotton sticks, tweezers, petri dish, baker glass, pipette 1 ml, stirring glass, microslide, microscope, vortex, centrifuge, incubator, and autoclave.

Milk samples were collected from 5 locations dairy farms in Surabaya such as B, K, P dairy farms were collected 10 samples and T and W dairy farm 5 samples. These research were used purposive sampling method technique. Criteria of raw milk samples was raw milk that ready to sold to consumer. The samples were taken after milking time in the afternoon on 2.00 pm.

Isolation and Identification of Staphylococcus aureus

The homogenized raw milk samples were taken used 1 ml pipette as much as 2 ml than put on test tube containing Buffer Pepton Water 1% 5 ml. It was rotated by vortex. The presence of Staphylococcus aureus can be confirmed by examination of Gram staining, catalase test, coagulase test and produce β-hemolysis in Blood Agar. Milk samples grown on Mannitol Salt Agar (MSA) that incubated at temperature 37°C for 24 hours, yellow colonies that grow and suspected Staphylococcus sp aureus replanted on MSA and incubated at temperature 37°C for 24 hours as subculture. After obtaining a separated colony of Staphylococcus aureus then the colonies can be identified.

The aim of staining was to differentiate bacteria in absorbing colour matter and known Gram staining characteristic and morphology of Staphylococcus aureus in microscopic. Gram positive bacteria will keep purple colour of crystal violet that looks purple colouring under the microscope although stain with Safranin, while Gram negative bacteria crystal violet will dissolve by the addition of 96% alcohol and binding on both of safranin/fuchsin so under the microscope will look red colouring (Sarudji et al, 2010). Catalase test use yellow colonies of MSA mixed with H₂O₂, on glass objects, if there any Staphylococcus sp signalment by the presence of gas bubbles.
Catalase test use for differentiated from *Streptococcus sp* (Tirnata, 2007).

Coagulase test is done using tubes containing rabbit blood plasma, according to the method Bruckler. Bacteria grown in tubes containing blood plasma, incubated for 6 to 18 hours at 37 °C. Coagulase test was positive if there was a clot in the coagulase test tube and negative when it was not happen clot in the tube (Purnomo et al, 2006). Hemolysin production is determined based on the presence of hemolysis zone formed by *Staphylococcus aureus* on blood agar plate. Bacteria grown in the plate agar (agar base, Oxoid, Germany) with the addition of sheep blood, and incubated for 18-24 hours at 37°C (Purnomo et al, 2006). *Staphylococcus aureus* in Blood Agar there were clear zone means there is complete hemolysis of blood cells (β-hemolysis). Blood Agar for growth of microorganisms that are difficult to bred and also to differentiate groups of microorganisms that produce red blood cells lyse or not (Mulyadi, 2011).

Antibiotic Sensitivity Test

Bacterias culture was obtained from separated colony in Blood Agar, then planted in test tubes containing BPW 1% incubated at 37°C for 24 hours, or until the turbidity obtained with standard Mc.Farland II then gently rubbed on the entire surface of Mueller Hinton Agar. Bacterias are left attached for 5 minutes, then Tetracycline 30 μg, Erythromycin 15 μg and Gentamicin 10 μg antibiotic disk be placed on the MHA and incubated at 37°C temperature for 24 hours. Observations done by measuring the diameter of zone inhibition of bacterial growth using the zone interpretations list of inhibition based on Clinical and Standard Laboratory Institue (2007).

Result and Discussion

Criteria observed sample were colonies of bacteria that can fermented mannitol on Mannitol Salt Agar. The positive results shown by the yellow colonies on MSA.
Table 1. Results isolation of bacteria from raw milk samples

<table>
<thead>
<tr>
<th>Dairy farm</th>
<th>Number of samples</th>
<th>Positive result on MSA</th>
<th>Negative result on MSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>K</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>P</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>T</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>W</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

Based on Gram staining and microscopic observing of colonies from MSA positive inoculation results were obtained 23 positive samples had the form grape, clustered, purple coloring colonies means Gram positive bacteria indicated characteristic of *Staphylococcus sp* and 2 samples showed negative results. Based from microscopic view these negative result showed that have chain bacillus. Catalase test results showed 23 positive samples have catalase enzyme that distinguished the bacteria with *Streptococcus sp*.

The observation result was made against 23 positive samples of *Staphylococcus sp* showed 7 (30.44%) samples were coagulase positive *Staphylococcus* and 16 (69.56%) samples were coagulase negative *Staphylococcus*. Subculture of *Staphylococcus sp* on MSA then inoculated on Blood Agar (BA). The results showed that 7 (30.44%) samples had β hemolysis and 8 (34.78%) samples had α-hemolysis whereas 8 (34.78%) samples were not hemolysis erythrocytes in BA. Produce of rapid and reliable identification of *Staphylococcus aureus* need to combine between β-hemolysis test and coagulase test with incubation time 4 hours (Effendi et al., 2005).

Non β-lactam antibiotics resistance test performed on 7 isolates of *Staphylococcus aureus* from raw milk. Inhibition zone diameters of antibiotic disk measuring on millimetre size. The measurement results of diameter inhibition zone can be seen in the table.2.
Table 2. Result of Inhibition zone diameter (mm) of non β-lactam antibiotics on Mueller Hinton Agar against *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Tetracycline 30 μg</th>
<th>Erythromycin 15 μg</th>
<th>Gentamicin 10 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3</td>
<td>28 (S)</td>
<td>26 (S)</td>
<td>23 (S)</td>
</tr>
<tr>
<td>B9</td>
<td>16 (I)</td>
<td>21 (I)</td>
<td>22 (S)</td>
</tr>
<tr>
<td>K8</td>
<td>11 (R)</td>
<td>13 (R)</td>
<td>22 (S)</td>
</tr>
<tr>
<td>P2</td>
<td>24 (S)</td>
<td>23 (S)</td>
<td>20 (S)</td>
</tr>
<tr>
<td>P4</td>
<td>10 (R)</td>
<td>29 (S)</td>
<td>25 (S)</td>
</tr>
<tr>
<td>P6</td>
<td>16 (I)</td>
<td>20 (I)</td>
<td>13 (I)</td>
</tr>
<tr>
<td>W2</td>
<td>20 (S)</td>
<td>11 (R)</td>
<td>20 (S)</td>
</tr>
</tbody>
</table>

Note: S: Sensitive, I: Intermediate, R: Resistant

From the results of research known that isolates *Staphylococcus aureus* from raw milk there were 2 (28.57%) samples which resistant, 2 (28.57%) samples which intermediates and 3 (42.86%) sample sensitive against Tetracycline, whereas 2 (28.57%) samples which resistant, 2 (28.57%) samples which intermediate and 3 (28.57%) sensitive against Erythromycin also it was obtained 6 (85.71%) samples which sensitive and 1(14.29%) samples which intermediates against Gentamicin.

Results of this research illustrate the relationship with the report about the bacteria isolated from beef and chicken meat circulating in Surabaya 92% were resistant to Erythromycin, and also supported that *Staphylococcus aureus* isolates from Surabaya is the resistant bacteria against Erythromycin and can happened Erythromycin resistance transfer of *Staphylococcus aureus* from chickens to *Staphylococcus aureus* bacteria that are in humans (Effendi, 2009).

Erythromycin is a macrolide also frequently used in the intramamary treatment and available in the treatment during lactation or dry period. Erythromycin binding 50 S ribosomal on Gram positive bacteria.
**Staphylococcus aureus** performing ribosome subunit formation and one of them is the 50 S ribosomal subunit. Erythromycin avoid the formation of 50 S ribosome formation. Translational inhibition associated with *peptidyltransferase* activity of the 50 S subunit, Erythromycin inhibition occurs in translational stages between initiation and elongation. Some *Staphylococcus aureus* strains had Erythromycin resistant methylation genes (ermA, ermB and ermC) that encode ribosomal RNA removal in the function of preventing binding of macrolides and produce high levels of resistance. ermA displacement of chromosomal genes associated with Tn. 554 transposon indicating displacement drug resistance mediated by the transposon (Effendi, 2009).

Tetracycline resistance occurs when cells membrane impermeable to the drug or there is an efflux increasing. Four genes, tet (L), tet (K), tet (M) and tet (O) coding for Tetracycline resistance have been identified in *Staphylococcus*. This fourth gene plays an important role in resistance mechanisms such as efflux pump activation and ribosomal protection (Pieshesa, 2011). One of the factors that can cause high resistance to Tetracycline in humans is the use of antibiotics for livestock. A study by the Massachusetts based Union of Concerned Scientists found that Tetracycline, Penicillin, Erythromycin, Sulphamethazine, and other antibiotics beneficial to humans is widely used for non therapeutic purposes in livestock production, which if consumed by humans will rapidly absorbed in the gastrointestinal tract (Nurhani, 2010).

Gentamicin has a high sensitivity in *Staphylococcus aureus* isolates from raw milk likely caused infrequent use of these types of antibiotics on the dairy farm in Surabaya. While the approach is intuitive and consistent with pharmacodynamic principles, limited data are available to describe the pharmacodynamic activity of Aminoglycosides against Gram positive bacteria (e.g., *Staphylococcus aureus*, viridans group streptococci,
and *Enterococcus* spp.). Aminoglycosides (e.g., Gentamicin) are often used clinically in combination with other antimicrobial agents such as Betalactams or lycopeptides for the treatment of serious infections with Gram negative and Gram positive organisms (Tam et al., 2006).

**Conclusion** According to the result, there were found resistance result on antibiotic resistance test against non β-lactam antibiotics from 7 isolates of *Staphylococcus aureus* isolated from raw milk but some isolates showed still had sensitivity levels on Tetracycline, Erythromycin and Gentamicin. Gentamicin also have potential to be antibiotics against *Staphylococcus aureus*, however the present resistance of *Staphylococcus aureus* on raw milk from this research warned to probability of transfer resistant from foodstuff (milk) to human.

**References**


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