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Phenotypic detection strategies of multidrug-resistant *Staphylococcus aureus* isolated from cat nasal swab in Madiun city, Indonesia

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Abstract. *Millannia SK, Khairullah AR, Effendi MH, Utama S, Kurniawan SC, Afnani DA, Silaen OSM, Ramadhani S, Ramandinianto SC, Waruwu YKK, Widodo A, Putra GDS, Farizqi MTI, Riwu KHP. 2023. Phenotypic detection strategies of multidrug-resistant* Staphylococcus aureus *isolated from cat nasal swab in Madiun city, Indonesia. Biodiversitas 24: 940-946.* Over the past ten years, the rise of bacterial disease resistance to antibiotics has presented challenges for veterinarians treating pets. Every day direct contact between pets and their owners is a risk for the spread of harmful bacteria. One of the most frequent patients in veterinary hospitals or clinics is cats. The prevalence of S. aureus infection is rising and becoming more serious as a result of antibiotic resistance, or what is known as multi-drug resistance (MDR). Pet owners and their pets may carry MDR S. aureus through zoonotic transmission. In 2018, MDR events were reported to infect ICU patients at Madiun City Hospital, East Java, Indonesia. Based on this backdrop, the authors decided to perform a study on the S. aureus bacteria resistance test of cefoxitin, tetracycline, erythromycin, gentamicin, and ampicillin bacteria obtained from cat nasal swabs in Madiun City. Making an MSA medium is the first step in bacterial isolation. Then, the Gram stain test identifies the species of Gram bacteria present in the colony. The following step is the catalase and coagulase assays, pinpointing the S. aureus bacterium. The Kirby Bauer disc diffusion method was used to conduct an antibiotic sensitivity test. With the aid of tweezers, paper discs containing the medicines cefoxitin 30 µg, tetracycline 30 µg, erythromycin 15 µg, gentamicin 10 µg and ampicillin 10 µg, are set apart from one another by 25 to 30 mm on an MHA medium. According to sample examination findings, S. aureus was present in 80 (80%), and six isolates (7.5%) were confirmed to be Multi-drug Resistance (MDR). The spread of MDR bacteria in people and animals can result in recurrent infections, a rise in complications, and an increase in morbidity and mortality. Furthermore, rational antibiotic use must be made for both humans and animals to stop the emergence of antibiotic resistance in the veterinary clinic in Madiun City.

Keywords: Cat, MDR, public health, *Staphylococcus aureus*

INTRODUCTION

A severe concern to people, animals, and the environment worldwide is antibiotic resistance (AMR) (Prestinaci et al. 2015). Over the past ten years, antibiotic-resistant bacterial diseases have presented challenges for veterinarians treating pets (Palma et al. 2020). Monitoring degrees of resistance in different organisms and assuring the long-term efficacy of an antibiotic product are based on knowledge about bacterial resistance to antibiotics throughout time (Grenni 2022). Unfortunately, there aren't many ongoing studies on antibiotic-resistant bacteria (Fair and Tor 2014). Therefore, it's critical to conduct ongoing research to track the emergence of bacterial antibiotic resistance (Murugaiyan et al. 2022).

Animals like dogs and cats are frequently considered family members (Menchetti et al. 2020). Almost every family owns a cat, a common pet worldwide (Amiot et al. 2022). Daily direct contact between pets and their owners is a risk for spreading harmful bacteria (Varela et al. 2022). After being domesticated since ancient Egypt, cats are susceptible to many ailments (Kurushima et al. 2012). Viruses, parasites, and bacteria can cause cat diseases (Maggi et al. 2022). Pets may serve as a reservoir for disease transfer to people (Stull et al. 2015). Checking into a clinic or animal hospital is one way to practice maintenance by keeping an eye on a pet's health (Afnani et al. 2022). Cats are among the most frequent patients in veterinary hospitals or clinics (Rodan and Sparkes 2012).

Staphylococcus aureus is a member of the Gram-positive bacterial group, the main causative agent of nosocomial infections (Khairullah et al. 2022). In addition, *S. aureus* is an opportunistic pathogen that is also a part of the body's normal flora (Hardy et al. 2020). *S. aureus* typically inhabits people's noses, throats, skin, and various animal species (Ramandinianto et al. 2020). According to research by Kaspar et al. (2018), 25% of *S. aureus* was found in the mouth and 55% in the nose.

An *S. aureus* infection can significantly affect agriculture and public health (Decline et al. 2020). *S. aureus* is a significant foodborne disease pathogen and can cause mastitis, tick pyemia, pyoderma, and botryomycosis (Kumari et al. 2020). In addition, *S. aureus* causes simple to severe and even fatal skin and soft tissue infections, like bacteremia or septicemia, which can occur (Tong et al. 2015; Yunita et al. 2020*).*

The prevalence of *S. aureus* infection is rising and becoming more serious as a result of antibiotic resistance or what is known as multi-drug resistance (MDR) (Guo et al. 2020). There have been reports of MDR *S. aureus* infections in people and pets such as dogs, cats, and horses (János et al. 2021; Gandolfi-Decristophoris et al. 2013; Adams et al. 2018). In addition, pet owners and their pets may contract MDR *S. aureus* through zoonotic transmission (Overgaauw et al. 2020). In 2018, MDR events were reported to infect ICU patients at Madiun City Hospital (Farida et al. 2018).

One of the uses of antibiotics in people and animals is to treat bacterial infections (Marshall and Levy 2011). As the population grows, antibiotic use frequently rises (Olesen et al. 2018). According to Aslam et al. (2018), factors contributing to antibiotic resistance formation include overcrowding, subpar sanitation, and waste disposal systems, increased use of antibiotics in hospitals and farms, and even inappropriate use of antibiotics.

The positive result is present in cat-derived *Staphylococcus* isolates for coagulase high resistance to ampicillin, tetracycline, amoxicillin, and penicillin (Qekwana et al. 2017). A similar result was noted that cat-derived *S. aureus* isolates were resistant to gentamicin, cefoxitin, ampicillin, and cefoxitin (Li et al. 2021).

Based on this backdrop, the authors decided to perform a study on the *S. aureus* bacteria resistance test of cefoxitin, tetracycline, erythromycin, gentamicin, and ampicillin bacteria obtained from cat nasal swabs in Madiun City.

MATERIALS AND METHODS

Study area and sample collection

The research was carried out from March to June 2022. Bacterial isolation and sensitivity tests were carried out at the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya, East Java, Indonesia. In contrast, cat nasal swab sampling was carried out at four animal clinics in Madiun City, East Java, Indonesia, namely the Heathy Pet Veterinary Clinic, Naureen Petcare Veterinary Clinic, Ichiyo Petcare Veterinary Clinic, and Hurera Care Veterinary Clinic. Using a sterile cotton swab and Amies transport medium, sampling was done on the mucosa of the cat's nasal cavity. The Amies transport medium tube was filled with the cotton swab, labeled, and kept in a coolbox with an ice pack.

Isolation and identification of *Staphylococcus aureus*

Making an MSA medium is the first step in bacterial isolation. First, the sample was obtained from the Amies transport medium tube with a burnt-in loop to make it sterile after being removed from the coolbox. Then, a streak injection of bacteria onto sterile MSA media in a Petri plate was carried out. Next, the MSA medium was incubated for 24 hours at 37°C (Effendi et al. 2019). On MSA media, the bacterial colonies that develop a yellow color are thought to be *S. aureus*. Then, the Gram stain test identified the species of Gram bacteria in the colony, followed by the catalase and coagulase assays to pinpoint the *S. aureus* bacterium.

The first step in the Gram staining procedure is to make the thinnest preparation from bacterial colonies on MSA media that are thought to contain Staphylococcus by combining a small amount of the bacterial culture with one drop of physiological NaCl and fixing it on a bunsen. The crystal violet dye was then drip-applied to the fixed preparation and was allowed to stand for two minutes. After removing the leftover color, the preparations were washed under running water. Next, the Lugol was applied to the preparation and left for one minute. After that, the Lugol was dumped and rinsed into the preparations under running water. Next, the mixtures were diluted with 96% alcohol, left to sit for 10-20 seconds, and then rinsed under running water. After waiting for two minutes, the preparation was next dyed with safranin. The dye was taken off, cleaned under running water, and dried. Finally, the immersion oil was sprayed onto the preparation, and a 1000x magnification microscope was used to observe the bacteria's morphology (O'toole 2016).

A single loop of a bacterial culture thought to be *Staphylococcus* on MSA media was used for the catalase test. Then, 3% hydrogen peroxide (H_2O_2) is drip-applied to a glass object before one layer of the bacterial culture is spread over it and mixed. The existence of gas bubbles (O_2) produced by the genus *Staphylococcus* is a sign that the catalase test was successful (Yuan et al. 2021). The coagulase test extracted plasma from rabbit blood using ethylene diamine tetra acetic acid (EDTA) anticoagulant and next, followed by 15-minute centrifugation at 10,000 rpm. Using a loop, the bacterium culture on MSA media

that was observed as *S. aureus* was removed and placed in 1 ml of nutrient broth for further incubation for 24 hours at 37°C. The next step was to use a syringe to inject 1 cc of rabbit plasma into the nutrient broth that already includes microorganisms with 4-24 hours of incubation (Rahmaniar et al. 2020).

Antibiotic sensitivity test

The Kirby Bauer disc diffusion method was used to conduct an antibiotic sensitivity test. After being isolated and identified, *S. aureus* bacteria were prepared into a suspension by transferring the bacterial culture using a loop into a test tube containing 9 ml of physiological NaCl and homogenizing with a vortex. Comparison of suspension turbidity to the McFarland criterion of 0.5. After carefully cleaning the MHA media's whole surface with a cotton swab to inoculate the suspension, leave for 15 minutes to allow the bacteria to adhere (Gautam et al. 2013). Then, using tweezers, paper discs containing the antibiotic cefoxitin 30 µg, tetracycline 30 µg, erythromycin 15 µg, gentamicin 10 µg, and ampicillin 10 µg were applied to the media's surface. At 37°C, the bacterial culture was cultured for a whole day.

A clean, clear area surrounding the paper disk—referred to as the zone of inhibition of bacterial growth—indicates the success of this method's tests. Next, Vernier calipers are used to measure the antibiotic disc's circumference in millimeters and the diameter of the zone that inhibits bacterial growth. The inhibitory zone's diameter will then be classified as Sensitive (S), Intermediate (I), and Resistant (R). After that, the measurement findings are compared to the CLSI (Clinical and Laboratory Standard Institute) 2020 norms.

RESULTS AND DISCUSSION

Bacterial isolates

According to sample examination findings, *S. aureus* was present in 80 (80%) of the 100 isolated cat nasal swab samples (Table 1) based on morphological culture features, Gram staining, and biochemical testing. Yellow bacterial colonies on MSA media indicated a positive morphological culture of *Staphylococcus aureus* (Figure 1.A). Purple colonies and grouped cocci indicated a positive Gram stain result, shown in Figure 1.B. In addition, the emergence of bubbles on the object glass in the catalase test (Figure 1.C) and the appearance of clout at the bottom of the microtube in the coagulase test both signify a positive biochemical test for *S. aureus* (Figure 1.D).

Antibiotic resistance of *Staphylococcus aureus*

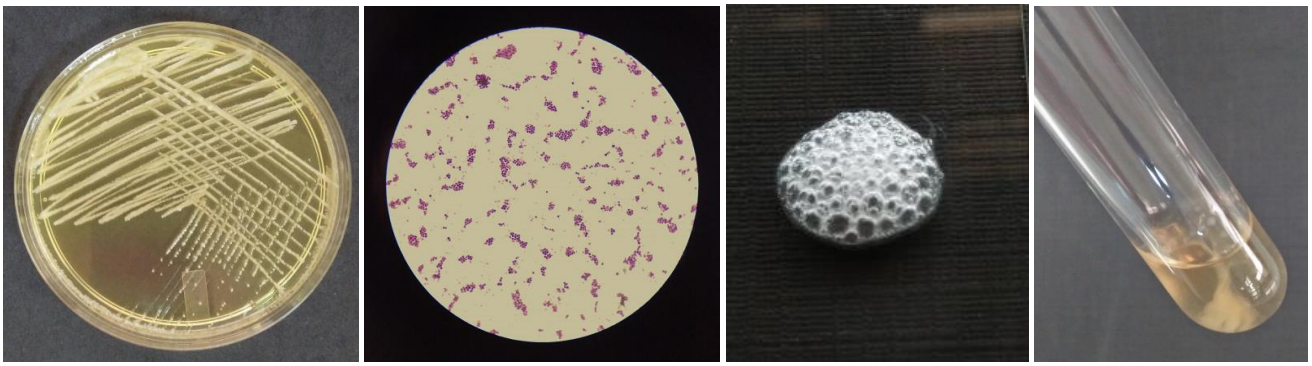
Antibiotic resistance profile from the results of the *S. aureus* resistance test to antibiotics showed that out of a total of 80 *S. aureus* isolates, 31 isolates (38.75%) were detected as resistant to a single class of antibiotics tested. In contrast, 18 isolates (22.5%) were resistant to two classes of antibiotics, and six isolates (7.5%) were confirmed to be multi-drug resistant (MDR) because they were resistant to 3 to 4 antibiotic classes (Figure 2). Those MDR were dominated by the pattern of antibiotic resistance FOX-TE-E-AMP (Cefoxitin, tetracycline, erythromycin, ampicillin) and the pattern of FOX-E-GM-AMP (Cefoxitin, erythromycin, gentamicin, ampicillin), namely two isolates (2.5%) each (Table 2).

Staphylococcus aureus MDR isolates were most frequently discovered in cat nasal swab samples from the Naureen Petcare and Ichiyo Petcare veterinary clinics, with two isolates each (Table 3). This could account for the six isolates of MDR *S. aureus* that were discovered from 100 cat nasal swab samples analyzed in multiple veterinary clinics in the city of Madiun. Of 47 isolates, ampicillin had the highest level of *S. aureus* resistance in this investigation.

Table 1. Isolation of *S. aureus* from cat nasal swab samples at each veterinary clinic

Veterinary clinic	Sample code	Sample size	*S. aureus* (%)
Heathy Pet	HP	25	22 (88%)
Naureen Petcare	NP	25	18 (72%)
Ichiyo Petcare	IP	25	18 (72%)
Hurera Care	HC	25	22 (88%)
Total		**100**	**80 (80%)**

Note: % (Percentage of positive).



A **B** **C** **D**

Figure 1. A. *Staphylococcus aureus* colonies in MSA, B. Gram-stained *Staphylococcus aureus* colonies under a microscope, C. Catalase test results indicate *Staphylococcus aureus* positivity, D. Coagulation test results indicate *Staphylococcus aureus* positivity

Table 2. Isolated *Staphylococcus aureus* resistance profile by antibiotic group

Group of antibiotics	Resistance profile	Number of isolates (n=80) Resistant isolates (%)	Total number of isolates (%)
0	No one is resistant	25 (31.25%)	25 (31.25%)
1	AMP	23 (28.75%)	31 (38.75%)
	E	7 (8.75%)	
	TE	1 (1.25%)	
2	TE-AMP	4 (5%)	18 (22.5%)
	E-AMP	8 (10%)	
	GM-AMP	1 (1.25%)	
	FOX-E-AMP	4 (5%)	
	FOX-GM-AMP	1 (1.2%)	
≥3	TE-E-AMP	1 (1.25%)	6 (7.5%)
	FOX-TE-E-AMP	2 (2.5%)	
	FOX-E-GM-AMP	2 (2.5%)	
	TE-E-GM-AMP	1 (1.25%)	

Note: FOX: Cefoxitin, E: Erythromycin, GM: Gentamicin, TE: Tetracycline, P: Penicillin.

Table 3. *Staphylococcus aureus* isolates with a profile MDR

Veterinary Clinic	Sample code	Resistance profile	Antibiotic				
			FOX	TE	E	GM	AMP
Heathy Pet	HP 25	TE-E-GM-AMP	-	✓	✓	✓	✓
Naureen Petcare	NP 12	FOX-TE-E-AMP	✓	✓	✓	-	✓
	NP 20	FOX-TE-E-AMP	✓	✓	✓	-	✓
Ichiyo Petcare	IP 3	FOX-E-GM-AMP	✓	-	✓	✓	✓
	IP 10	FOX-E-GM-AMP	✓	-	✓	✓	✓
Hurera Care	HC 14	TE-E-AMP	-	✓	✓	-	✓

Note: ✓: Resistant, FOX: Cefoxitin, E: Erythromycin, GM: Gentamicin, TE: Tetracycline, AMP: Ampicillin.

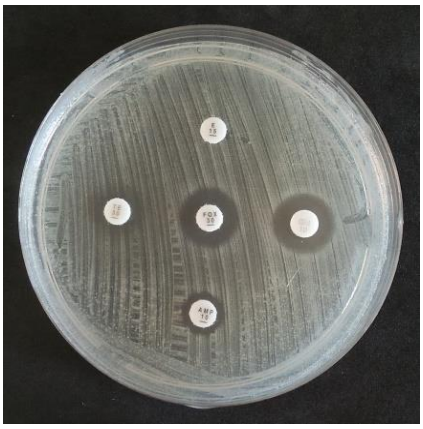


Figure 2. Analyze the susceptibility to antibiotics of an *S. aureus* isolate cultured on MHA

Discussion

Bacterial isolates

After performing several isolation and identification steps, it was determined that *S. aureus* was present in up to 80 of 100 samples collected from four veterinary clinics in Madiun City. The finding of *S. aureus* in a cat's nasal is connected to *S. aureus*, a typical microbial flora in animals (Bierowiec et al. 2016). In addition, a common commensal bacterium called *S. aureus* can be found in humans' noses, throats, skin, and many other animal species (Heaton et al. 2020).

Amies transport media was used as the transport medium for the sample collection and sent swab samples to the lab. In Amies transport medium, pathogenic bacteria can live longer, albeit, after a while, their viability may start to decline (Rosa-Fraile et al. 2005).

Following sample collection, MSA media is used to isolate the samples. Furthermore, this isolation seeks to breed and separate bacteria using culture media to acquire pure isolates. To isolate *S. aureus*, MSA was used as a selective and differential medium. *Staphylococcus* is the only bacteria that can grow in an MSA (7.5%) salt environment because it is the only one that ferments the mannitol substrate in this medium, producing fatty acids. Fatty acids convert the medium into acid, changing its appearance to yellow (Budiarso et al. 2019).

The findings of presumed *Staphylococcus* isolation, namely isolates of bacteria capable of digesting mannitol, were examined under a microscope. Moreover, to make the identification procedure more thorough and precise, this analysis tried to identify the morphological properties of cells. Microscopic analysis revealed that *Staphylococcus*, all clustered cocci and purplish color, was present in every cat nasal swab sample (Rasheed and Hussein 2021). Like clustered cocci resembling grapes and Gram-positive characteristics, *S. aureus* colonies have this characteristic.

All *Staphylococcus* isolates demonstrated positive findings in the catalase test by producing gas bubbles. In addition, the hydrolysis of hydrogen peroxide (H_2O_2) into

water (H_2O) and oxygen (O_2) produced gas bubbles. Because of the catalase enzyme that *Staphylococcus* produces, H_2O_2 is hydrolyzed. Staphylococcus produces catalase enzymes as a defensive mechanism against hydrogen peroxide, which is poisonous to bacteria (Wang et al. 2015).

The coagulase test aimed to identify whether the Staphylococcus coagulase enzyme was present. The production of coagulase enzymes was then utilized to distinguish between Coagulase-Negative *Staphylococcus* (CoNS) bacteria such as *S. epidermidis* and *S. aureus*. The bacteria *S. aureus*, also known as Coagulase-Positive *Staphylococcus*, can produce coagulase enzymes (CoPS). A total of 80 positive samples were obtained based on the coagulase test results, which were demonstrated by the presence of gel-like clots of rabbit plasma. The *S. aureus*-produced coagulase enzyme binds to the coagulase-responding factor in plasma to generate plasma clots (McAdow et al. 2012). The thrombin complex is created from these connections. Afterward, thrombin transforms fibrinogen into fibrin, which causes the plasma to clot (Kattula et al. 2017).

Antibiotic resistance of Staphylococcus aureus

Antibiotics' use increases in proportion to population growth, which also directly correlates with antibiotic resistance emergence (Klein et al. 2018). Increased population, improved access to veterinary services, and the use of antibiotics for various health concerns have all contributed to an increase in the frequency of antibiotic usage in pets (Rhys-Davies and Ogden 2020). As a result, antibiotic use has increased, resulting in the emergence of bacterial resistance to antibiotics (Cantón and Morosini 2011). An antibiotic sensitivity test can be used to track the emergence of antibiotic resistance (Khan et al. 2019). The antibiotic sensitivity test results are now referred to as the antibiotic resistance profile, which can be used as scientific data by practitioners to treat illnesses and by connected parties to keep track of an antibiotic's level of resistance (Gajic et al. 2022).

S. aureus frequently develops resistance to regularly used antibiotics (Deyno et al. 2017). In addition, different antibiotic classes may each have their own specific resistance mechanisms (Reygaert 2018). In general, a pathogenic bacteria's method for resisting antibiotics can be either innate (natural) or acquired (acquired) (Peterson and Kaur 2018). Two different mechanisms of acquired resistance exist in *S. aureus*, including bacterial gene chromosomal mutation and genetic exchange with other bacteria to acquire resistant genes (conjugation, transduction, or transformation) (Yılmaz and Aslantaş 2017).

In this investigation, the number of isolates with ampicillin resistance reached high in 47 isolates. According to research by Malik et al. (2005), *Staphylococcus* resistance in cats and dogs is comparable to that in people, which exhibits a high value of ampicillin resistance. Ampicillin is an antibiotic used to treat *S. aureus*, a broad-spectrum penicillin derivative (Jubeh et al. 2020). Penicillinase (class A β-lactamase) is an extracellular enzyme responsible for *S. aureus* resistance to ampicillin (Bush and Bradford 2020). The amide link in the ampicillin β-lactam ring will be hydrolyzed by the penicillinase enzyme (Bonomo 2017). This eventually resulted in ampicillin losing its antimicrobial effectiveness.

In the four animal clinics where samples for this study were taken, *S. aureus* resistance to several drugs was discovered. Unlike other veterinary clinics in the city of Madiun, the veterinary clinic does not accept grooming and caring for healthy cats, which can lead to this. Cats at daycare and grooming facilities can be classified as healthy cats if the veterinarian so declares at the time of patient registration. Resistance to antibiotics caused by *S. aureus* in domestic cats is largely tied to earlier treatments given to the animal or its owner (Vitale et al. 2006). *S. aureus*, including strains resistant to antibiotics, can spread more easily when pets and their owners are nearby (Smith 2015). Antibiotic resistance has been found in pets, which poses a risk to public health (Marshall and Levy 2011).

Multi-drug resistant (MDR) is a condition in which bacteria are resistant to at least one antibiotic from three or more classes of antibiotics (Widodo et al. 2022). Six of the 80 isolates tested positive for *S. aureus* and were resistant to three of the four types of antibiotics (β-lactam, tetracycline, macrolides, and aminoglycosides) during the sensitivity test. The accumulation of resistance coding genes for each antibiotic in R plasmids frequently contributes to MDR characteristics in bacteria (Nikaido 2009). Transposons, integrons, and integrons and insertion sequence common (ISCR) elements provide a way by which the resistance gene on a single R plasmid is acquired (Pérez-Etayo et al. 2018). Integrons, for instance, are highly effective at producing MDR because they may combine several resistance genes at the appropriate target and offer a powerful promoter to express them (Gillings 2014). In addition, the resistance gene can easily join another integron since it is marked after joining with an integrin (Sarkar et al. 2015).

The spread of MDR bacteria in people and animals can result in recurrent infections, complications, and morbidity and mortality increases (van Duin and Paterson 2016). Additionally, the choice of different medicines for treating bacterial infections may be limited in the presence of microorganisms having MDR characteristics (Terreni et al. 2021). Therefore, rational antibiotic use must be made for both humans and animals to stop the emergence of antibiotic resistance in the veterinary clinic in Madiun City.

To conclude, in Madiun City, Indonesia 80 out of 100 cat nasal swab samples tested positive for *S. aureus*, and 6 of those isolates were MDR. Therefore, antibiotic use in animals must be assessed to administer medication and prevent the development of antibiotic resistance appropriately.

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