Fw: Acknowledgement Letter # 177/19

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ACKNOWLEDGEMENT

Reg. No: 177/19 Dated : 20/05/2019

Dear Dr. Mustofa Helmi Effendi,

We acknowledge the receipt of the following articles entitled “Antibiotic Resistant on Staphylococcus aureus and Methicillin Resistant Staphylococcus aureus (MRSA) Isolated from Several Dairy Farms in Surabaya, Indonesia.” (Mustofa Helmi Effendi, et al.).

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Dr. Mustafa Helmi Effendi

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177/19
II
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Antibiotic Resistant on Staphylococcus aureus and Methicillin Resistant Staphylococcus aureus (MRSA) Isolated from Several Dairy Farms in Surabaya, Indonesia

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Abstract

The purpose of this study was to isolate and identify the strain of *methicillin-resistant Staphylococcus aureus* (MRSA) from raw milk in Surabaya, Indonesia. Raw milk samples of 80 samples obtained from four dairy farms. Bacterial identification was based on the growth in Mannitol Salt Agar (MSA) and Gram staining and catalase, & coagulase tests. 14 (17.5%) out of 80 milk samples were for positive *Staphylococcus aureus* isolation. Antibiotic sensitivity testing using Cefoxitin, Penicillin, Amphicillin, Oxacillin and Tetracyclin antibiotics showed 14(100%), 14(100%), 12(85.7%), 9(64.3%) and 0(0%) isolates were resistant to the antibiotics, respectively. MRSA isolates showed that 9 isolates were positive by using Cefoxitin disc diffusion (DD) combined with Oxacillin disc diffusion (DD) Test. It was concluded that the raw milk can be a potential reservoir for MRSA strains to threat public health.

Key words: Staphylococcus aureus, Antibiotic Sensitivity Test, MRSA, Raw Milk, Public Health, Indonesia

Introduction

Milk is a good medium for the growth and development of these bacteria. S. aureus contamination can occur due to the presence of these bacteria in raw milk, during milking or processing. The main S. aureus reservoir is found in infected quarters (Akineden et al., 2001). Staphylococcus aureus (S. aureus) is an important pathogenic bacterium that causes mastitis in ruminants (Salasia et al., 2004). This organism is the main agents of subclinical or chronic mastitis in dairy cows which cause considerable losses in the dairy industry (Katsuda et al., 2005). Mastitis caused by S. aureus shows symptoms of subacute or chronic inflammation.

S. aureus infection in humans, especially Methicillin Resistant S. aureus (MRSA) is an infection that is difficult to overcome because these germs are known to be resistant to various antibiotics(Hata et al., 2010). Staphylococcal infections include the effects of postoperative injuries, pollution during hemodialysis, bacteremia, and pneumonia (Fournier et

al., 2008). The potential of S. aureus in causing various diseases and food poisoning is very large in both animals and humans(Salasia et al., 2011).
In Surabaya, it is a little source data of the antimicrobial pattern, especially from beta lactam antibiotics and the distribution of Methicllin Resistant S. aureus (MRSA) isolated from raw cow's milk. Therefore, the study was to identify the antimicrobial resistance of S. aureus isolated from raw cow's milk sample from four dairy farms in Surabaya, East Java, Indonesia based on its antibiotic sensitivity test and to understand the MRSA strains distribution.

Materials and Methods

Sampling

Milk samples taken from Kaliwaron Farm, WonocoloFarm, Pogot Farmand Bendul Merisi Farm20 samples each. With a total sample of 80 samples of cow's milk per individual. Sampling uses purposive sampling technique that is sampling which is based on the consideration of the researcher itself with specific aims and objectives. Milk sampling is carried out during the morning milking time.

Isolation of Staphylococcus aureus

The steps taken to isolate and identify Staphylococcus aureus is to prepare Manitol Salt Agar (MSA) media and 10 ml cow milk samples in a test tube. Prepare a bunsen that has been lit, then the inoculating loop to be used is burned first until it glows. Dip the inoculating loop in the sample, then streak in the form of a zig-zag line on the MSA isolation media. Incubating the media at 37°C for 24 hours. Then observe separate colonies, and showed yellow colony color, shown on figure 1. Yellow colonies taken from the MSA media are then carried out microscopic observations by performing a Gram staining test.

Identification with Gram staining

One eye over the bacterial colony is taken aseptic, dry and fixation on a bunsen lamp. After dry, drop 2-3 drops of crystal violet and let stand for 1 minute. Wash with running water and dry then drop again with lugol solution, and leave for 1 minute, then wash with running water, and dry it. Wash again with 70% alcohol for 30 seconds, wash and dry. Give safranin solution for 2 minutes, washed with running water and dried, shown on figure 2. (Effendi et al., 2018)

Identification by catalase and coagulase test

catalase test, by taking a yellow bacterial colony on the MSA media, then putting it on a glass object and adding with H_2O_2 3% on the glass object, if there is Staphylococcus aureus it is indicated by gas bubbles, shown on figure 3. Followed by a coagulase test, namely by taking a colony taken from the MSA media using an ose, then inserting it into Nutrient Brooth media and incubating it for 24 hours at 37°C. After incubation, prepare 1 ml rabbit plasma and then mix until evenly then incubated for 4 to 24 hours. The presence of Staphylococcus aureus is characterized by plasma clotting, shown on figure 4. (Effendi et al., 2019).

Antibiotic Sensitivity Test

The antibiotic sensitivity test of the Staphylococcus aureus in this study was to use beta-lactam antibiotics including Cefoxitin, Penicillin, Amphicillin, Oxacilin, andnon beta-lactam used Tetracyclin,by Kirby-Baurer method. Prepare the MHA media on the petri disk, take the bacterial suspension with a 0.2 ml sterile pipette and pour it on the MHA media. Then, prepare a disk containing Cefoxitin, Penicillin, Amphicillin, Oxacilin, and Tetracyclin. Place the disc on the surface of the MHA media using tweezers. Incubation for 24 hours at 37°C. The results are seen by the presence of a clear area around the paperdisk as a barrier area for

bacterial growth, and the clear zone is measured using a caliper, shown on figure 5.(CLSI, 2017).

Results and Discussion

Based on the results of isolation and identification carried out on 80 samples of raw cow's milk per individual from 4 dairy farms in Surabaya there were 14 (17.5%) positive samples of Staphylococcus aureus (tabel 1.).

Table 1. Isolation and identification of Staphylococcus aureusfrom milk samples in Surabaya, Indonesia.

Location of farm	Sample size	(+) MSA media	(+) Gram staining	(+) Catalase	(+) Coagulase
Kaliwaron (K)	20	5	5	5	2
Pogot (P)	20	5	5	5	4
Wonocolo (W)	20	6	6	4	2
Bendul Merisi (B)	20	7	7	6	6
Total	**80**	**23**	**23**	**20**	**14 (17.5%)**

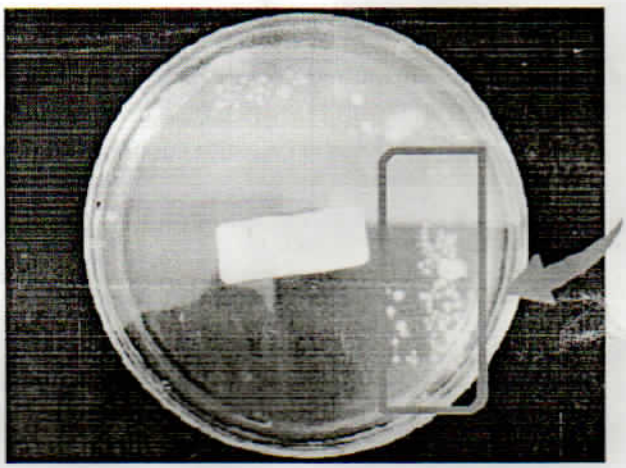

Figure 1. Staphylococcus aureus on MSA media

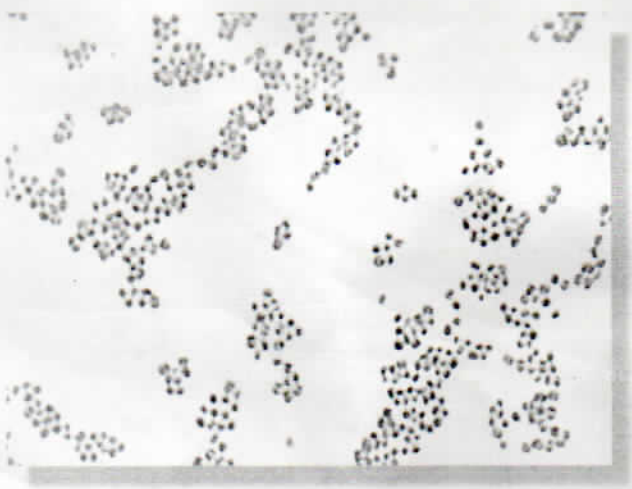

Figure 2. Microscopic examination of Gram-positive Staphylococcus aureus and clustered cocci

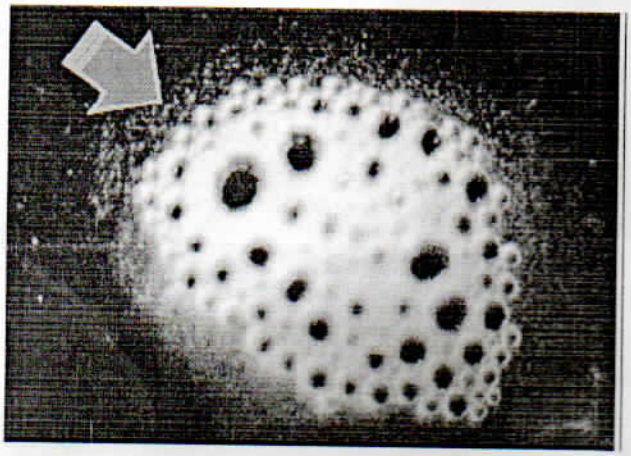

Figure 3. Catalase test on Staphylococcus aureus shows positive bubbles

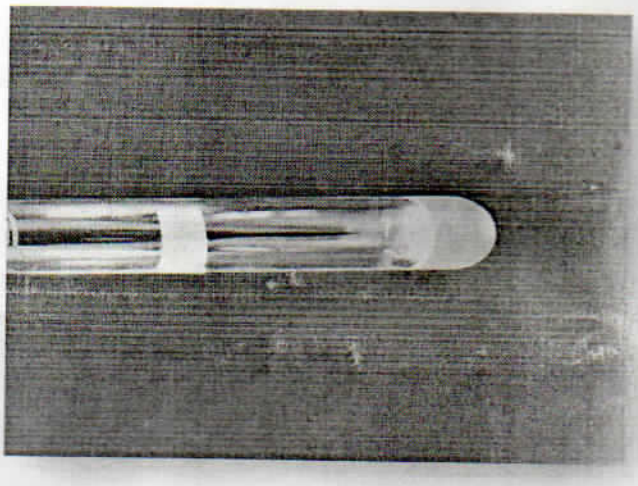

Figure 4. Coagulase test for confirmation of Staphylococcus aureus isolate

Fourteen positive samples of Staphylococcus aureus in the bacterial identification test were followed by antibiotic sensitivity test using five antibiotics in the form of discs including Oxacillin, Penicillin, Amphicillin, Cefoxitin, and Tetracyclin carried out on Mueller Hinton Agar (MHA) media with diffusion method, shown on figure 5. The results showed on table 2.

Table 2. Measurement of inhibition zone on antibiotic sensitivity test of Staphylococcus aureus

No	Isolates Code	Diameter of inhibition zone of antibiotics in mm				
		Cefoxitin 30 µg	Penicillin 10 µg	Amphicillin 10 µg	Tetracyclin 30 µg	Oxacillin 10 µg
1.	B1	15 (R)	26 (R)	27 (R)	22 (S)	21 (R)
2.	B2	16 (R)	26 (R)	20 (R)	22 (S)	15 (R)
3.	B3	20 (R)	24 (R)	19 (R)	23 (S)	16 (R)
4.	B4	20 (R)	22 (R)	19 (R)	15 (I)	22 (S)
5.	B5	18 (R)	20 (R)	19 (R)	20 (S)	27 (S)
6.	B6	20 (R)	25 (R)	29 (S)	17 (I)	15 (R)

7.	K1	18 (R)	19 (R)	15 (R)	18 (I)	27 (S)
8.	K2	20 (R)	26 (R)	22 (R)	23 (S)	25 (S)
9.	P1	21 (R)	20 (R)	18 (R)	17 (I)	24 (S)
10.	P2	20 (R)	17 (R)	14 (R)	23 (S)	19 (R)
11.	P3	20 (R)	24 (R)	21 (R)	23 (S)	21 (R)
12.	P3	17 (R)	25 (R)	29 (S)	17 (I)	14 (R)
13.	W1	18 (R)	22 (R)	18 (R)	23 (S)	11 (R)
14.	W2	19 (R)	20 (R)	18 (R)	16 (I)	21 (R)

Information: R: Resistant I: Intermediate S: Sensitive

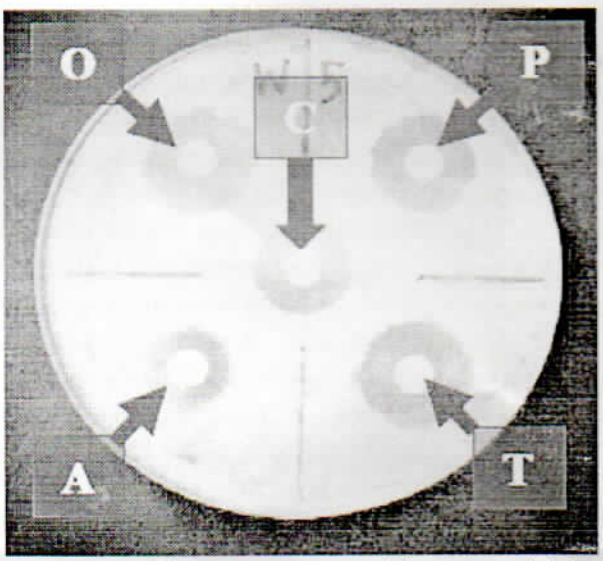

Figure 5. Inhition zone on antibiotic sensitivity test ofisolate code W2

Information : C = Cefoxitin 30 µg P = Penicillin 10 µg
A = Amphicillin 10 µg O = Oxacillin 10 µg
T = Tetracyclin 30 µg

The prevalence of S. aureus in milk showed a high yield of 14 (17.5%). These results indicate that good hygiene practices in every step of milk production from the housing system to milking are very important and needed to reduce the contamination of S. aureus. The prevalence of S. aureus in this study is comparable with studies conducted in Ethiopia (Ayano et al., 2013)reported a prevalence of 13.8%. The lower prevalence of 6.6% and 10.8% was reported in India (Kumarand Prasad, 2010)and Brazil (Fagundes et al., 2010).
While a higher prevalence of 40%, and 100% respectively has been reported in Morocco (Bendahou et al., 2008), and South Africa (Ateba et al., 2010). The prevalence observed can be caused by the presence of subclinical infected cows and negligence of hygienic conditions such as improper milking procedures, milk handling techniques, and improper storage that increases S. aureus in milk.
S. aureus is often isolated from cattle with subclinical mastitis (Mdegela et al., 2004) and previous studies revealed that S. aureus infection originating from dairy products is a public health problem throughout the world (Tarekgne et al., 2015, De Buyser et al., 2001). The causes mentioned are improper food handling, unclean production environment, storage, transportation, and personal hygiene.

The level of bacterial resistance to antibiotics according to the assessment standards of antibiotic inhibitory zone based on Clinical Laboratory Standards Institute(CLSI) are grouped

into three categories, namely sensitive, intermediate, and resistant(CLSI, 2017). A bacterium is said to be sensitive to antibiotics if the bacteria can be inhibited properly and formed a clear zone when tested, intermediate categories if the bacteria can be inhibited but with a weaker inhibitory power, and a resistance category if the bacteria can be inhibited but shows a very weak inhibition or no inhibition is formed at all (Effendi et al., 2019).

Penicillin, Amphicillin, Cefoxitin and Oxacillin are antibiotics belonging to the class of β-lactam antibiotics. B-lactam antibiotics are antibiotics that are often used in the treatment of mastitis cases in dairy cows. The beta-lactam group has the ability to inhibit bacterial growth by influencing bacterial cell wall synthesis. This antibiotic has activity in S. aureus through the interaction of three heavy molecules and one mild molecule in penicillin binding proteins. The mechanism of action of antibiotics β-lactam begins with penicillin binding protein (PBPs) in bacteria (Quinn et al., 2002). The function of penicillin binding protein is to have an effect on the synthesis of peptidoglycan cell walls and cell growth. B-lactam antibiotics bind and inhibit penicillin binding protein (PBPs), which is an enzyme for the synthesis of Peptidoglycan (Effendi, 2009). The resistance to β-lactam can be caused by S. aureus being able to produce β-lactamase which can break up the β-lactam ring or the expression of PBP 2a which has a low affinity for oxacillin and other β-lactams.

Sensitivity test on Cefoxitin antibiotics showed fourteen isolates 100% resistant to Cefoxitin. Cefoxitin is a second generation cefamycin antibiotic with broad spectrum activity that is also used as a test antibiotic to detect the resistance properties of antibiotic substances Cefoxitin and methicillin to S. aureus (Datta et al.,2011). Isolates showed resistance to Cefoxitin and Penicillin which was also used in this study as a marker of Staphylococcus aureus in milk which isolates from several dairy farms in the Surabaya were resistant to beta-lactam antibiotics.

Sensitivity test results for penicillin antibiotic as described in table 2. showed Staphylococcus aureus bacteria 100% resistant to penicillin. Whereas amphicillin and oxacillin antibiotics show varying resistance results. The amphicillin antibiotics showed that Staphylococcus aureus bacteria were 87.5% resistant and 14.3% sensitive. In oxacillin antibiotics Staphylococcus aureus bacteria were resistant 64.2% and 35.8% were sensitive of the 14 isolates tested. The resistance of Staphylococcus aureus to β-lactam antibiotics is a problem that is quite common in several places, this is becoming more prevalent if treatment with β-lactam antibiotics is not based on dosage and appropriate use (Effendi, 2009). The development of bacterial resistance to antibiotics is influenced by the intensity of antibiotic exposure in an area, uncontrolled use of antibiotics tends to increase the resistance of germs that were originally sensitive (Shryock and Richwine, 2010).

The sensitivity test conducted on tetracyclin antibiotic from 14 isolates showed 42.8% intermediate isolates and 57.2% were sensitive isolates. This shows that tetracyclin antibiotics can still be used in cases of infection by Staphylococcus aureus because they still have sensitivity to several S. aureus isolates. Tetracyclin has a broad spectrum that is active against gram-positive and gram-negative bacteria by working to inhibit protein synthesis (Velhner and Milanov, 2015).

In this study, different inhibition areas were found because of the different abilities of tetracyclin in each isolate of Staphylococcus aureus. Some sensitive bacterial isolates showed that these bacteria still had a side introduction to the tetracyclin target. While bacterial isolates classified as intermediates should receive special attention if the bacteria are still induced by tetracyclin. Bacteria undergoing intermediate conditions are caused by the ineffectiveness of tetracyclin caused by mutations in the recognition and binding side of tetracyclin. Mutations that occur may be silent mutation until a codon bias occurs which causes a disturbance in tRNA movement (Zibandeh et al., 2016).

Resistance to antibiotics caused by bacteria can be divided into three, among others, innate resistance (primary), acquired resistance (secondary), and episomal resistance. Innate (primary) resistance due to the presence of antibiotic decomposing enzymes in bacteria so that naturally these bacteria can break down antibiotics, resistance can be obtained (secondary) due to mutations in bacteria that occur quickly and can also occur for a long time and episomal resistance where bacteria have a factor R on plasmids that can be transmitted to other bacteria that have species links through conjugate or transduction cell contact (Jagielski et al.,2014). The overall discussion of this study is that antibiotic β-lactam in this study showed that Cefoxitin and penicillin were ineffective for the treatment of S. aureus infection because based on the results of the study, 14 samples were resistant to these antibiotics. While for Oxacillin also showed that5 samples that were still sensitive in samples code B4, B5, K1, K2 and P1. Samples code B6 and P4 were samples still sensitive against amphicillin. Samples that are still sensitive to β-lactam show that β-lactamase is still capable of hydrolyzing the β-lactam ring which can cause sensitivity (Elsayed et al., 2009). While tetracyclin is still possible to be effectively used in the treatment of S. aureus infections. Although in this study there were 6 intermediate isolates against tetracyclin which were thought to be resistant. However, there were still 8 sensitive isolates.

We found that cefoxitin discs, as recommended by Jain et al., 2008, is a good method for detecting MRSA by combining oxacillin discs so that no MRSA is missed. It is always recommended to combine the two method, one with high sensitivity and the other with high specificity. According to our results, 9 (64.28%) included MRSA. We conclude that the disc diffusion (DD) oxacillin test is more specific but less sensitive than the cefoxitin DD test. This finding is important to confirm the existence of MRSA and encourage the government to control MRSA sourced from raw milk.

Summary

MRSA is a bacterium that is resistant to antibiotic treatment. The presence of MRSA in raw milk in Surabaya requires the government to respond to encourage antibiotic use in livestock to be appropriate and rational. Which is an important step to reduce the incidence of MRSA sourced from animal origin, especially milk.

Acknowledgement

This study was supported in part with the PENELITIAN UNGGULAN FAKULTAS Funds from Airlangga University,Indonesia.

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Abbreviation

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Dr. S. SUKUMAR
MANAGING EDITOR

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Dated : June 26, 2019

ACCEPTANCE LETTER

The following article has been accepted and will be published in **NOVEMBER, 2019** issue of Indian Veterinary Journal.

Article No.	Title	Author (s)
177/19	Antibiotic Resistance to Staphylococcus aureus and Methicillin Resistant Staphylococcus aureus (MRSA) Isolated from Dairy Farms in Surabaya, Indonesia.	Wiwiek Tyasningsih, **Mustofa Helmi Effendi**, Budiarto Budiarto, Indra Raja Syahputra

Sd/-

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Indian Veterinary Journal

To,

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