Bacterial species causing subclinical mastitis in dairy cows: rapid identification and antimicrobial susceptibility testing

Son H. Ly, Tuan T. Pham, Han M. N. On, Bao D. Truong, & Thuong T. Nguyen*

Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Thi Thuong
Email:
thuong.nguyenthi@hcmuaf.edu.vn

ABSTRACT

This study aimed to determine subclinical mastitis (SCM) caused by bacterial species, using chromogenic culture media and to assess the antimicrobial resistance rate in the isolated bacteria. From March to December 2023, 143 milk samples were collected from 71 Holstein Friesian cows with SCM across seven dairy farms in Ho Chi Minh City and Binh Duong province. Milk samples were incubated in triplicate chromogenic culture media to identify SCM caused by microorganisms. Our study revealed that 39.2% (56/143) of the samples had the growth of a single morphology, 26.6% (38/143) exhibited growth of two distinct morphologies, 9.0% (13/143) were found to be contaminated, and 25.2% (36/143) showed no growth. The isolated *Streptococcus* species were *Strep. agalactiae* 34.3% (49/143), *Strep. uberis* 22.4% (32/143), and *Enterococcus* spp. 1.4% (2/143). Besides, *S. epidermidis* 20.3% (29/143), *S. saprophyticus* 14.7% (21/143), and *S. aureus* 4.2% (6/143) were frequently isolated among *Staphylococcus species*. For gram-negative bacteria causing SCM, *E. coli* 2.8% (4/143), *Klebsiell*a spp. 1.4% (2/143), and *Pseudomonas* spp. 4.2% (6/143) were the most isolated. Regarding antimicrobial susceptibility testing, the resistance rate of each bacterial species to each antibiotic tested differed for *Staphylococcus*, *Streptococcus*, and gram-negative bacteria. *Staphylococcus aureus* was not resistant to gentamycin, florfenicol, and marbofloxacin. The resistance rate of *S. epidermidis* to gentamycin, florfenicol, trimethoprim-sulfadiazine, and amoxicillin-clavulanic acid varied from 10.3% to 17.2%. Marbofloxacin and trimethoprim-sulfadiazine were excellent choices in treating SCM caused by *S. saprophyticus* because of their low resistance rate (10.3 - 13.3%). *Streptococcus uberis* was sensitive to the combined antibiotic amoxicillin-clavulanic acid. The resistance rate of *Strep. agalactiae* to this combined antibiotic (amoxicillin-clavuclanic acid) was the lowest (10%). *Pseudomonas* spp. was resistant to the tested antibiotics. Our study suggests that identifying bacterial species and conducting antimicrobial susceptibility tests play a crucial role in improving the treatment effectiveness for bovine SCM.

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1. Introduction

Bovine mastitis is the inflammation of one or more of the four udders, primarily caused by a bacterial infection (Ruegg, 2011). Subclinical mastitis (SCM), one of the types of mastitis, results in decreased milk quality and quantity and increased veterinary costs (Bar et al., 2008; Hagnestam-Nielsen & Ostergaard, 2009). If left uncontrolled or untreated, it can progress to clinical mastitis, leading to more serious economic losses and compromising animal welfare. Previous studies have indicated that approximately 90% of microorganisms isolated from infected milk were environmental or infectious bacteria, such as *Staphylococcus aureus*, Negative Coagulase *Staphylococcus* (NCS), *Streptococcus agalactiae*, *Streptococcus uberis*, *Escherichia coli*, *Klebsiella* spp. (Bar et al., 2008; Hagnestam-Nielsen & Ostergaard, 2009). Each bacterial species has a different pathogenic mechanism (Côté-Gravel & Malouin, 2019). Therefore, identifying the causative agents of SCM plays an essential role in treatment efficacy. Previous studies showed that mastitis caused by *E. coli* and *Staphylococcus aureus* was not recommended of local intramammary antibiotic therapy except in severe cases (Pinzón-Sánchez et al., 2011; Suojala et al., 2013). Currently, the traditional methods for diagnosing SCM-causing bacteria rely on bacterial culture and biochemical tests. However, these methods have limitations, such as the requirement for aseptic sample collection and the time delay between sample sending and receiving results (Adkins & Middleton, 2018). Consequently, routine diagnosis of the microorganisms causing SCM is not performed on most dairy farms, leading to antimicrobial treatment without prior knowledge (Ly et al., 2022). This approach can contribute to the development of antibiotic resistance. CHROMagar is a type of chromogenic culture media developed to identify microbial pathogens based on the colors exhibited by microbial colonies. Compared to traditional methods, this chromogenic media helps rapid identification, reducing the biochemical tests to determine bacterial species. Indeed, the efficacy of CHROMagar for the rapidly identifying bacteria isolated from mastitis cows in Brazil has been published (Granja et al., 2021). However, studies on the use of CHROMagar to determine the SCM-causing agents in Vietnamese dairy farms are still limited. Thus, our study aimed to identify SCM caused by bacterial species using the chromogenic media, CHROMagar, and to assess the antimicrobial resistance rate of these isolated agents.

2. Materials and Methods

2.1. Time and locations

Our study was conducted from March 2023 to December 2023. Milk samples were collected from seven farms in Binh Chanh district and Cu Chi district of Ho Chi Minh city, and Tan Uyen city in Binh Duong province. The selected farms operated on an industrial scale, with the number of cows ranging from 200 to 2,000. Cows were fed using a total mixed ration and milked with a milking system. Bacterial identification and antimicrobial susceptibility testing were conducted at the Veterinary Hospital of Nong Lam University following the guidelines provided by NMC (2017).

2.2. Milk sample collection

In the present study, 143 milk samples were collected from 71 Holstein Friesian cows with subclinical mastitis. The California Mastitis Test (CMT) was used to detect subclinical mastitis udder. Initially, the teats were immersed in a teat disinfectant solution and then dried with paper towels. After discarding the initial three milk

streams, 2 mL from each udder was collected into the shallow cups on the paddle and mixed with 2 mL of CMT solution (DeLaval CMT®, DeLaval company, France). Milk samples were considered subclinical mastitis when the CMT results were more significant than 1+ (Kandeel et al., 2018). The subclinical mastitis milk samples were collected aseptically, with the teat ends disinfected using 70% iodized alcohol, and the milk was then collected in a sterile tube 15 mL (Aptaca®, Aptaca company, Italy) following the guidelines provided by NMC (2017). The samples were stored at 4°C and sent to the Veterinary Hospital of Nong Lam University for diagnosis.

2.3. Microbial identification by chromogenic culture media

Three chromogenic culture media (CHROMagar company, France) were utilized to identify subclinical mastitis-causing pathogens. The mechanism involves the chromogenic substrate being hydrolysed to release a coloured product that remains highly localized on microbial colonies. This allows clear differentiation of microbes producing the target enzyme from those that do not (Perry, 2017). The diagnostic performance (accuracy, sensitivity, and specificity) of chromogenic culture media in identifying microorganisms isolated from cows with clinical and subclinical mastitis has been demonstrated (Granja et al., 2021). The first medium specifically targeted *Staphylococcus* (CHROMagarTM *Staphylococcus*), the second medium selectively identified *Streptococcus* (CHROagarTM *Streptococcus*), and the third medium was designed to isolate gram-negative bacteria and yeasts (CHROMagarTM GramNeg). These culture media were plated in 90 x 15 mm tri-plate petri dishes. The milk samples were inoculated into the culture media using sterile swabs and then placed in an incubator at 37°C for 24 h under aerobic conditions. After the incubation period, the growth of colonies and microbiological identification were visually evaluated based on the colony-staining characteristics, according to the manufacturer's instructions: (1) pink = *Staphylococcus aureus*, (2) colorless to pinkish = *S. epidermidis*, (3) turquoise blue = *S. sarprophyticus* for *Staphylococcus plates*; (4) blue = *Streptococcus agalactiae*, (5) metallic blue = *Streptococcus uberis*, (6) mauve = *Enterococcus* spp. for *Streptococcus plates*; (7) mauve = *E. coli*, (8) colorless = *Pseudomonas* spp. for gram-negative plates (Figure 1).

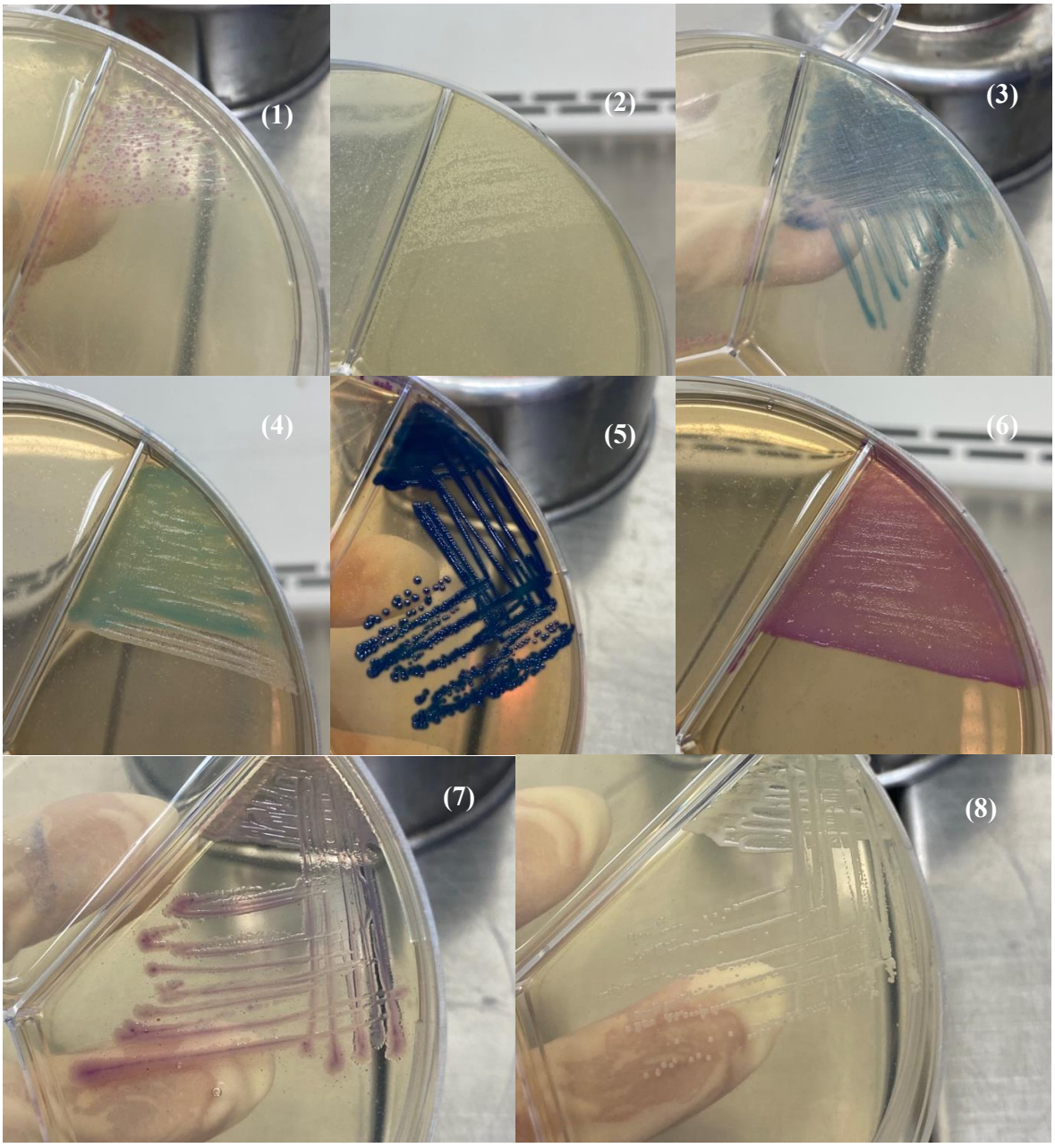

Figure 1. Colonies of subclinical mastitis-causing microorganisms inoculated in triplicate of chromogenic culture media: (1) *Staphylococcus aureus*, (2) *Staphylococcus epidermidis*, (3) *Staphylococcus sarprophyticus*, (4) *Streptococcus agalactiae*, (5) *Streptococcus uberis*, (6) *Enterococcus* spp.; (7) *Escherichia coli*, (8) *Pseudomonas* spp.

2.4. Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was applied to evaluate the antimicrobial susceptibility testing. In short, from 5 to 7 separate colonies of one species were picked and suspended in a 5 mL saline solution, resulting in 0.5 McFarland. The suspension was used for flooding the Mueller Hinton agar plates (MHA, Merck company, Germany), and the redundant solution was discarded. Streptococcus was examined on MHA plates supplemented with 5% sheep blood. The antimicrobial agents were selected according to their occurrence in commercially available products for mastitis treatment. Antibiotic discs (Oxoid company, United Kingdom) used including cetiofur (30 µg), amoxicillin (20 µg) + clavulanic acid (10 µg), ampicillin (10 µg), oxacillin (1 µg), cephalexin (30 µg), cefotaxim (30 µg), penicillin (10 UI), erythromycin (15 µg), trimethroprim (1.25 µg) + sulfadiazine (23.75 µg), tetracylin (30 µg), kanamycin (30 µg), gentamycin (10 µg), florfenicol (30 µg), marbofloxacin (5 µg), enrofloxacin (5 µg), clindamycin (2 µg), cefoperazone (30 µg). After 24 h incubation at 37oC, plates were read by measuring the inhibition zone diameters. Inhibition zone diameters were first evaluated by clinical breakpoints and provided by CLSI (2018) to determine resistant strains (Table 1).

2.5. Statistical analysis

Data were expressed as the parameters estimates and 95% confidence intervals using R version 4.2.3 (https://cran.r-project.org/). Confidence intervals (CI) were calculated based on the standard error obtained from a bnomial distibution following the formulas:

Standard Error (SE) $= \sqrt{\frac{p(1-p)}{n}}$ and CI 95% = estimate ± 1.96 x SE.

3. Results

3.1. Microbial identification by chromogenic culture media

A total of 143 subclinical mastitis samples were assessed. Among these, 39.2% (56/143) had growth of a single morphology, 26.6% (38/143) exhibited growth of two distinct morphologies, 9.0% (13/143) were found to be contaminated, and 25.2% (36/143) showed no microbial growth. Regarding the subclinical mastitis-causing agents, *Streptococcus* accounted for the majority at 62.9% (90/143). Within this group, *Strep. agalactiae* 34.3% (49/143) and *Strep. uberis* 22.4% (32/143) were the most frequently isolated strains. *Staphylococcus* was also isolated in 44.7% (64/143) of the samples. Specifically, negative coagulase *Staphylococcus*, such as *S. epidermidis* and *S. saprophyticus*, represented 35% of this group. Among the gram-negative bacteria, *Pseudomonas* spp., *E. coli*, and *Klebsiella* spp. were isolated in proportions of 4.2%, 2.8%, and 1.4%, respectively (Table 2).

3.2. Antimicrobial susceptibility testing

The percentage of resistant strains per species is provided in Table 3. The antibiotic resistance rate differed for each bacterial species within the *Staphylococcus* group on the tested antibiotics. *S. aureus* was utterly resistant to ampicillin, oxacillin, cephalexin, and cefotaxim. Conversely, gentamycin, florfenicol, and marbofloxacin were effective against *S. aureus in vivo*. The resistance rate of *S. epidermidis* varied widely from 10.3% to 93.1%. *S. epidermidis* resisted ampicillin, cephalexin, cefotaxim, and oxacillin, while this bacterial species was sensitive to gentamycin, florfenicol, and trimethoprim sulfadiazine. Similarly, *S. saprophyticus* was resistant to cefotaxime, cephalexin, ampicillin, and penicillin, with only marbofloxacin and

Table 1. Available clinical breakpoints for the pathogen in combination with the tested antimicrobials for a specific disk content (in µg)

Antibiotic	Disk content (µg)	*S. aureus*	*S. epidermidis*	*S. saprophyticus*	*Strep. uberis*	*Strep. agalactiae*	*Enterococcus* spp.	*E. coli*	*Pseudonomas spp.*
	RIS (mm)	RIS (mm)	RIS (mm)	RIS (mm)	RIS (mm)	RIS (mm)	RIS (mm)	RIS (mm)	RIS (mm)
Cetiofur	30	17 - 21	17 - 21	17 - 21	17 - 21	17 - 21	13 - 17	17 - 21	14 - 18
Amox/clav*	20/10	19 - 20	19 - 20	19 - 20	13 - 18	13 - 18	13 - 18	13 - 18	NA
Amoxicillin	10	28 - 29	28 - 29	28 - 29	18 - 26	18 - 26	16 - 17	13 - 17	NA
Oxacillin	1	17 - 18	17 - 18	17 - 18	≥ 20	≥ 20	NA	NA	NA
Cephalexin	30	22	22	22	≥ 24	≥ 24	NA	NA	NA
Cefotaxim	30	22	22	22	NA	NA	NA	22 - 26	NA
Penicillin	10 UI	28 - 29	28 - 29	28 - 29	≥ 24	≥ 24	14 - 15	NA	NA
Erythromycin	15	13 - 23	13 - 23	13 - 23	15 - 21	15 - 21	13 - 23	NA	NA
Tri-sufa**	1.25/23.75	10 - 16	10 - 16	10 - 16	10 - 16	10 - 16	NA	10 - 16	NA
Tetracylin	30	14 - 19	14 - 19	14 - 19	14 - 19	14 - 19	14-19	14 - 19	NA
Kanamycin	30	13 - 18	13 - 18	13 - 18	NA	NA	NA	13 - 18	NA
Gentamycin	10	12 - 15	12 - 15	12 - 15	NA	NA	NA	12 - 16	12 - 16
Florfenicol	30	12 - 18	12 - 18	12 - 18	18 - 22	18 - 22	NA	12 - 18	NA
Marbofloxacin	5	14 - 20	14 - 20	14 - 20	14 - 20	14 - 20	NA	14 - 20	20 - 25
Enrofloxacin	5	16 - 23	16 - 23	16 - 23	16 - 23	16 - 23	NA	16 - 23	16 - 23
Clindamycin	2	14 - 21	14 - 21	14 - 21	14 - 21	14 - 21	NA	NA	NA
Cefoperazone	30	17 - 23	17-23	17 - 23	≥ 18	≥ 18	NA	17 - 23	NA

R: resistant, I: intermediate, S: sensitive, NA: not available.

**Amoxicillin + clavulanic acid.*

***Trimethroprim sulfadiazine.*

Table 2. Distribution of subclinical mastitis-causing agents identified by CHROMagar

Variable	Number of samples	%	CI 95%
Total samples	143	100	
No growth	36	25.2	18.1 - 32.3
Colonies with one morphology	56	39.2	31.1 - 47.2
Colonies with two morphology	38	26.6	19.3 - 33.8
Contamination	13	9.0	4.3 - 13.7
Staphylococcus	64	44.7	36.5 - 52.8
Staphylococcus aureus	6	4.2	0.9 - 7.5
Staphylococcus epidermidis	29	20.3	13.6 - 26.7
Staphylococcus saprophyticus	21	14.7	9.0 - 20.5
Other *Staphylococcus*	8	5.6	1.8 - 9.3
Streptococcus	90	62.9	54.9 - 70.8
Streptococcus uberis	32	22.4	15.6 - 29.2
Streptococcus agalactiae	49	34.3	26.5 - 42.1
Enterococcus spp.	2	1.4	0.5 - 3.0
Other *Streptococcus*	7	4.9	1.3 - 8.4
Gram-negative bacteria	12	8.4	3.8 - 12.9
Escherichia coli	4	2.8	0.9 - 5.5
Klebsiella spp.	2	1.4	0.5 - 3.0
Pseudomonas spp.	6	4.2	0.9 - 7.5

CI 95%: confidence interval 95%.

Table 3. Antimicrobial resistance of isolated pathogens from bovine subclinical mastitis

Antibiotic tested	Disk content (µg)	*S. aureus* (n = 5)	*S. epidermidis* (n = 29)	*S. saprophyticus* (n = 15)	*Strep. uberis* (n = 22)	*Strep. agalactiae* (n = 30)	*Enterococcus* spp. (n = 2)	*E. coli* (n = 1)	*Pseudonomas* spp. (n = 5)
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Cetiofur	30	3 (60)	12 (41.4)	4 (26.7)	8 (36.3)	10 (33.3)	0	0	5 (100)
Amox/clav*	20/10	3 (60)	5 (17.2)	6 (40.0)	0	3 (10.0)	0	0	NA
Ampicillin	10	5 (100)	25 (86.2)	12 (80.0)	9 (40.9)	10 (43.3)	0	1 (100)	NA
Oxacillin	1	5 (100)	18 (62.0)	10 (66.7)	21 (95.4)	28 (93.3)	NA	NA	NA
Cephalexin	30	5 (100)	25 (82.7)	14 (93.3)	18 (81.8)	26 (86.7)	NA	NA	NA
Cefotaxim	30	5 (100)	27 (93.1)	15 (100)	NA	NA	NA	1 (100)	NA
Penicillin	10 UI	4 (80)	23 (79.3)	12 (80.0)	15 (68.1)	23 (76.6)	0	NA	NA
Erythromycin	15	3 (60)	13 (44.8)	9 (60.0)	19 (86.3)	24 (80.0)	2 (100)	NA	NA
Tri-sufa**	1.25/23.75	2 (40	5 (17.2)	3 (20.0)	20 (90.9)	23 (76.6)	2 (100)	0	NA
Tetracylin	30	1 (20)	16 (55.2)	8 (53.3)	15 (68.1)	24 (80.0)	2 (100)	0	NA
Kanamycin	30	1 (20)	11 (37.9)	10 (66.7)	NA	NA	2 (100)	0	NA
Gentamycin	10	0	3 (10.3)	3 (20.0)	10 (45.4)	19 (63.3)	NA	0	0
Florfenicol	30	0	3 (10.3)	5 (33.3)	6 (27.2)	12 (40.0)	NA	0	NA
Marbofloxacin	5	0	8 (27.6)	2 (13.3)	NA	NA	NA	NA	5 (100)
Enrofloxacin	5	2 (40)	10 (34.4)	5 (33.3)	8 (36.3)	14 (46.6)	NA	NA	2 (40)
Clindamycin	2	3 (60)	9 (31.0)	5 (33.3)	10 (45.4)	17 (56.6)	NA	NA	NA
Cefoperazone	30	3 (60)	6 (20.7)	5 (33.3)	8 (36.3)	9 (30.0)	NA	NA	NA

**Amoxicillin + clavulanic acid, ** Trimethroprim sulfadiazine, NA: not available.*

ceftiofur remaining effective against this species. For *Streptococcus*, the antibiotic resistance rate also varied among bacterial species. *S. uberis* resisted oxacillin, trimethoprim-sulfadiazine, cephalexin, erythromycin, penicillin, and tetracycline. Conversely, this strain showed sensitivity to amoxicillin/clavulanic acid. *S. agalactiae* was resistant to oxacillin, cephalexin, erythromycin, tetracycline, and penicillin, while the most effective antibiotic was ampicillin. *Enterococcus* spp. exhibited complete resistance to erythromycin, trimethoprim. In contrast, amoxicillin/clavulanic acidsulfadiazine, tetracycline, kanamycin, and ampicillin remained sensitive to this bacterial species. The number of isolated gram-negative bacteria was not substantial. Initially, *Pseudomonas* spp. showed intensely extreme resistance to ceftiofur and marbofloxacin, while gentamicin was effective against this bacterium.

4. Discussion

The present study used a triplate containing chromogenic culture media to rapidly identify microorganisms in subclinical mastitic milk samples. Of 143 samples, it observed a prevalence of positive samples was 65.8%. This result aligns with the findings of Granja et al. (2021), who also used chromogenic culture media to isolate agents-causing subclinical mastitis in Brazil. Among these positive samples, it found that the microorganisms with the highest prevalence were *Strep. agalactiae*, *Strep. uberis*, and negative coagulase *Staphylococcus*. The results of this study were like those described for the distribution of microorganisms causing subclinical mastitis in dairy cows in Southern Vietnam (Östensson et al., 2013). The results of contamination (9.0%) were higher than those described by previous studies from 0.6 - 2.9% (Cameron et al., 2013; Ganda et al., 2016; Granja et al., 2021). This differentiation could be associated with practical skill, although the milk sample protocol was followed by the NMC (2017). In this study, 25.2% of the samples had no microbial growth. Previous studies showed that the percentage of subclinical mastitis milk samples isolated without colonies varied between 28.6% and 31.3% (McCarron et al., 2009; Ganda et al., 2016; Granja et al., 2021). It could be due to a low bacterial concentration to be detected by the culture method, or belong to other bacteria species, such as *Mycoplasma* (Fox, 2012). Regarding the *Staphylococcus* group isolated from SCM samples, the chromogenic culture media differentiated contagious pathogens (*S. aureus*, 4.2%) and environmental pathogens (NCS). Similarly, the differentiation capacity among *Streptococcus* group isolated in the chromogenic media. In this case, rapid identification results could be used to separate cows with contagious transmission (*Strep. agalactiae*) and environmental transmission (*Strep. uberis*). One of the limitations of using the CHROMagar *Streptococcus* is the lack of differentiation of *Strep. agalactiae* and *Strep. dysgalactiae* because they have similar colony color. In our study, gram-negative bacteria constituted an insignificant proportion of subclinical mastitis cases. This finding was identical to those reported by Ashraf and Imran (2018). Indeed, gram-negative bacteria, such as *E. coli*, are often the cause of severe clinical mastitis. Thus, the assessment of the effectiveness of chromogenic media in SCM-caused by gram-negative bacteria was limited. It is necessary to conduct another research on milk samples from cows with clinical mastitis to further evaluate the efficacy of CHROMagar in identifying gram-negative bacterial species.

For the *Staphylococcus* group, the results showed that the resistance rate of each bacterial species to each antibiotic tested was different.

This finding suggested that it was essential to identify bacterial species causing SCM could help to select more sensitive antibiotics, thereby increasing treatment effectiveness. For example, ceftiofur, which has been widely used on dairy farms, had resistance rates to *S. saprophyticus* (26.7%), *S. epidermidis* (41.8%), and *S. aureus* (60%). In our study, *S. aureus* was highly resistant to the β-lactam antibiotic group, while this bacterial species was sensitive to gentamycin, florfenicol, and marbofloxacin. Our findings were like those of previous studies (Erskine et al., 2002; Roesch et al., 2006; Botrel et al., 2010). The antimicrobial resistance rate of *S. aureus* from bovine mastitis for gentamycin in the United States, Chile, Europe, and Iran varied between 0 and 6.8% (Oliver & Murinda, 2012). For *S. epidermidis*, we found that antibiotics with low resistance rates for this bacterium, including gentamycin, florfenicol, and trimethoprim-sulfadiazine, varied from 10.3 % to 17.2%. The previous studies from Botrel et al. (2010), Kalmus et al. (2011), and Persson et al. (2011) were also similar. In the current study, marbofloxacin was the best choice in the treatment of subclinical mastitis caused by *S. saprophyticus* because of the lowest resistance rate (13.3%), followed by trimethoprim sulfadiazine (20%) and ceftiofur (26.7%). Cetiofur and trimethoprim sulfadiazine were found also sensitive to this species in the research of Suriyasathaporn (2010), with a resistance rateo of 16.7%. In a systemic review, Peter et al. (2012) indicated that *S. saprophyticus* was sensitive to cetiofur and trimethoprim sulfadiazine, with a resistance rate of 0% and 17%, respectively.

Like the *Staphylococcus* group, the results of the present study indicated that different bacterial species within the *Streptococcus* group had different rates of resistance to various antibiotics. Florfenicol, enrofloxacin, gentamycin, clindamycin, and tetracycline were effective against *Strep. uberis*, while *Strep. agalactiae* was sensitive to others such as trimethoprim sulfadiazine, erythromycin, cefoperazone. Those findings confirmed the importance of identifying bacterial species in selecting antibiotic treatment. In this study, the resistance rate of the combined antibiotic amoxicillin-clavulanic acid to *Strep. uberis* and *Strep. agalactiae* was the lowest, 0% and 10% respectively. Similar results were described by Petrovski et al. (2015) that all isolated *Strep. uberis* strains were susceptible to the amoxicillin-clavulanic acid in the United States and New Zealand. Bacteria *Enterococcus* spp. recorded to be sensitive to some beta-lactam antibiotics commonly used to treat mastitis, including ceftiofur, amoxicillin-clavulanic, amoxicillin-clavulanic acid, and ampicillin. Ampicillin resistance rates were also reported by Ebrahimi et al. (2007) and Nam et al. (2009). However, a limitation encountered in our study was the small number of antibiotic samples for *Enterococcus* spp. Similarly, although the number of isolated gram-negative bacteria was insignificant, our results showed that *Pseudomonas* spp. resisted tested antibiotics. of the report ed by Rajala-Schultz et al. (2004), which mentioned that resistant ability. Another limitation of our study was that *Klebsiella* spp. did not grow on Mueller Hinton agar plates for the antimicrobial susceptibility testing.

5. Conclusions

The chromogenic media, CHROMagar, are an option for rapid species-level identification of bacteria causing subclinical mastitis without biochemical tests in Vietnam livestock conditions. Isolated colonies from this chromogenic media can be utilized in antimicrobial susceptibility testing. Most isolated bacterial pathogens were resistant to β-lactam antibiotics. Additionally,

varying levels of antibiotic resistance were observed across different bacterial species. Thus, identifying the bacterial species and conducting antimicrobial susceptibility tests play a crucial role in improving the effectiveness of treatment for bovine subclinical mastitis.

Conflict of interest

The authors have no conflicts of interest to declare.

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