

Identification Coagulase-Negative Staphylococci's Species and Antibiotic Sensitivity Isolates from Cow Milk with Subclinical Mastitis

*(IDENTIFIKASI SPESIES STAPHYLOCOCCI KOAGULASE
NEGATIF DAN UJI KEPEKAAN ANTIBIOTIK ISOLAT
ASAL SUSU SAPI MASTITIS SUBKLINIS)*

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ABSTRACT

Coagulase-negative *Staphylococci* (CoNS) were bacteria that had initially been considered harmless but were identified as pathogenic in some cases. Recently, CoNS became the most commonly isolated bacteria from cow's milk, especially in cows with subclinical mastitis. The CoNS species have been the most frequently identified bacteria over the past several decades, exhibiting diverse characteristics that may impact antimicrobial resistance profiles. This study was aimed to identify CoNS bacterial species originated from preserved milk isolates from subclinical mastitis in cows and their sensitivity patterns to several antibiotics. A total of 15 preserved isolates were used in this study. Species identification was done with a phenotypic approach using the VITEK-2 compact tool. The species identification results found *Staphylococcus sciuri*, *S. saprophyticus*, *S. cohnii*, *S. vitulinus*, *S. arlettae* and *S. xylosus*. Antibiotic resistance tests showed five out of 13 CoNS species were resistant. The antibiotics used are ampicillin, cefoxitin, oxacillin, penicillin G, erythromycin, gentamicin, tetracycline and ciprofloxacin. Penicillin G is not recommended for treating mastitis infections caused by CoNS.

Keywords: Antimicrobial resistance; CoNS species; Mastitis treatment; Subclinical mastitis

ABSTRAK

Coagulase Negative Staphylococci (CoNS) merupakan bakteri yang awalnya dianggap tidak berbahaya. Namun, belakangan ini telah teridentifikasi sebagai bakteri patogen pada beberapa kasus. Beberapa waktu terakhir CoNS merupakan bakteri yang paling sering diisolasi dari susu sapi terutama yang mengalami kasus mastitis subklinis. Faktor virulensi yang dimiliki CoNS menyebabkan bakteri-bakteri tersebut bersifat persisten pada kulit termasuk pada ambing sapi perah yang memungkinkan terjadinya resistansi antibiotik. Penelitian ini bertujuan untuk mengidentifikasi spesies bakteri CoNS yang berasal dari isolat tersimpan asal susu sapi perah yang mengalami mastitis subklinis beserta pola kepekaannya terhadap beberapa sediaan antibiotik. Isolat tersimpan yang digunakan sebanyak 15 isolat. Identifikasi spesies dilakukan dengan pendekatan fenotipik yaitu konfirmasi kembali dan menggunakan perangkat vitek-2 compact. Uji kepekaan antibiotik dilakukan dengan menggunakan metode Kirby-Bauer. Hasil identifikasi spesies bakteri ditemukan spesies *Staphylococcus sciuri*, *S. saprophyticus*, *S. cohnii*, *S. vitulinus*, *S. arlettae*, dan *S. xylosus*. Pengujian kepekaan antibiotik menunjukkan lima dari 13 isolat spesies CoNS mengalami resistansi antibiotik. Antibiotik yang digunakan yaitu ampicillin, cefoxitin, oxacillin, penicillin G, erythromycin, gentamicin, tetracycline dan ciprofloxacin. Penicillin G tidak direkomendasikan untuk pengobatan infeksi mastitis yang disebabkan oleh bakteri CoNS.

Kata-kata kunci: mastitis subklinis; pengobatan mastitis; resistansi antibiotik; spesies CoNS

INTRODUCTION

Coagulase-negative *Staphylococci* (CoNS) are a bacteria genus with a large population of species identified in recent decades. The incidence of disease caused by CoNS is increasing, primarily due to environmental contamination (Michels *et al.*, 2021). The CoNS is also increasingly found in samples associated with clinical relevance (Galvani *et al.*, 2020). Such interspecies differences have found some species are more virulent than others, particularly those found in food and can cause infections in hosts with low immunity (Franca *et al.*, 2021; Michels *et al.*, 2021). The CoNS are normal microorganisms commonly found on human and animal skin, such as udder skin and cow nipples (Michels *et al.*, 2021; Ruizripa *et al.*, 2020).

Recently, CoNS has become the most frequently isolated bacterium from cow's milk, especially from those with subclinical mastitis (Idamokoro, 2022; Sender *et al.*, 2017). The problem of CoNS in milk is closely related to mastitis. Mastitis can cause a decrease in milk production ranging from

2.6 to 43.1% and potentially lead to food-borne disease (Marti-de *et al.*, 2020; Pole *et al.*, 2023). Antibiotics used in mastitis cases can contribute to the development of antibiotic resistance (Pole *et al.*, 2023; Syahputra *et al.*, 2020). Antibiotic resistance refers to a condition in which antibiotics are no longer able to effectively inhibit or eliminate bacterial growth. Resistance can occur due to the improper or indiscriminate use of antibiotics (Syahputra *et al.*, 2020). Several studies have found that animals infected with CoNS experience antibiotic resistance (Ruizripa *et al.*, 2020). This resistance has become a frequently found characteristic of CoNS, especially those isolated from animals and clinical samples. Different CoNS species exhibit varying levels of virulence, resulting in distinct responses to antibiotics for each species (Michels *et al.*, 2021; Garbacz *et al.*, 2021). This study was conducted to identify CoNS species derived from preserved isolates collected from cows' milk with subclinical mastitis and their resistance patterns to several antibiotic preparations. It shows antibiotics that can effectively treat subclinical mastitis caused by CoNS.

RESEARCH METHODS

This study is a descriptive investigation aimed at providing data on CoNS bacterial species found in preserved isolates derived from subclinical mastitis milk samples and the species' sensitivity pattern to antibiotics. The preserved isolates used have been identified as CoNS totalling 15 samples derived from milk of dairy cows with subclinical mastitis at Mekar Bakti Farm and Putra Saluyu Farm, of Pamulihan District, Sumedang Regency, West Java Province with a minimum positive of California Mastitis Test (CMT) result of 2 (++), Gram-positive cocci, positive catalase, negative coagulase, and have been preserved on Skim Milk, Tryptone, Glucose, Glycerol (STGG) media and 30% glycerol at the Microbiology Laboratory of the Faculty of Medicine, Universitas Padjadjaran.

Species identification in this study was carried out by phenotypic means. Preserved isolates were reconfirmed through Gram staining tests with Gram-positive cocci, differential media in the form of Mannitol Salt Agar (MSA), catalase test (+), and coagulase test (-). After being confirmed as CoNS, testing continued using the compact tool to determine automatically the species of bacteria (VITEK-2[®], bioMérieux, Marcy-l'Étoile, France). The VITEK-2 compact is an instrument for bacterial species identification and can also be used for automated bacterial susceptibility testing. This tool utilizes the phenotypic principle by comparing the biochemical reaction results of the sample with the database and has a confidence rate of 99.3% (Monteiro *et al.*, 2021). Isolates identified species will then undergo antibiotic sensitivity testing using the *Kirby-Bauer* method. The antibiotics used are ampicillin, cefoxitin, oxacillin, penicillin G, erythromycin, gentamicin, tetracycline and ciprofloxacin. This study obtained research ethics from the Research Ethics Commission of the Faculty of Medicine, Universitas Padjadjaran, with number 848/UN6.KEP/EC/2024.

RESULTS AND DISCUSSION

The reconfirmation revealed that one of the 15 isolates did not exhibit the characteristics of CoNS bacteria, as it showed negative catalase results. Testing continued using the VITEK-2 Compact system. The results from the VITEK-2 Compact indicated that two of the 15 isolates were not CoNS bacteria (Table 1). A total of 13 isolates identified as CoNS were continued with the antibiogram test using the Kirby-Bauer method. The test results showed that some bacteria were resistant, even isolates that were resistant to multiple drugs (Table 2).

The re-identification results in this reveal show discrepancies compared to the test outcomes conducted approximately two years earlier, in 2022, particularly in isolate four, which shows negative catalase. The difference can be caused by genetic mutations, weakened virulence, or even contamination during storage. The VITEK examination results indicated that isolate four was identified as *Enterococcus faecium*. *Enterococcus faecium* enables survival for extended periods and can potentially infect mammary glands (Kim *et al.*, 2022). Biofilm formation is one of the dominant virulence in this bacterium (Hayati *et al.*, 2022).

Isolate 1 showed non-CoNS results on the VITEK-2 compact test, *Leuconostoc mesenteroides subsp. dextranicum* is a non-motile, Gram-positive coccus with alpha or beta hemolytic on blood agar plate (BAP). *Leuconostoc mesenteroides subsp. Dextranicum* is often found in dairy farms and fermented milk products as an adventitious non-starter lactic acid bacteria (NSLAB) population. These bacteria in milk samples may be due to environmental contamination during sampling (Almeida *et al.*, 2024).

The species identification results of CoNS bacterial isolates showed that 13 out of 15 samples were CoNS bacteria. These species consist of *Staphylococcus sciuri*

Table 1. Coagulase Negative Streptococci (CoNS) species identification results

Sample Code	Hemo-lysis	Gram Strain	MSA	Catalase	Coagulase	Interpretation	Vitek-2 Results
Isolat 1	Beta	Coccus +	-	+	-	CoNS	<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>
Isolat 2	Gamma	Coccus +	+	+	-	CoNS	<i>Staphylococcus sciuri</i>
Isolat 3	Gamma	Coccus +	+	+	-	CoNS	<i>S. sciuri</i>
Isolat 4	Gamma	Coccus +	+	-	-	Non-CoNS	<i>Enterococcus faecium</i>
Isolat 5	Gamma	Coccus +	+	+	-	CoNS	<i>S. sciuri</i>
Isolat 6	Gamma	Coccus +	+	+	-	CoNS	<i>S. vitulinus</i>
Isolat 7	Gamma	Coccus +	-	+	-	CoNS	<i>S. saprophyticus</i>
Isolat 8	Gamma	Coccus +	+	+	-	CoNS	<i>S. sciuri</i>
Isolat 9	Gamma	Coccus +	+	+	-	CoNS	<i>S. saprophyticus</i>
Isolat 10	Gamma	Coccus +	-	+	-	CoNS	<i>S. cohnii</i>
Isolat 11	Gamma	Coccus +	+	+	-	CoNS	<i>S. arlettae</i>
Isolat 12	Gamma	Coccus +	+	+	-	CoNS	<i>S. sciuri</i>
Isolat 13	Gamma	Coccus +	+	+	-	CoNS	<i>S. sciuri</i>
Isolat 14	Gamma	Coccus +	+	+	-	CoNS	<i>S. xylosus</i>
Isolat 15	Gamma	Coccus +	+	+	-	CoNS	<i>S. sciuri</i>

Note: Coccus + = Coccus Gram Positive, Coccus - = Coccus Gram Negative, + = Positive Result, and - = Negative Result

Table 2. Sensitivity test results of coagulase negative streptococci (CoNS) bacteria to antibiotics

No	CoNS bacterial species	Number of sensitive samples							
		Amp	Fox	Ox	P	E	Cn	Te	Cip
1	<i>Staphylococcus sciuri</i> (n= 7)	7	7	7	6	7	7	7	7
2.	<i>S. saprophyticus</i> (n=2)	2	2	2	2	2	2	1	2
3.	<i>S. cohnii</i> (n=1)	1	1	1	0	1	1	1	1
4.	<i>S. vitulinus</i> (n=1)	1	1	1	1	1	1	1	1
5.	<i>S. arlettae</i> (n=1)	0	1	1	0	0	1	1	1
6.	<i>S. xylosus</i> (n=1)	1	1	1	0	1	1	1	1
To	13	12	13	13	9	12	13	12	13
tal									

Note: n represents the total number of samples. Antibiotics: Amp = Ampicillin, Fox = Cefoxitin, Ox = Oxacillin, P = Penicillin G, E = Erythromycin, Cn = Gentamicin, Te = Tetracycline, Cip = ciprofloxacin.

(7 isolates), *S. saprophyticus* (2 isolates), *S. cohnii* (1 isolate), *S. vitulinus* (1 isolate), *S. arlettae* (1 isolate), and *S. xylosus* (1 isolate). Antibigram testing of the isolates showed differences in sensitivity patterns among the species. The results prove that different species with varying levels of virulence can exhibit distinct responses to antibiotics. Penicillin G became the antibiotic with the highest level of resistance in four samples; *S. cohnii* (Isolate 10), *S. arlettae* (Isolate 11), *S.*

sciuri (Isolate 13), and *S. xylosus* (Isolate 14). Ampicillin resistance in one isolate, *S. arlettae* (Isolate 11). These results follow previous studies showing that penicillin G and ampicillin, which belong to the β -Lactam group, showed a high percentage of resistance in CoNS bacteria. Rahmawati *et al.*, (2019). The β -lactam antibiotics are selective inhibitors of bacterial cell wall synthesis (Wanger *et al.*, 2017).

Tetracycline in this study showed that

isolates experienced resistance in isolate 7 (*S. saprophyticus*). This antibiotic inhibits protein synthesis in bacteria by binding the 30S subunit of bacterial ribosomes thereby inhibiting the aminoacyl-tRNA attachment process. Resistance can occur due to the emergence of bacteria with resistant genes. Genes have been found to encode efflux pumps that will remove antibiotics from bacterial cells, preventing antibiotic from actively entering the cell. Besides being resistant, some isolates exhibit intermediate stages of resistance to erythromycin antibiotics in isolate 11 (*S. arlettae*). Erythromycin belongs to the macrolide group. This group also works by inhibiting protein synthesis. Resistance can occur due to changes in the binding site through mutation (Wanger *et al.*, 2017).

The resistant pattern of CoNS bacteria in this study showed multidrug resistance in isolate 11 (*S. arlettae*), which was observed in combination with ampicillin, penicillin G, and erythromycin. Bacteria resistant to antibiotics such as *Staphylococcus* sp., have an increased ability to spread significantly if the strain's virulence genes increase (Akindolire *et al.*, 2015). Several studies have found that *S. arlettae* has a series of antibiotic resistance genes and is even located in high-mobility genomic islands that allow lateral mobilization and gene transfer (Lavecchia *et al.*, 2019). Cefoxitin, oxacillin, gentamicin and ciprofloxacin showed 100% susceptibility in this test. Ciprofloxacin exhibited significant overall efficacy, as evidenced by the size of the antibiotic inhibition zone.

Coagulase-negative *Staphylococci* are an opportunistic bacterium that generally does not cause disease but can lead to health problems when the host's immune system is compromised. This condition is often triggered by infections with other pathogens, causing CoNS to frequently appear as a secondary infection. In several animal cases, CoNS are recognized as major causative agents, particularly in bovine mastitis (Cheung and Otto, 2023). The situation becomes more serious when this bacterium develops

resistance to antibiotics, particularly in multidrug-resistant (MDR) cases, as treatment becomes increasingly difficult (Meza *et al.*, 2024). The difficulty in treating resistant infections can affect food-producing animals, such as cattle, and may ultimately influence the quality of animal products, including milk. In certain situations, these infections may also contribute to the occurrence of foodborne disease, which is transmitted through food and is generally caused by bacteria, although other agents may also be involved. Foodborne diseases associated with multidrug-resistant bacteria are of particular concern because they complicate the selection of effective treatment options in humans (Farrukh *et al.*, 2025).

CONCLUSION

The identification results of CoNS bacteria from 15 preserved isolates of cow milk affected by subclinical mastitis, 13 samples were CoNS. The CoNS bacteria identified were *S. sciuri*, *S. saprophyticus*, *S. cohnii*, *S. vitulinus*, *S. arlettae*, and *S. xylosus*. Each species showed differences in sensitivity to antibiotics, and even the same species showed differences in sensitivity to antibiotics. The test results showed that five out of 13 CoNS species were resistant. Antibiotics that yield 100% susceptible results include cefoxitin, oxacillin, gentamicin and ciprofloxacin, therefore these types of antibiotics can still be recommended in antibiotic treatment for cases of bacterial CoNS, one of which is subclinical mastitis. Penicillin G is not recommended for treating mastitis infections caused by CoNS bacteria. Antibiotic resistance in CoNS requires serious attention because it has the potential to significantly impact animal health and veterinary public health.

SUGGESTION

The following recommendations may

be considered for future research: 1). Perform identification of isolates from sources other than milk, including the barn environment, farm equipment, and farmers' hands; 2). Conduct further studies on virulence factors in each CoNS species that are associated with the occurrence of antibiotic resistance.

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