

Profile of Multidrug Resistance (MDR) and Methicillin Resistant *Staphylococcus aureus* (MRSA) on Dairy Farms in East Java Province, Indonesia

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Abstract

Staphylococcus aureus is one of the causes in the case of Foodborne Disease, with the resulting toxin triggering the occurrence of Foodborne Intoxication. The purpose of this study was to identify multidrug resistant patterns and detect Methicillin Resistance *Staphylococcus aureus* (MRSA) in cow's milk in East Java, Indonesia. A total of 170 cow's milk samples were taken from dairy farms on 3 Districts, then the bacteria were cultured and purified using Mannitol Salt Agar media. Resistance tests were carried out with 5 antibiotics on Mueller Hinton Agar media and Methicillin Resistant *Staphylococcus aureus* (MRSA) by Oxacillin Resistance Screen Agar Base (ORSAB). The results showed there were 76 (44.7%) *S. aureus*, then antibiotic testing showed 13 (17.1%) *S. aureus* isolates had multidrug resistant (MDR) properties on the antibiotics tested and 84.6% isolates with MDR confirmed MRSA properties by ORSAB. It was concluded that the raw milk can be a potential reservoir for MDR and MRSA strains to threat public health.

Keywords: Foodborne Disease, Raw Milk, MDR, MRSA, Public health.

Introduction

Foodborne disease is a major public health problem throughout the world and is defined as an infectious or toxic disease due to consuming contaminated food or water¹. *Staphylococcus aureus* (*S. aureus*) is an opportunistic pathogen and one of the causes of foodborne intoxication / poisoning which has a high incidence rate related to consumption of raw milk and milk products², it is caused by ingestion of food contaminated by *Staphylococcal* enterotoxins (SE)

strains of *Staphylococcus*³. The enterotoxins produced by *S. aureus* can cause poisoning even at very low doses, which are 20 ng -1 µg / ml⁴. SE is very stable to heat, resistant to environmental conditions such as freezing, drying and resistant to proteolytic enzymes such as pepsin or trypsin and low pH, enabling them to function fully in the digestive tract after consumption⁵.

The main treatment for staphylococcal infection is oxacillin preparations, but the development of antibiotics and misuse in agriculture, stock farming, veterinary medicine, and human diseases treatment increases the potential for antibiotic resistance by bacteria^{6, 7}. This study aimed to evaluate the antibiotic resistance from raw milk on dairy farms in East Java. The antibiotic resistance that have impact in human health as well as in dairy cows health as such a potential transmission of MRSA strains with MDR properties in several

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antibiotics.

Material and Methods

The total sample of 170 dairy cows were taken on 3 dairy farms in the districts of Kediri, Probolinggo and Blitar during September - November 2019. Dairy Cows milk was taken at milkcan deposited by farmers at 25 ml milk storage posts, put in a 50 ml centrifuge tube setril (Biologix, BD-T0034). A total of 1 ml of each sample was taken aseptically using Syringe 3CC (AKD 20902900277) and placed in a 10 ml vaculab containing 4 ml of media broth Buffered Peptone Water (Oxoid, CM0509) referring to the enrichment method from research in *Veterinary World* and *Journal of Veterinary Medicine* which was modified^{8, 9}. Vaculab containing enrichment media was incubated in an incubator (Isuzu Model 2-2195, Jica) at 37°C for 24 hours. Samples were cultured and purified using Mannitol Salt Agar (HiMedia Pvt. Ltd, M118) and then incubated at 37°C for 24 hours.

Microscopic examination of the colony was carried out by Gram's method of staining to produce a display of Gram positive bacteria in the form of coccus and clustered. Biochemical examination was carried out by Catalase test and Coagulase test. Catalase tests were carried out by dripping hydrogen peroxide (HO) 3% on clean glass objects^{10, 11}. Coagulase tests were carried out by modifying the method from *Journal of Clinical Microbiology*¹², Coagulase slide test / clumping factor gave 50 µl rabbit blood plasma dripped on a glass object, then mixed with 1 ose of bacterial colony, Coagulase tube test using 200 µl blood plasma was added with as many as 3-4 isolate colonies then incubated 37°C for 24 hours.

The resistance pattern of *S. aureus* was determined by Disk-diffusion test¹³. 5 antibiotics such as Tetracycline 30 µg, Erythromycin 15 µg, Gentamicin 10 µg, Cefoxitin 30 µg and Oxacillin 30 µg (Oxoid) were selected based on various data on the use of antibiotics in human medicine, national veterinary therapy and various epidemiological purposes studies. Isolates that are resistant to 30 µg Oxacillin (Oxoid) will be confirmed with Oxacillin Screen Agar Base (HiMedia Pvt. Ltd., M1415) added Oxacillin Resistance Selective Supplement (HiMedia Pvt. Ltd., FD191) to detect MRSA strains.

Results and Discussion

Sample test results showed that from 170 milk samples taken in 3 districts, there were 76 samples (44.7%) positive for of *S. aureus* based on morphological cultural characteristics and biochemical tests. This number is distributed with the number of different *S.aureus* positive samples in each location, such as in Kediri found 20 (40%) *S.aureus* positive isolates from 50 samples, while in Probolinggo found 30 (60%) *S.aureus* positive isolates from 50 samples and Blitar 26 (37%) positive isolates of *S. aureus* from 70 (Table 1). Basically *S. aureus* can be transmitted to humans through contamination of milk or milk products, but also milk contamination can be sourced from humans¹⁴. The positive amount of *S. aureus* which is more than 40% can be caused by a variety of factors, which are mainly due to the hygiene milking factor played by the milker. A researcher from *International journal food contamination* states that contamination in milk can be sourced from post-harvest handling which is categorized into 3 main sources including udder health and udder surfaces to teat, milk handling and storage equipment¹⁵.

Table 1. Isolation of *S. aureus* according to sampling location

Location	Location Code	Sample size	Positive <i>S. aureus</i> (%)
Kediri	Kr	50	20 (40%)
Probolinggo	Pb	50	30 (60%)
Blitar	Bl	70	26 (37%)
Total		170	76 (44.7%)

Note : % (Percentage of *Staphylococcus aureus* in all milk samples).

Profile of antibiotic resistance from antibiotic susceptibility test it was found that as many as 25 (32.9%) *S.aureus* isolates had resistance to 1 class of antibiotics tested, while 21 (27.6%) isolates were resistant to 2 classes

of antibiotics and 13 (17.1%) isolates were confirmed to be multidrug resistant (MDR) because it has resistance to ≥ 3 classes of antibiotics (Fig 1.). There are 5 MDR *S.aureus* patterns in which predominantly have 3 (3.9%) isolate patterns of Cefoxitin, Oxacillin, Tetracyclin, and Erythromycin, followed by a pattern of resistance to Cefoxitin, Oxacillin, Tetracyclin, Erythromycin and Gentryn in a number of 3 (3.9%) isolate. While those who have resistant patterns of Cefoxitin, Oxacillin, Tetracyclin and Gentamicin are 3 (3.9%) isolates (Table 2).

Table 2. Resistance profile of isolated *S. aureus* by Group of Antibiotic.

Group of Antibiotic	Resistance profile	Number of <i>S. aureus</i> isolates	Total
		(n = 76)	Number of Isolates (%)
		Resistant Isolates (%)	
1	OX	2 (2.6)	25 (32.9)
	TE	16 (21.1)	
	E	6 (7.9)	
	CN	1 (1.3)	
2	FOX, OX, TE	3 (3.9)	21 (27.6)
	OX, TE	5 (6.6)	
	OX, E	2 (2.6)	
	TE, E	10 (13.2)	
	TE, CN	1 (1.3)	
≥ 3	FOX, OX, TE, E	3 (3.9)	13 (17.1)
	FOX, OX, TE, CN	3 (3.9)	
	FOX, OX, TE, E, CN	4 (5.3)	
	OX, TE, E	2 (2.6)	
	TE, E, CN	1 (1.3)	

Note : FOX=Cefoxitin 30 μ g, OX= Oxacillin 30 μ g, TE=Tetracycline 30 μ g, E=Erythromycin 15 μ g, CN=Gentamicin 10 μ g, % (percentage).

Info: Total 13 isolate Resistance with ≥ 3 Group of Antibiotic are Multidrug resistant (MDR).

S.aureus actually has intrinsic susceptibility to antibiotics, but has tremendous potential to develop, or obtain resistance to almost all antimicrobials exposed to these bacteria¹⁶. Actually the resistance of pathogenic bacteria to antibiotic agents is a natural mechanism for survival¹⁷, but its presence has an adverse effect on public health.

Table 3. Profile Multidrug Resistance of *S. aureus* isolates by location.

Location	Sample code	Resistance profile				
		FOX 30 μ g	OX 30 μ g	TE 30 μ g	E 15 μ g	CN 10 μ g
Kediri	Kr 05	✓	✓	✓	✓	-
	Kr 37	✓	✓	✓	-	✓
Probolinggo	Pb 04	✓	✓	✓	✓	✓
	Pb 07	✓	✓	✓	✓	✓
	Pb 17	✓	✓	✓	-	✓
	Pb 21	✓	✓	✓	✓	✓
	Pb 31	✓	✓	✓	-	✓
	Pb 35	✓	✓	✓	✓	-
	Pb 45	✓	✓	✓	✓	✓
Blitar	Bl 11	-	✓	✓	✓	-
	Bl 30	-	-	✓	✓	✓
	Bl 31	✓	✓	✓	✓	-
	Bl 46	-	✓	✓	✓	-

Note : ü= Resistant, FOX= Cefoxitin 30 µg, OX= Oxacillin 30 µg,

TE= Tetracycline 30 µg, E= Erythromycin 15 µg, CN= Gentamicin 10 µg.

Researcher from *BMC Microbiol* believes that the presence of multidrug resistant (MDR) by *S.aureus* is due to genomic integrated genetic transfer from its main source, Coagulase negative staphylococcus (CNS)¹⁸. Where CNS itself has a low pathogenetic level compared to *S. aureus* but has a high tendency to have MDR properties¹⁹. *S.aureus* positive isolates that are MDR are

found most at Probolinggo location, which is 7 isolates or 53.8% of *S.aureus* isolates that are MDR (Table 3) and shown in Fig. 2. The most isolated isolates found by *S.aurus* with MDR characteristics were at the location of Kediri, namely 2 isolates or 15.4% of *S.aureus* isolates that were MDR. This can be caused by differences in the management of post-harvest handling and post-mastitis treatment in each region. Agree with this *Food Control Journal* states that the percentage of *S.aureus* resistance to several antibiotics tested can result from misuse and miss control of antibiotic use for the treatment of infections in dairy farming areas, where risk factors can be sourced from the farmer, veterinarian in question²⁰.

Table 4. Total Number Confirmed MRSA by ORSAB

Location	Resistance Group of Antibiotics	Positive ORSAB Test	Number of MRSA by location
Kediri	1	1	5
	2	2	
	≥3	2	
Probolinggo	1	-	8
	2	2	
	≥3	6	
Blitar	1	1	6
	2	2	
	≥3	3	
Total Number of MRSA			19

Info : Total number MRSA isolates were 19 isolates.

Testing for MRSA was carried out on oxacillin resistant isolates, of which a total of 24 oxacillin resistant isolates contained 19 (79.2%) strains of MRSA confirmed by ORSAB (Table 4) and shown in Fig. 2. Not all MRSA strains in this study were MDR on the antibiotics tested, where there were 11 MRSA isolates that were MDR while 8 other MRSA isolates were not MDR.

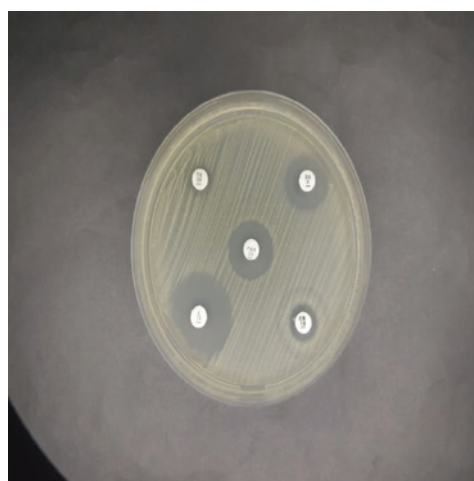


Figure 1. Antibiotic susceptibility test of *Staphylococcus aureus* isolate of MDR in Mueller Hinton Agar Media.

Legend : FOX=Cefoxitin, OX= Oxacillin, TE=Tetracycline, E=Erythromycin, CN=Gentamicin (Cannon 600D DSLR).

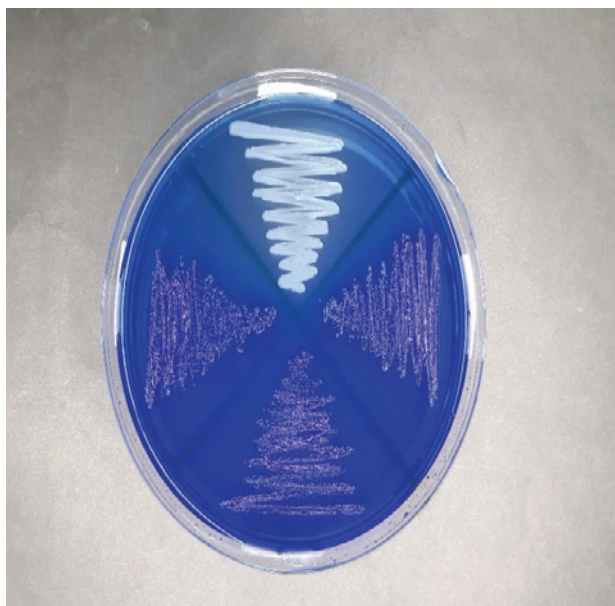


Figure 2. Confirmation for MRSA by Oxacillin Screen Agar Base (ORSAB) (HiMedia Pvt. Ltd, M1415). Blue color shows positive confirmation results, white results show negative confirmation results. (Cannon 600D DSLR).

Detection of MRSA resistance can be achieved by using oxacillin disk or cefoxitin followed by ORSAB test¹¹. Oxacillin is a class of lactam antibiotics and is the first choice drug in the treatment of mastitis infections caused by *S. aureus* bacteria. The mechanism of action of penicillin inhibits bacterial growth by interfering with the transpeptidation reaction in cell wall synthesis⁸. There are mechanisms of resistance to beta-lactam namely: inactivation of antibiotics with beta-lactamase; modification of the Penicillin-Binding-Protein (PBP) target; impaired drug penetration to achieve PBP goals²¹.

We found that cefoxitin discs, as recommended by *Journal of Medical Microbiology*, is a good method for detecting MRSA by combining oxacillin discs to confirm MRSA presence²². It is always recommended to combine the two methods, oxacillin with high sensitivity and the cefoxitin with high specificity. According to the present results, 19 (25%) included MRSA of *S. aureus* isolates based ORSAB test. It is concluded that the ORSAB test is more sensitive but less specific compared with the cefoxitin test. This finding is important to confirm the existence of MRSA and advice the government to take steps to control MRSA sourced from raw milk.

Conclusion

This is the high incidence of MDR and MRSA from *Staphylococcus aureus* in dairy cows. ORSAB test showed the MRSA were higher compared to the MDR cases. The MRSA and MDR of *Staphylococcus aureus* showed the potential for rapid and wider dissemination and poses a threat to animal health and public health. This is an alarming high prevalence of MRSA from dairy farms in East Java Province, Indonesia.

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Designation :

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Ethical Clearance: Raw milk were used in this study, hence ethical clearance was not necessary. Raw milk samples were collected from dairy farms in East Java province, Indonesia.

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Conflict of Interest: Nil

References

1. Ed-Dra, A., Filali, F. R., Bouymajane. A., Benhallam, F., El Allaoui, A., Chaiba, A., Giarratana, F. Antibiotic Susceptibility profile of *Staphylococcus aureus* isolated from sausages in Meknes, Morocco. *Veterinary World*, 2018. **11** (10): 1459-1465.
2. Spanu, V., Spanu, C., Viridis, S., Cossu, F., Scarano, C., and de Santis, E. P. L. Virulence factors and genetic variability of *Staphylococcus aureus* strains isolated from raw sheep's milk cheese.

- International Journal of Food Microbiology*. 2012. **153** : 53–57.
3. Saadat, Y. R., Fooladi, A. A. I., Shapouri, R., Hosseini, M. M., and Khiabani, Z. D. Prevalence of enterotoxigenic *Staphylococcus aureus* in organic milk and cheese in Tabriz, Iran. *Iranian Journal of Microbiology*. 2014. **6** (5): 345-349.
 4. Pelisser, M. R., Klein, C. S., Ascoli, K. R., Zotti, T. R., and Arisil, A. C. M. Occurrence of *Staphylococcus aureus* and multiplex PCR detection of classic enterotoxin genes in cheese and meat products. *Brazilian Journal of Microbiology*. 2009. **40** (1): 145-148.
 5. Hennekinne, J.A., de Buyser M.L., and Dragacci S. *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiology Reviews*, 2012.**36** : 815–836.
 6. Waters, A. E., Contente-Cuomo, T., Buchhagen, J., Liu, C. M., Watson, L., Pearce, K., Foster, J. T., Bowers, J., Driebe, E. M., Engelthaler, D. M., Keim, P. S., Price, L. B. Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clinical Infectious Diseases*. 2011. **52** (10): 1227-1230.
 7. Rahmaniar RP, Yunita MN, Effendi MH, Yanestria SM. Encoding Gene for Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolated from Nasal Swab of Dogs. *Indian Veterinary Journal*. 2020 . **97** : 37 – 40.
 8. Thaker, H. C., Brahmabhatt, M. N., and Nayak, J. B. Isolation and identification of *Staphylococcus aureus* from milk and milk products and their drug resistance patterns in Anand, Gujarat. *Veterinary World*. 2013. **6** (1) : 10-13.
 9. Effendi, M. H., Oktavianto, A and Hastutiek, P. Tetracycline Resistance Gene In Streptococcus Agalactiae Isolated From Bovine Subclinical Mastitis In Surabaya, Indonesia. *Philipp. Journal of Veterinary Medicine*. 2018. **55** (SI): 115-120.
 10. Effendi M. H., Hisyam M. A. M., Hastutiek P., Tyasningsih W. Detection of coagulase gene in *Staphylococcus aureus* from several dairy farms in East Java, Indonesia, by polymerase chain reaction, *Vet. World*, 2019. **12**(1): 68-71.
 11. Tyasningsih, W., Effendi, M. H., Budiarto, B., and Syahputra, I. R. (2019) Antibiotic Resistance to *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolated from Dairy Farms in Surabaya, Indonesia. *Indian Veterinary Journal*. 2019. **96** (11) : 27 – 31.
 12. Jungkind, L. J., Torhan, N. J., Corman, K. E., and Bondi, J. M. Comparison of Two Commercially Available Test Methods with Conventional Coagulase Tests for Identification of *Staphylococcus aureus*. *Journal of Clinical Microbiology*. 1984. **19** (2) : 191-193.
 13. CLSI. M100 Performance Standards for Antimicrobial. 27th ed. Clinical and Laboratory Standards Institute, USA. 2017.
 14. Sasidharan, S., Prema, B., and Latha, Y. L. (2011) Antimicrobial drug resistance of *Staphylococcus aureus* in dairy products. *Asian Pacific Journal of Tropical Biomedicine*. **1** (2): 130-132.
 15. Reta, M. A., Bereda, T. W., and Alemu, A. N. Bacterial contaminations of raw cow's milk consumed at Jigjiga City of Somali Regional State, Eastern Ethiopia. *International journal food contamination*. 2016. **3** (1):1–9.
 16. Schmidt, T., Kock, M. M., and Ehlers, M. M. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *Journal of Dairy Science*. 2015. **98** (9): 2 – 14.
 17. Fischbach M. A., and Walsb C. T. Antibiotics for Emerging Pathogens. American Association for the Advancement of Science. *Science*, 2009. **325** (5944): 1089-1093.
 18. Vitali, L. A., Petrelli, D., Lamikanra, A., Prenna, M., and Akinkunmi, E. O. Diversity of antibiotic resistance genes and staphylococcal cassette chromosome mec elements in faecal isolates of coagulase-negative staphylococci from Nigeria. *BMC Microbiol*. 2014. **14** (1) :106.
 19. Taponen, S., and Pyörälä, S. Coagulase-negative staphylococci as cause of bovine mastitis □ not so different from *Staphylococcus aureus*. *Veterinary Microbiology*. 2009. **134** : 29–36.
 20. Jamali, H., Paydar, M., Radmehr, B., Ismail, S., and Dadrasnia, A. Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control*, 2015. **54**: 383-388.
 21. Broekema, N.M., Van, T.T. Monson, T.A. Marshall S.A. and Warshauer, D.M. Comparison

- of Cefoxitin and Oxacillin Disk Diffusion Methods for Detection of *mecA* Mediated Resistance in *Staphylococcus aureus* in a Large-Scale Study. *J. Clin. Microbiol.*, 2009. 47: 217-219.
22. Jain A., Agarwal, A. and Verma, R. K. Cefoxitin disc diffusion test for detection of methicillin-resistant staphylococci. *Journal of Medical Microbiology*, 2008. 57 : 957–961.