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ANTIBIOTIC RESISTANCE ANALYSIS OF *Staphylococcus aureus* AS A MAIN CAUSE OF MASTITIS IN THE SOUTHERN REGION OF BANDUNG REGENCY

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Abstract

Pangalengan in Southern Bandung Regency, was known as a center for dairy farming. Mastitis caused by Staphylococcus aureus is a disease that often arises and difficult in treatment, because of lack of knowledge that causes arise resistance to antibiotics. Therefore, observation of milk samples, udder swabs, milking utensils, milking buckets and stable floors, from the Warnasari, Babakan Kiara and Tarumajaya small holder ranches, were tested using antibiotic discs. Results observations showed that Ampicillin, Oxytetracycline and Sulfametoxazole - Trimethoprim, ciprofloxacine and Chloramphenicol are five groups of antibiotics that are often used for the treatment of mastitis, and are now experiencing resistance. The highest percentage of resistance was to Ampicilin which reached 100% and the lowest was Sulfametoxazole - Trimethoprim, Ciprofloxacine and Chloramphenicol which reached 3.12%. Generally, the percentage of resistance of these five antibiotic group, shows the highest number in Babakan Kiara and the lowest in the Tarumajaya region. The factors that support increased antibiotic resistance in the treatment of mastitis are the use of antibiotics that is continuous and uncontrolled, poor farm management and the cage very close to farmer houses.

Key words: *antibiotics, dairy cows, milk, resistance, Staphylococcus aureus.*

INTRODUCTION

The rules for setting up farms, animal hygiene, shelters, milking and milk hygiene are important concerns of dairy farming. Due to different kind of microorganisms, the number of germs in milk may increase by exceeding the norms allowed for milk consumptions. The average total number of bacteria in milk (Total Plate Count) in the working area of the Southern Bandung Dairy Cattle Cooperative (KPBS) in Bandung Regency is 2.59 million Colony Forming Units (CFU) (KPBS, 2019). The low quality of milk produced due to high total bacteria, is inseparable from the production patterns of small holder farms, namely the number of livestock, the small size of land and the very limited skills of farmers (Mauludin et al., 2017). To avoid this situations, antibiotic are sometimes used excessively and uncontrollably, which causes the animals to develop resistance to their action. In addition, poor maintenance management affects up to 70% of milk production (Suprayogi et al., 2019). Distance of the cage adjacent to the farmer's house, as well as dairy cattle dung is usually collected in the cage, which often causes pollution of equipment used for milking, is one of the causes of infection of the udder/mastitis (Kero et al., 2002).

For this reason, research is needed on the level of antibiotic resistance and the use of antibiotic for the treatment of mastitis infections in dairy cattle farms in the Bandung Region, by analyzing the resistance level of *Staphylococcus aureus* bacteria and the factors that influence mastitis. *Staphylococcus aureus* is a major bacterium that causes mastitis in West Java, especially in the Bandung regency. The prevalence of subclinical mastitis in Bandung Regency reaches 53.1% (Supar and Ariyanti, 2008; Afnita, 2016). Bacteria *Staphylococcus aureus*, the cause of mastitis has become resistant to antibiotics Penicillin G, Ampicillin, Vancomycin, Basitrasin and, Ciprofloxacin (Campion et al., 2004), but are still very sensitive to the antibiotics Gentamicin

and Oxacillin (Meng et al, 2018). Poor livestock environmental factors can also increase bacterial resistance to antibiotics (Collignon and Scott, 2019). For this reason, research on how antibiotic resistance levels are used for the treatment of mastitis infections in dairy farms in Bandung Regency, by analyzing the resistance level of *Staphylococcus aureus* bacteria and the factors that influencing of mastitis.

MATERIALS AND METHODS

Sample preparation and isolation of Staphylococcus aureus

This study used 192 subclinical mastitis milk samples, 48 udder swabs, 18 milking utensils swabs, 18 dairy bucket swabs and 18 stable floor swabs, which were isolated from stable farms in Warnasari and Babakan Kiara areas in Pengalengan District and Tarumajaya in Kertasari areas, Bandung district. Determination of subclinical mastitis is done according to the Californian Mastitis Test (CMT). Isolation and identification of *Staphylococcus aureus* was done through bacterial growth screening on Mannitol Salt Agar (MSA), Gram staining, catalase test, coagulase, Voges-Proskauer test and growth properties on 5% sheep blood agar plates (PAD) conducted at the Processing Technology Laboratory, Faculty of Animal Husbandry, University of Padjadjaran. Isolate *Staphylococcus aureus* positive 23S rRNA with code American Type Culture Cell (ATCC) 25923 (Mbrio).

Phenotypic Test of Antibiotic Resistance Properties

This test was determined using a standard inhibition zone test on Müller Hinton agar (Himedia) using Oxoid disk, consisting of: Ampicillin (AMP 10 mcg), Gentamicin (CN 10 mcg), oxytretracycline (OT 30 mcg), Chloramphenicol (C 30 mcg), Sulphamethoxazole - trimethroprim (S x T 25 mcg) and Ciprofloxacine (CIP 5 mcg). *Staphylococcus aureus* ATCC 25923 (Mbrio) was used as a reference strain. Furthermore, isolates are categorized as vulnerable and resistant based on interpretive criteria developed by The Clinical and Laboratory Standards Institute (CLSI) 2019 (Table 1).

Table 1. Standardization of the MIC Zone Antibiotics CLSI 2019

Antibiotic	Diameter (mm)		
	Sensitive	Intermediate	Resistance
Peniciline	≥ 29	–	≤ 28
Gentamicine	≥ 15	13-14	≤ 12
Ciprofloxacine	≥ 21	16-20	≤ 15
Sulfa dan Thrimeth	≥ 16	11–15	≤ 10
Chloramphenicol	≥ 18	11–17	≤ 12
Oxytetrasikline	≥ 19	11–18	≤ 14
Ampiciline	≥ 26	–	≤ 25

Source: CLSI 2019

RESULTS AND DISCUSSION

The results showed that all of 192 milk samples studied, 48 udder swabs, 18 milking utensils swabs, 18 dairy bucket swabs and 18 stable floor swabs, positively contained *Staphylococcus aureus*. This was confirmed phenotypically based on identification results stating that the bacteria were Gram-positive, able to ferment mannitol on MSA media, compact white colonies on Blood Agar Plate media by hemolysis of sheep blood, showing positive reactions on catalase test, positive coagulase test. This is in accordance with the references of Quinn et al. (2002) and Todar (2005), which stated that *Staphylococcus aureus* is Gram-positive, capable of fermenting mannitol, a compact white colony with variations in hemolysis properties, positive on the catalase, coagulase test (Figures 1, 2 and 3).

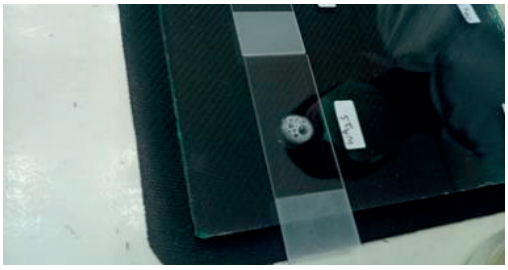

Figure 1. Catalase Test on *Staphylococcus aureus*

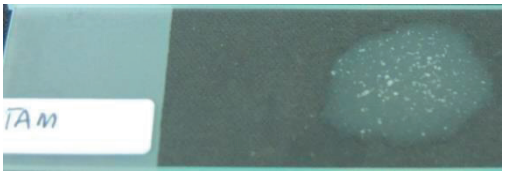

Figure 2. Coagulase Test on *Staphylococcus aureus*

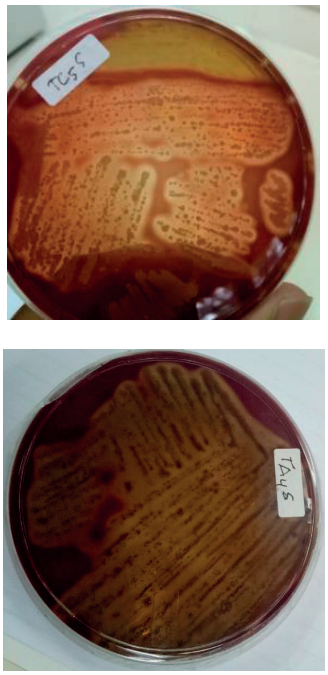

Figure 3. Growth of *Staphylococcus aureus* bacteria on Sheep Blood Agar Media

Based on the nature of bacterial hemolysis isolation in sheep blood agar media, Staphylococcus aureus can lyse sheep blood. Thus these bacteria have virulence factors including alpha hemolysis (hlα) and beta hemolysis (hlβ). Hassuny (2014) mentions that in general the virulence factor of exotoxin haemolisin works by lysis of red blood cells and cause damage to mammalian epithelial cells.

In Gram staining, the bacterium *Staphylococcus aureus*, shows a violet color and is shaped like grapes (Figure 4), is in accordance with the statement of Carter and Cole (2019), that *Staphylococcus aureus* bacteria are Gram-positive, violet-colored bacteria and are shaped like clustered grapes.

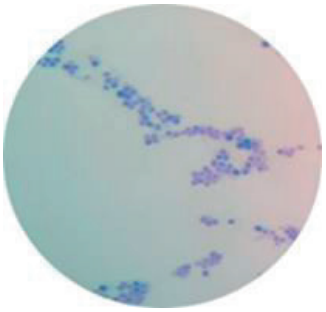

Figure 4. Gram staining of *Staphylococcus aureus*

Staphylococcus aureus, is a major bacterium that causes mastitis in West Java, especially in the Bandung Regency (Sugiri and Anri, 2010). This bacterium often causes mastitis infections both clinically and subclinically.

In dairy farming, *Staphylococcus aureus*, is a threat in increasing the quality and quantity of cow's milk. This threat is due to the difficulty in handling and preventing mastitis infection.

In the observation of this study, it showed that Ampicillin, Oxytetracycline, Sulfametoxazole-Trimethoprim, Ciprofloxacine and Chloramphenicol are five groups of antibiotics for the treatment of mastitis which have become resistant.

The lowest percentage of resistance level occurred in Sulfametoxazole - Trimethoprim, Ciprofloxacine and Chlorampenicol antibiotics (3.12%) and the highest was Ampicilin (100%) (Figure 5, Table 2).

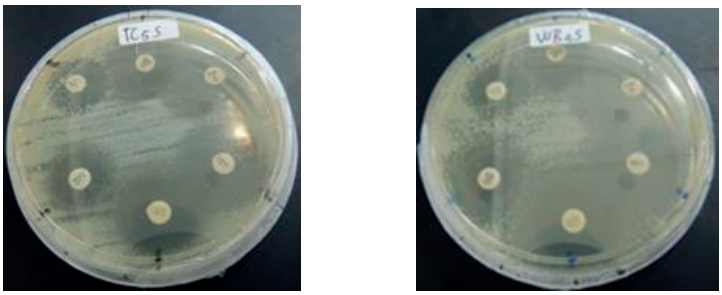

Figure 5. Observation Results of Growth Inhibition Zones (MIC), *Staphylococcus aureus* bacteria in S x T Antibiotic Discs, Cip 5, C30, OT30, CN 50 and AMP 10.

Notes: S x T: Sulfametoxazole - Trimethroprim; Cip 5: Ciprofloxacine; C30: Chloramphenicol; OT30: Oxitetracycline; CN50: Gentamicin; AMP 10: Ampicillin

Table 2. Percentage Data (%) of Antibiotic Resistance Levels in Milk, Udder, Milking utensils, Milking buckets and Stable floor in Small holder Dairy Farming in the KPBS Pangalengan Area

No	Area	Milk		Udder Swabs		Milking utensils		Milking Buckets		Stable Floors	
		Sample	%	Sample	%	Sample	%	Sample	%	Sample	%
1	**Warnasari**										
	S x T	16/64	25	2/16	12,5	3/6	50	0/6	0	2/6	33,33
	C30	0/64	0	0/16	0	0/6	0	0/6	0	0/6	0
	OT30	4/64	6,25	5/16	31,25	3/6	50	3/6	50	2/6	0
	CN 50	0/64	0	0/16	0	0/6	0	0/6	0	0/6	0
	AMP 10	64/64	100	16/16	100	6/6	100	6/6	100	6/6	100
	Cip 5	0/64	0	2/16	12,5	0/6	0	0/6	0	0/6	0
	Total	64		16		6		6		6	100
2	**Babakan Kiara**										
	S x T	23/63	36,51	2/16	12,5	3/6	50	3/6	50	4/6	66,67
	C30	3/63	4,77	0/16	0	0/6	0	0/6	0	0/6	0
	OT30	0/63	0	8/16	50	5/6	83,3	4/6	66,67	4/6	66,67
	CN 50	0/63	0	0/16	0	0/6	0	0/6	0	0/6	0
	AMP 10	63/63	100	16/16	100	6/6	100	6/6	100	6/6	100
	Cip 5	0/63	0	1/16	6,25	0/6	0	0/6	0	0/6	0
	Total	63		16		6		6		6	
3	**Tarumajaya**										
	S x T	2/64	3,12	1/16	6,25	0/6	0	0/6	0	0/6	0
	C30	2/64	3,12	1/16	6,25	0/6	0	1/6	16,67	0/6	0
	OT30	0/64	0	4/16	25	1/6	16,67	0/6	0	1/6	16,67
	CN 50	0/64	0	0/16	0	0/6	0	0/6	0	0/6	0
	AMP 10	64/64	100	16/16	100	6/6	100	6/6	100	6/6	100
	Cip 5	2/64	3,12	3/16	18,75	3/6	50	1/6	16,67	1/6	16,67
	Total	64		16		6		6		6	

Notes: S x T: Sulfametoxazole – Trimethroprim; Cip 5: Ciprofloxacine; C30: Chloramphenicol; OT30: Oxitetracycline; CN50: Gentamicin; AMP 10: Ampicillin

The observations results of antibiotic resistance levels against *Staphylococcus aureus* bacteria (Table 2) show that in general the lowest antibiotic resistance occurs in milk samples, from the stable groups in the Tarumajaya Region against the Cloramphenicol (C30) antibiotic group, the Ciprofloxacine (CIP 5) group, and the Sulfametoxazole - Trimethroprim antibiotic group (Cimetroprimic) S x T) were 3.12% respectively, and the highest was on the antibiotic Ampicillin (AMP 10) of 100% in milk, udder, milking devices, milk bucket and stable floor samples.

The low resistance of *Staphylococcus aureus* to Sulfametoxazole - Trimethoprim (S x T), Ciprofloxacine (CIP 5) and Cloramphenicol (C30) antibiotics (3.12%) occurred in stables in the Tarumajaya region, Kertasari District. Stables in the Tarumajaya area are groups/collective enclosures, with locations far from settlements, bordered by protected forests and with a clean housing system, separated between lactation stables and heifer stables, the feed warehouse is separated from the location of livestock waste disposal. This is consistent with the statement conveyed by Rahayu (2015), that the high level of mastitis infection is highly correlated with cleanliness of the stables, and tends to reduce the use of antibiotics that also to reduce the antibiotic resistance. However, the highest percentage of resistance of *Staphylococcus aureus* to antibiotics is Ampicillin (100%), occurring in all three study areas and in all isolate samples. Ampicillin (AMP 10) is the most studied antibiotic group and has become resistant. This is in accordance with the data submitted by CIVAS (2016) that the highest resistance and most found on animal husbandry is the antibiotic group of Sulphamethoxazole - Trimethoprim, Ampicillin and Tetracycline. This occurs due to several factors that support the inappropriate use of antibiotics and too often using of monotherapy, the very long use and uncontrolled of antibiotics and irrational usages (Utami, 2011).

Dairy stables in the Babakan Kiara and Warnasari area are integrated with farmers homes or only a few meters away, tend to be more humid and with very densely populated

settlements. Cow dung is usually stacked in one corner of the stable and then discharged directly into the water channel. Even so, although the Tarumajaya enclosure is relatively cleaner, in fact the ampicillin (AMP 10) has become resistant. Such conditions, if uncontrolled will result in the spread of antibiotic resistance and residues into the surrounding environment, including water sources.

Based on the isolation, identification and detection of bacterial resistance of *Staphylococcus aureus,* milk isolates from the working area of the KPBS Pangalengan, which is positive for mastitis, indicate a real threat to dairy farms in Bandung Regency, due to the presence of *Staphylococcus aureus*, not only causing clinical and subclinical mastitis but also the potential for resistance to antibiotics that are commonly and uncontrolled used (the Ampicillin, Tetracycline, Quinolone, Aminoglycoside and Chloramphenicol groups). *Staphylococcus aureus* infections treatments, that cause mastitis is known to be very difficult because these bacteria have virulence factors (hlα and hlβ) and genetic changes, especially in the encoding gene for penicillin binding protein, in the use of beta-lactam class antibiotics (Hartman and Alexander, 1984; Olsen et al., 2006) and the presence of the dfrG gene which decreases the affinity of antibiotics, so that it quickly leads to resistance in the aminoglycoside group (Lowy, 2003). *Staphylococcus aureus* infection is also known to be contagious, so that it spreads quickly between the nipples and even between cows. The spread of bacterial infections that cause mastitis in the field, mainly due to poor milking management, location of farms that is close to settlements and poor environment, so the spread of infection between individuals in a farm is increasing.

The observational data on the percentage of antibiotic resistance in the three regions, showed that in small holder dairy farming in Babakan Kiara Region, in general had the highest level of antibiotic resistance compared to the other two regions. The percentage of antibiotic resistance that occurs between 4.77% in Chloramphenicol (C30) antibiotics to 100% in Ampicillin (Amp 10) antibiotics. The lowest resistance was found in the milk sample, then increased in the udder, milking bucket, milking devices, and the highest in the stable floor samples. However, all samples were resistant to Ampicillin antibiotics (AMP 10) (Table 2). The Babakan Kiara region, is known as a dairy farming area located in a densely populated area. Even the stables are very close to their homes and the sewage is directly into the Cileunca lake as water sources, which is less than 100 meters away, so that the spread of bacteria that are resistant to antibiotics in the environment will increase.

This is consistent with the statement of Frieri et al. (2017) that the spread of antibacterial resistant bacteria in the environment is currently increasing and bacteria become resistant due to horizontal gene transfer (HGT). HGT between bacterial species that can cause the spread of Antibiotic Resistance Genes (ARGs) is becoming easier and through various media in the environment, such as in water, soil, air, food, and living things. Facts have been found that the resistance to antibiotics is very large in the environment, due to poor environmental management (Hadi et al., 2018). This bacterial resistance can then spread to animals, humans and the surrounding environment. Bacterial resistance in animals can spread to other animals that are genetically diverse. Whereas in humans, the spread of resistant bacteria can spread directly between patients in the hospital or indirect transmission through health workers. This case, greatly influences the epidemic pattern and transmission of *Staphylococcus aureus* (FOPH, Switzerland, 2019).

CONCLUSIONS

Based on the observations of the resistance of *Staphylococcus aureus* bacteria from milk, udder, milking utensils, milking buckets and stable floors, in the KPBS working area of Bandung Regency, it was concluded that antibiotics (Ampicillin, Oxytetracycline, Sulfamethoxazole - Trimethoprim and Chloramphenicol) that are become resistant, while the Gentamicin are still sensitive.

Therefore, to prevent the increasing resistance of *Staphylococcus aureus* bacteria to antibiotics used for the treatment of mastitis, it is necessary to take steps among others, the use of

wise and well-controlled antibiotics, implementing good farm management by paying attention to environmental health aspects.

Collective system dairy farms stables must far from human settlements; to be one of the livestock systems that can be chosen to minimize the increase the mastitis and also in antibiotic resistance. Antibiotics that can still be recommended for use in dairy farms in the KPBS region, Bandung Regency are antibiotics of the Gentamicin group (CN 50).

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