ORIGINAL ARTICLE

COMPARISON OF PHENOTYPIC AND GENOTYPIC METHODS OF DETECTION OF TWO BETA-LACTAM RESISTANCE GENES IN *Staphylococcus aureus* ISOLATES OF MASTITIC MILK FROM BATANGAS, PHILIPPINES

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ABSTRACT

The study was conducted to compare phenotypic and genotypic methods of detection of two beta-lactam resistant genes in *Staphylococcus aureus* isolates from mastitic milk. Nine (9/92) *S. aureus* isolates were revived and used. The isolates were subjected to phenotypic prediction of *blaZ* gene using the penicillin disk diffusion method and nitrocefin test while prediction of *mecA* gene was observed using the cefoxitin disk diffusion method. Both the penicillin disk diffusion method and nitrocefin test detected three (33.33%) isolates to be producing beta-lactamase while PCR detected two (22.22%) isolates positive for *blaZ* gene. Kappa coefficient revealed perfect agreement (1.00) between phenotypic methods and substantial agreement (0.7270) between the phenotypic methods and PCR. In the detection of *mecA*-mediated methicillin resistance, none of the isolates resulted positive for *mecA* gene for both tests hence, no methicillin-resistant *S. aureus* (MRSA) was detected. The results of the study show that there are *S. aureus* isolates from mastitic milk that expresses beta-lactam resistance through beta-lactamase production however, while there was no MRSA detected, the study highly suggests the monitoring of the antimicrobial usage in the farms and their management practices to avoid further development of resistance and possible transfer of resistance from animals to humans and/or humans to animals.

Keywords: *blaZ* gene*, dairy cattle, mecA* gene*, methicillin resistance, mastitis, Staphylococcus aureus*

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INTRODUCTION

Mastitis is an economically important disease responsible for several problems in dairy production worldwide (Asfour and Darwish, 2011). Staphylococci are identified to be the most prevalent mastitis-causing pathogens in dairy cows (Pitkala *et al.*, 2004; Wald *et al.,* 2019) and they are classified accordingly as coagulase-positive and coagulase-negative staphylococci (CoNS) (Taponen and Pyorala, 2009). Although CoNS are the predominantly isolated group (Pitkala *et al.,* 2004; Haguingan *et al.,* 2010), coagulase-positive *Staphylococcus aureus* is said to be the most frequently isolated microorganism from mastitis in dairy cattle (Cantekin *et al.,* 2014).

On dairy farms, beta-lactam antibiotics such as penicillin are used for the treatment and prevention of the disease (Soares *et al.,* 2012; Robles *et al.,* 2014). Antibiotic treatment of *S. aureus* infections is considered poor primarily due to the bacteria's resistance against a wide range of antimicrobial classes (Cantekin *et al.,* 2014). Resistance to beta-lactams is mediated by either beta-lactamases expressed by *blaZ* and/or *mecA*-encoded penicillin-binding protein (PBP2a) which shows reduced binding to beta-lactam antibiotics (Soares *et al.*, 2012). Moreover, *S. aureus* can acquire methicillin resistance by insertion of staphylococcal cassette chromosome

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(SCC*mec*), carrying the *mecA* gene, into the chromosome (Lakhundi and Zhang, 2018; Schnitt and Tenhangen, 2020). A drastic rise in the number of methicillin-resistant *Staphylococcus aureus* (MRSA) has been reported in domestic dairy cattle (Mohana *et al.,* 2016). Cross infection between humans and animals being present makes it a public health concern as this can cause a broad spectrum of diseases (Cantekin *et al.,* 2014; Abdel-Tawab *et al.,* 2018).

Detection of methicillin resistance in *S. aureus* can be done through phenotypic and genotypic methods of which the phenotypic methods include the disk diffusion method and minimum inhibitory concentration (MIC), while the genotypic method includes the polymerase chain reaction (PCR). PCR is considered to be the gold standard in the detection of the *blaZ* and *mecA* genes coding for resistance in the pathogen (Pitkala *et al.,* 2007; Kaase *et al.,* 2008; Pereira *et al.,* 2014). In the Philippines, published literature about comparison of tests for detection of resistant genes in staphylococci is limited, same with studies about the occurrence of MRSA isolated from mastitic milk in dairy cattle being scarce.

The study aims to compare phenotypic and genotypic methods of detection of *blaZ* and *mecA* genes mediated beta-lactam resistance in *S. aureus* isolates of mastitic milk from Batangas, Philippines. The result of the study will be of assistance in determining the occurrence of MRSA in mastitis cases and will help the local industry and farmers create an effective milking management and mastitis prevention and control program for their farms which can facilitate the reduction of the occurrence of antimicrobial resistance. Additionally, the efficiency of the phenotypic detection methods used in the study can be assessed which can help in the reliable detection of these microorganisms aside from PCR.

MATERIALS AND METHODS

Staphylococcus aureus isolates

Bacterial isolates from another study (unpublished reference), which were detected, isolated, and identified using conventional microbiological culture method, were used in the present study. The bacterial isolates were detected coming from mastitic milk in selected dairy cattle farms in Batangas, Philippines.

From the mastitic milk collected from four dairy cattle farms, 92 bacterial isolates were identified using the conventional microbiological culture method. The 92 bacterial isolates were from subclinical mastitic milk coming from 61 quarters from 33 animals. Out of the 92 bacterial isolates in mastitic milk, nine (9.78%) were phenotypically identified as *Staphylococcus aureus*.

Revival and re-confirmation of identification of *S. aureus* isolates

Nine (9) *S. aureus* isolates were revived and re-confirmed of their identification. The isolates were stock culture samples preserved in a -80°C freezer.

The isolates were subcultured in blood agar plates (BAP) supplemented with defibrinated sheep blood and aerobically incubated at 37°C for 24-48 hours to determine purity. After incubation, well-isolated colonies showing morphologic characteristics of *S. aureus* (Markey *et al.*, 2013) were selected and were subcultured into new nutrient agar (NA) slants and nutrient broths (NB). Subcultures were aerobically incubated at 37°C for 18-24 hours.

After incubation, a loopful of each sample isolates from NBs were streaked in Baird-Parker agar plates supplemented with potassium egg yolk tellurite emulsion using quadrant streak method. Plates were then aerobically incubated at 37°C for 18-24 hours. Plates containing colonies that were black, convex, shiny, 1.0-1.5 mm in diameter, and surrounded by a clear (sometimes opaque) zone/ halo of lipolysis, were considered positive for *S. aureus* (Corry *et al.*, 2003; Markey *et al.*, 2013).

Likewise, a commercial identification system for the identification and differentiation of *Staphylococcus* species (RapID™ STAPH PLUS System, Remel, Thermo Fisher Scientific, MA, USA) was used in the study following the manufacturer's instruction for further confirmation of the identity of the presumably *S. aureus* revived isolates. Quality control for both conventional and commercial identification of the revived isolates was done through the use *S. aureus* reference strain from American Type Culture Collection (ATCC 25923).

All sample isolates that were confirmed to be *S. aureus* using both methods (conventional and commercial) were subcultured into new BAPs and aerobically incubated at 37°C for 18-24 hours. Colony growth of the revived isolates was then subjected phenotypic and genotypic methods for the detection of beta-lactamase production and methicillin resistance in *S. aureus*.

In vitro disk diffusion method

Antimicrobial susceptibility and resistance of *S. aureus* isolates to beta-lactams, specifically penicillin and cefoxitin, were determined using the Kirby-Bauer disk diffusion method as described by Markey *et al.* (2013) and the Clinical and

Laboratory Standards Institute (CLSI, 2017). A 24-hour pure culture of the revived *S. aureus* isolates was inoculated into trypticase soy broth (TSB) tubes and incubated aerobically at 37°C for four hours to achieve approximately 5 x 10^5 CFU/ml or a turbidity equivalent to a 0.5 McFarland standard. The enriched *S. aureus* isolates in TSB were inoculated in Mueller-Hinton agar (MHA) and antibiotic discs were placed accordingly and incubated aerobically at 35°C for 16-18 hours (CLSI, 2017).

For the detection of beta-lactamase production and methicillin resistance among *S. aureus* isolates, Penicillin G (10 IU) and Cefoxitin (30 µg) discs (Mast Diagnostics, Mast Group Ltd., Merseyside, UK) were used. The zone of inhibition measurements for both discs, as well as the penicillin zone edge evaluation, were recorded and interpreted according to the standards proposed by CLSI (2017).

Quality control of the *in vitro* disk diffusion method was done through the use of reference strains from ATCC including: *S. aureus* ATCC 25923 (beta-lactamase negative) and *S. aureus* ATCC 29213 (beta-lactamase positive).

Nitrocefin test

Phenotypic detection of beta-lactamase production among *S. aureus* isolates was also done through the use of a commercial kit utilizing a chromogenic cephalosporin (nitrocefin)-based detection method of beta-lactamase production (BD BBL™ DrySlide™ Nitrocefin, Becton, Dickinson and Company, Sparks, MD, USA) following the manufacturer's instruction. Quality control was performed using *S. aureus* ATCC 29213 (beta-lactamase positive) reference strain.

Genotypic detection method of beta-lactamase production and methicillin resistance

Genotypic detection of beta-lactamase production and methicillin resistance among *S. aureus* isolates was done through the use of duplex polymerase chain reaction (PCR) for the detection of *blaZ* and *mecA* gene, respectively.

DNA extraction

Well-isolated colonies from an 18 to 24-hour culture of *S. aureus* isolates were cultured and enriched into TSB tubes and aerobically incubated at 37°C for 18-24 hours before DNA extraction. The genomic DNA of *S. aureus* isolates was isolated using a commercial DNA extraction kit (PureLink® Genomic DNA Mini Kit, Invitrogen™, Thermo Fisher Scientific, MA, USA) following the manufacturer's instruction. The extracted DNA samples were stored at -80°C until use.

Duplex polymerase chain reaction

Genomic DNA of *S. aureus* isolates was subjected to duplex PCR. PCR amplification of the *blaZ* and *mecA* gene was carried out using the primers patterned after the study of Sampimon *et al.* (2011). The primers that were used in the study include: a) for *blaZ* gene: *blaZ*1 (5'-AAG AGA TTT GCC TAT GCT TC-3') and *blaZ*2 (5'-GCT TGA CCA CTT TTA TCA GC-3') which have a final product of 518 bp (Haveri *et al*., 2005), and b) for *mecA* gene: *mecA*174A (5'-AAC AGG TGA ATT ATT AGC ACT TGT AAG-3') and *mecA174B* (5'-ATT GCT GTT AAT ATT TTT TGA GTT GAA-3') which have a final product of 174 bp (Martineau *et al.*, 2000). The reaction of the primers was carried out using a final reaction volume of 10 µl containing: 2 µl of DNA template, 0.5 µl of 20 pmol of each primer, 1 µl of nuclease-free water, and 5 µl of 1X PCR master mix (GoTaq® G2 Green Master Mix, Promega Corporation, Madison, USA).

Amplification of DNA samples was carried out in the PCR thermal cycler machine (ProFlex PCR system, Thermo Fisher Scientific, MA, USA). The duplex PCR amplification conditions for *blaZ* and *mecA* gene were patterned after Arafa *et al.* (2016) which consist of four-minute initial denaturation step at 94°C followed by 35 cycles of 94°C for one minute, 55°C for one minute, and 72°C for one minute, and lastly, final elongation at 72°C for 10 minutes. The amplified PCR products were stored at -80°C until use.

Gel electrophoresis and visualization

The PCR products (518 bp and 174 bp) for *blaZ* and *mecA* gene were separated using agarose gel electrophoresis using 1.5% agarose gel and were stained using 0.5 µg/ml ethidium bromide for 30-60 minutes. PCR amplificons were visualized using a UV transilluminator (Quantum, Vilber Lourmat, France) and images were taken using a gel documentation software (Bio-Vision, Vilber Lourmat, France).

Data analysis

All data obtained in the study were recorded in a spreadsheet and statistically analyzed using a statistical software program (Epi Info™ ver. 7.2.2.16, CDC, Atlanta, GA, USA). All statistical analyses were evaluated at $\alpha = 0.05$ or 95% level of significance.

Positivity rate of *S. aureus* isolates

The positivity rate of revived *S. aureus*

isolates in the farms, in respect to a separate study (unpublished reference), was calculated as the total number of isolated *S. aureus* over the total bacterial isolates in the four samples farms. Positivity rate in animals was calculated as the proportion of animals with isolated *S. aureus* (with at least one quarter positive for *S. aureus*) over the total number of animals subjected for microbial culture detection and isolation, while the positivity rate in quarters was determined through the proportion of quarters positive for *S. aureus* over the total number of quarters utilized in the separate study (total mastitic milk samples used for microbial culture detection and isolation).

Validity and agreement of the phenotypic methods and genotypic method

Validity and agreement of the phenotypic methods and genotypic method in the detection of beta-lactamase production and methicillin resistance in *S. aureus* isolates in mastitic milk was evaluated through the determination of sensitivity (se), specificity (sp), positive predictive value (PPV) and negative predictive value (NPV), and through the determination of Kappa coefficient (Thrusfied, 2007; Pereira *et al.*, 2014; Russi *et al.*, 2015).

Positivity rate of beta-lactamase-producing and methicillin-resistant *S. aureus* isolates

The positivity rate of beta-lactamase-producing and methicillin-resistant *S. aureus* isolates was calculated as the total number of beta-lactamase-producing, and methicillin-resistant *S. aureus* isolates using PCR of *blaZ* and *mecA* gene as the reference method (Pitkala *et al.,* 2007; Kaase *et al.,* 2008; Pereira *et al.,* 2014) over the total *S. aureus* isolates, respectively.

RESULTS AND DISCUSSION

Out of the 92 bacterial isolates from another study (unpublished reference), nine (9.78%) *S. aureus* were isolated, revived, and confirmed of its identification using the conventional culture method and a commercial identification system. Both methods confirmed all isolates as *S. aureus*. The revived isolates were used for the detection of methicillin-resistant *S. aureus* (MRSA) using phenotypic and genotypic methods.

Positivity Rate of Isolated *Staphylococcus aureus*

The positivity rate of *Staphylococcus aureus* isolates in mastitic milk were 9.78% (9/92), 21.21% (7/33), and 14.75% (9/61) in farm-, animal-, and quarter-level, respectively (Table 1).

Table 1. Positivity rate of *Staphylococcus aureus* isolates in mastitic milk in farm-, animal-, and quarter-level.

Farm	Number of animals	Number of animals positive for *S. aureus*	Number of quarters	Number of quarters positive for *S. aureus*	Isolated *S. aureus*
B	3	0	3	0	0
D	16	5	33	7	7
E	8	0	13	0	0
F	6	2	12	2	2
TOTAL (%)	33	7 (21.21%)	61	9 (14.75%)	9/92 (9.78%)

In a parallel study conducted by Urriquia (2019), coagulase-negative staphylococci (CoNS) were the predominantly isolated group (42/92 or 45.65%). Similar studies around the world also reported CoNS as the most frequently isolated group from bovine mastitic milk (Pitkala *et al.,* 2004; Kirkan *et al.,* 2005; Haguingan *et al.,* 2010), nonetheless, *S. aureus* is still of particular importance in the dairy cattle industry due to its highly infectious nature (Gao *et al.,* 2011). Being often transmitted in milk and milk products, *S. aureus* poses great public health concern as it can cause a broad spectrum of diseases (Cantekin *et al.,* 2014; Abdel-Tawab *et al.,* 2018). Moreover, the aforementioned species varies in its antimicrobial susceptibility due to its resistance mechanisms. According to Lakhundi and Zhang (2018), the genetic determinant of methicillin resistance in MRSA is not a gene native to *S. aureus* but rather is an acquired gene from another source through an unknown mechanism. Concerning this, CoNS was believed to play a role in the development of resistance in the species under study (Irlinger 2008; Sampimon *et al.,* 2011; Becker *et al.,* 2014; Taponen *et al.*, 2016).

Taponen and Pyorala (2009) mentioned that CoNS tend to be more resistant to antimicrobials compared to *S. aureus* and can easily develop multi-resistance. CoNS are thought to be a reservoir of resistant genes not only for beta-lactam antibiotics but also for other antibiotic classes (Irlinger 2008; Becker *et al.,* 2014; Taponen *et al.,*

2016). This information is of utmost importance for both human and veterinary medicine because these resistant genes are mobile by nature hence, they may transfer into other significant staphylococcal species such as the more pathogenic *S. aureus* (Sampimon *et al.,* 2011; Becker *et al.,* 2014; Schnitt and Tenhagen, 2020). In a study by Frey *et al.* (2013), it was revealed that CoNS may harbor antimicrobial-resistant elements such as the staphylococcal cassette chromosome (SCC*mec*) element which contains the *mec* genes (*mecA* or *mecC*). This is of great concern as it may lead to the possible emergence of MRSA.

In the field, beta-lactam compounds such as penicillin continue to be the most frequently used drug for treating mastitis (Oliver and Murinda, 2012; Soares *et al.,* 2012; Robles *et al.,* 2014); the same goes with human medicine where penicillin remains to be the antimicrobial of choice for infected patients (Papanicolas *et al.,* 2014). However, the occurrence of penicillin-resistant *S. aureus* strains became rapidly spread due to the production of penicillinase encoded by the *blaZ* gene (Jarlov and Rosdahl, 1986; Pitkala *et al.,* 2007; Pereira *et al.,* 2014; Ferreira *et al.,* 2016). Four types of penicillinase (termed A-D) have been described in *S. aureus* (Kaase *et al.,* 2008; Pereira *et al.,* 2014). Production of beta-lactamase is considered to be the most frequent mechanism of penicillin resistance among *S. aureus* isolated from bovine intramammary infections (IMI) (Haveri *et al.,* 2005). The antimicrobial resistance developed by the pathogen is one of the main reasons for treatment failure of mastitis (Gao *et al.,* 2011; Wang *et al.,* 2015). With this, the emergence of MRSA has become of great concern and a growing threat to both human beings and dairy animals (Broekema *et al.,* 2008; Wang *et al.,* 2015; Lakhundi and Zhang, 2018). Accurate detection of these resistant genes through phenotypic and genotypic methods is significant to ensure the effective treatment of an affected patient, and to be able to determine appropriate treatment, management, and control programs on the farm.

Detection of Beta-lactamase Production

Beta-lactamase production was determined through phenotypic and genotypic methods. Phenotypically, the Kirby-Bauer disk diffusion method and nitrocefin test were performed. In this study (Table 2), using the penicillin disk diffusion method, 3/9 (33.33%) *S. aureus* isolates were identified as resistant to penicillin or showing beta-lactamase production for both penicillin zone diameter and penicillin zone edge test. Both tests had a 100% agreement with the result of the nitrocefin test (Fig. 1), identifying the same isolates positive for beta-lactamase production (3/9 or 33.33%). On the other hand, genotypic detection of beta-lactamase production was done using PCR resulting in the detection of the *blaZ* gene in 2/9 (22.22%) *S. aureus* isolates (Fig. 2).

In the study, both penicillin disk diffusion method and nitrocefin test produced a different result from that of PCR of the *blaZ* gene. Inconsistencies between phenotypic and genotypic results were observed as detection of phenotypic resistance in the absence of detected genotypic determinants. This can be explained by the possible involvement of other genes in the process of beta-lactamase resistance. According to studies, beta-lactamase phenotype could be a result of the expression of more than one gene as there is more than one mechanism that gives staphylococci resistance to beta-lactams other than the expression of *blaZ* gene (AbdelGhani *et al.,* 2013;

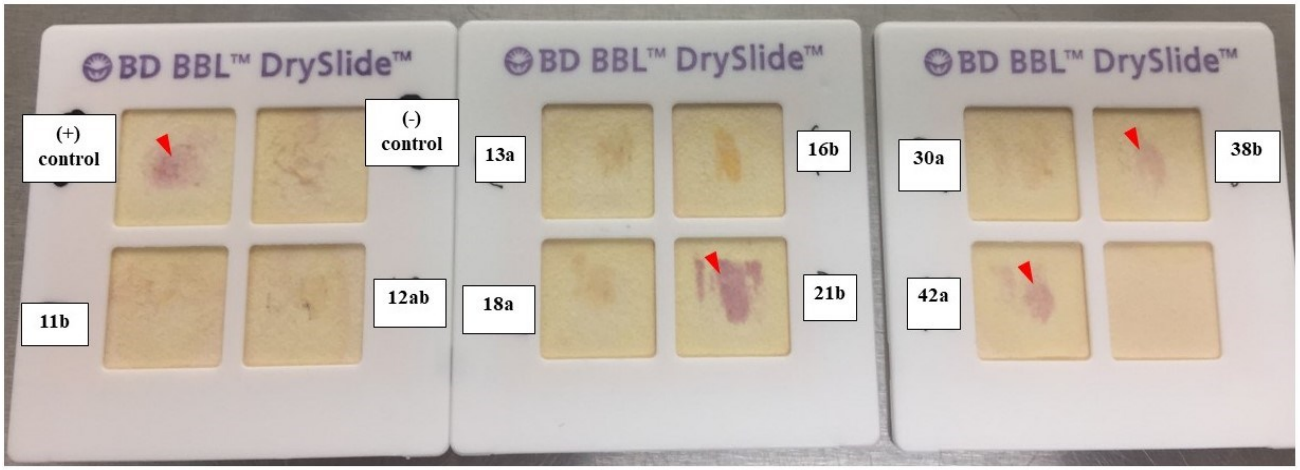

Figure 1. Nitrocefin test performed in the study showing positive test result indicated by a change in color from yellow to pink (red arrowhead) on the reaction area indicating beta-lactamase production among *S. aureus* isolates.

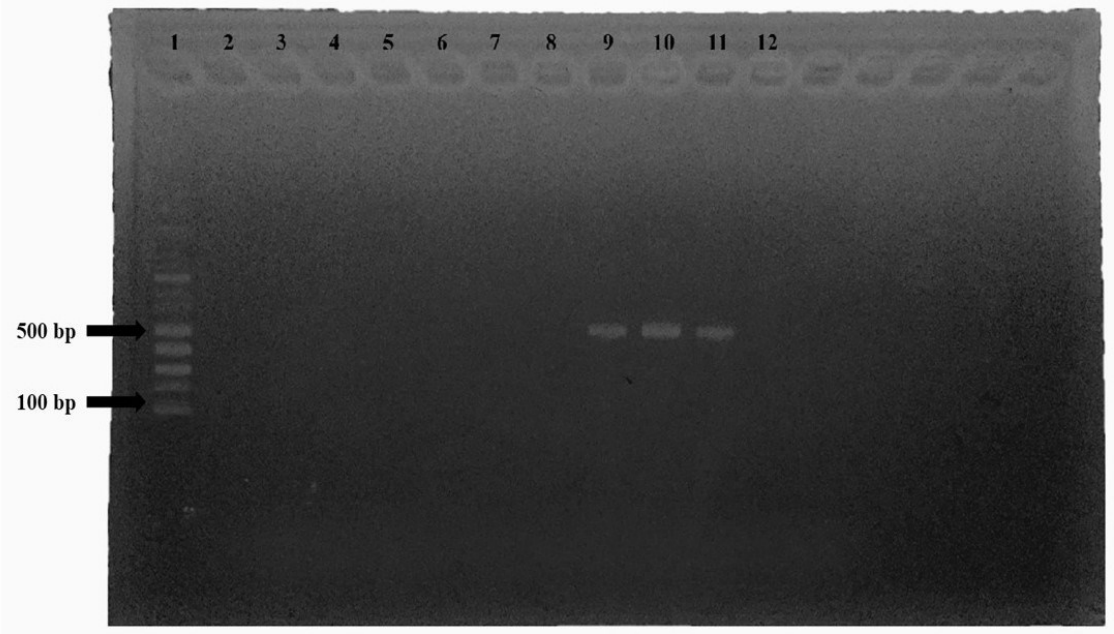

Figure 2. Genotypic detection of beta-lactamase production and methicillin-resistance using duplex PCR of *blaZ* (518 bp) and *mecA* (174 bp) gene, respectively. Lane 1: 100 bp DNA ladder; Lane 2-10: *S. aureus* isolates; Lane 11: positive control for *blaZ* gene (*S. aureus* ATCC 29213); Lane 12: negative control Lane 9 and 10: *blaZ* gene positive; Lane 2-8: *blaZ* gene negative; Lane 2-10: *mecA* gene negative

Malik *et al.,* 2007 as cited by Robles *et al.,* 2014).

Findings from previous studies reported poor sensitivity of chromogenic cephalosporin tests *i.e.,* nitrocefin test, making them inferior to disk zone edge tests in detecting resistance in *S. aureus* (Kaase *et al.,* 2008; Papanicolas *et al.,* 2014). In a study by Kaasee *et al.* (2008), penicillin zone edge determination was determined to be the most sensitive phenotypic test. sensitive phenotypic test. This is also supported by a more recent study by Ferreira *et al.* (2015) where the zone edge test was determined to be the most sensitive (90.3%), followed by MIC determination (85.5%), and lastly by the nitrocefin test (28.9%). The aforementioned study demonstrated that the zone edge test was the most sensitive phenotypic test for detection of beta-lactamase, although it is still not an ideal test to detect this type of resistance due to its low specificity (40.0%). In the present study, using penicillin disk diffusion method as the reference standard, the sensitivity and specificity of nitrocefin test in detecting the presence of beta-lactamase production were both at 100% and were in perfect correlation. Although some studies Haveri *et al.,* 2005; AbdelGhani *et al.*, 2013) considered nitrocefin test reliable for detection of beta-lactamase production in *S. aureus* based on high agreement with the (presence of the *blaZ* gene, discrepancies may still occur due to inaccuracy in interpreting results. A weak color change by some beta-lactamase producers can affect discernment in establishing results, indicating the need for quality controls (Pitkala *et al.,* 2007; AbdelGhani *et al.,* 2013; Papanicolas *et al.,* 2014). A weak reaction in the test may be caused by the weak affinity of beta-lactamase to nitrocefin or the low amount of beta-lactamase produced by specific strains (Jarlov and Rosdahl, 1986; Pitkala *et al.,* 2007; AbdelGhani *et al.,* 2013). Meanwhile, according to Haveri *et al.* (2005), the most common reason for the failure of the nitrocefin test is that some isolates may need prior exposure to beta-lactam antibiotics before producing beta-lactamase. In general, the nitrocefin test is considered a poor inducer and only reliable when staphylococci produce beta-lactamase constitutively (Livermore, 1993; Haveri *et al.,* 2005). Nonetheless, based on the obtained result in the present study, it can be said that the results were good since all isolates had a strong pink reaction and correlated with the disk diffusion test. It can be inferred that the high sensitivity and specificity of nitrocefin test could have been a result of the use of a kit with a different brand, and presumably with a different concentration (Kaase *et al.*, 2008; Papanicolas *et al.*, 2014)

Kappa agreement coefficient was used to determine the validity and agreement of the tests used in the study. A perfect agreement was established between the penicillin disk diffusion method and nitrocefin test (kappa score of 1.00). Also, substantial agreement (kappa score of

0.7270) indicating good clinical significance was determined between the phenotypic methods and PCR. Conventional PCRs have formerly been described and used as the gold standard when comparing phenotypic tests (Pitkala *et al.,* 2007; Kaase *et al.,* 2008; Pereira *et al.,* 2014). The results of the study show that the sensitivities and specificities of both phenotypic tests were the same, with PCR as the reference standard. This is contrary to a study conducted by Pitkala *et al.* (2007), where the sensitivities and specificities of the different methods used vary. Results in other studies (Gao *et al.,* 2011; Robles *et al*., 2014) on the other hand, suggested that there is no significant difference observed using the different tests in the detection of the production of beta-lactamase for *S. aureus*. Determining the appropriate and most efficient test in determining resistance in *S. aureus* species is vital and will be helpful to the researchers, veterinarians, and the dairy industry, in general.

Detection of Methicillin-resistant *Staphylococcus aureus* (MRSA)

Detection of MRSA among the bacterial isolates was done phenotypically using cefoxitin disk diffusion method and genotypically using PCR. No *S. aureus* isolates were determined to be resistant to cefoxitin indicating no oxacillin/ methicillin resistance in all samples. Similarly, using PCR, no isolates resulted positive for the *mecA* gene indicating the absence of methicillin resistance on all tested isolates (Fig. 2). Therefore, all *S. aureus* isolates (9/9) that were identified to have no oxacillin/methicillin resistance using the phenotypic method were also negative for the *mecA* gene using the genotypic method (PCR), thus, indicating the absence of the occurrence of MRSA among all isolates.

In the study, cefoxitin disks were used instead of oxacillin in the disk diffusion method as recommended by CLSI. The use of cefoxitin as a surrogate had been validated by a study conducted by Pottumarthy *et al.* (2005), where detection of oxacillin resistance in 304 staphylococcal isolates from 16 different countries using the cefoxitin disk test had an excellent sensitivity and specificity without interpretive error, as compared to ceftizoxime disk (1% major and 0.5% minor errors) and oxacillin disk test (4.4% major and 1.5% minor errors), with *mecA* gene PCR as the reference standard. Another study by Witte *et al*. (2007) revealed higher sensitivity of disk-diffusion using cefoxitin 30-µg discs (96%) compared to using oxacillin 1-µg discs (92%). It was established that cefoxitin results are much easier to interpret and are thus more sensitive for the detection of *mecA*-mediated resistance than oxacillin results (Pottumarthy *et al.,* 2005; Witte *et al.,* 2007; Broekema *et al.,* 2008). In the present study, the positivity rate of oxacillin/methicillin resistance in *S. aureus* isolates was not detected in using both cefoxitin disk diffusion and PCR resulting in a negative predictive value of 100%. Overall, the sensitivity and specificity of the phenotypic test used in the study were undefined and thus, unsuitable for comparison. Regardless, other studies (Reischl *et al.,* 2000; Asfour and Darwish, 2011) find phenotypic methods to be time-consuming and labor-intensive, and disadvantageous due to

Table 2. Absolute and relative (%) frequencies of beta-lactamase production detection among *Staphylococcus aureus* isolates (n = 9) in mastitic milk.

	Test	Result	P10 zone diameter				P10 zone edge				Nitrocefin test				PCR of *blaZ* gene			
			S	%	R	%	(+)	%	(-)	%	(+)	%	(-)	%	(+)	%	(-)	%
Beta-lactamase production	P10 zone diameter [a]	S	-	-	-	-	0 [b]	0.00	6 [b]	66.66	0 [c]	0.00	6 [c]	66.66	0 [a]	0.00	6 [a]	66.66
		R	-	-	-	-	3 [b]	33.33	0 [b]	0.00	3 [c]	33.33	0 [c]	0.00	2 [a]	22.22	1 [a]	11.11
	P10 zone edge [a]	(+)	0 [b]	0.00	3 [b]	33.33	-	-	-	-	3 [c]	33.33	0 [c]	0.00	2 [a]	22.22	1 [a]	11.11
		(-)	6 [b]	66.66	0 [b]	0.00	-	-	-	-	0 [c]	0.00	6 [c]	66.66	0 [a]	0.00	6 [a]	66.66
	Nitrocefin test [a]	(+)	0 [b]	0.00	3 [b]	33.33	0 [c]	0.00	3 [c]	33.33	-	-	-	-	2 [a]	22.22	1 [a]	11.11
		(-)	6 [b]	66.66	0 [b]	0.00	6 [c]	66.66	0 [c]	0.00	-	-	-	-	0 [a]	0.00	6 [a]	66.66
	PCR of *blaZ* gene [a]	(+)	0 [a]	0.00	2 [a]	22.22	2 [a]	22.22	0 [a]	0.00	2 [a]	22.22	0 [a]	0.00	-	-	-	-
		(-)	6 [a]	66.66	1 [a]	11.11	1 [a]	11.11	6 [a]	66.66	1 [a]	11.11	6 [a]	6.66	-	-	-	-

Means with different superscripts among rows between each detection methods are statistically significant (p-value = < 0.05) P10 - Penicillin G (10 IU), S – susceptible, R - resistant

possible false positive and false negative results.

Positivity rate of Beta-lactamase producing and Methicillin-resistant *Staphylococcus aureus* Isolates

The positivity rate of *S. aureus* isolates that produce beta-lactamase and show methicillin resistance was previously established. Three (3/9 or 33.33%) isolates were positive for beta-lactamase production using phenotypic methods, while 2/9 (22.22%) samples were positive using PCR. On the other hand, all samples were negative for the presence of methicillin resistance using both phenotypic and genotypic methods.

A study by Robles *et al.* (2014) mentioned the probability of beta-lactamase being herd dependent. In the present study, both farms with *S. aureus* isolates (2/4 or 50%) had animals positive for beta-lactamase-producing *S. aureus,* suggesting possible causes of the development of resistance in the herd. The treatment used in these farms was the same, that is a combination of penicillin and streptomycin (Pen-Strep). Recent studies show that there is an association between antimicrobial use and antimicrobial resistance in dairy farms (Saini *et al.,* 2012; Robles *et al.,* 2014). Dairy farms are said to form an ideal environment where bacteria are subjected to antimicrobial treatments, and the succeeding selection pressure may favor the selection and dissemination of resistant strains (Saini *et al.,* 2012). Studies suggest that intensive use of these antimicrobial agents plays a great factor in the emergence of antibiotic resistance (Preethirani *et al.,* 2015; Mohana *et al.,* 2016; Awad *et al.,* 2017; Pyatov *et al.,* 2017). Inappropriate use of these drugs leading to the development of resistance can also be attributed to the absence of a veterinarian in the farms affected.

Methicillin resistance was not detected in all *S. aureus* isolates used in the study. Methicillin resistance was said to be more prevalent in CoNS than in *S. aureus* (John and Harvin, 2007; Becker *et al.,* 2014). This holds true considering the parallel study by Urriquia (2019), where 25/42 (59.52%) of the CoNS isolated were reported positive for methicillin resistance. Although MRSA prevalence is said to be generally low in dairy herds as compared to other animal species (Schnitt and Tenhagen, 2020), it is still an important issue in veterinary medicine as treatment options for the pathogen are limited. In addition, aside from its zoonotic transmission through milk, documented evidence of MRSA transmission between cows and close human contacts exists (Haenni *et al.,* 2010 as cited by Schmidt *et al.,* 2017). A better understanding of factors that increase the risk for MRSA transmission into and within dairy herds is essential to develop effective prevention strategies and control the spread of the pathogen.

The results of the study show that *S. aureus* is becoming more resistant to the commonly used antimicrobials for the treatment of bovine mastitis due to the detection of beta-lactamase production among *S. aureus* isolates. Development of resistance may be attributed to the inappropriate use of antimicrobials brought about by the absence of a veterinarian in the farm. Despite the fact that there were no detected MRSA in the study, consistent monitoring is still important especially due to the reason that there were methicillin-resistant CoNS isolates observed from a parallel study. The use of a greater sample size is recommended in order to properly and more accurately establish a clinical picture. Detection of other *mec* gene homologues, specifically the *mecC* gene, can also be performed to possibly determine occurrence of *mecC* MRSA.

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STATEMENT ON COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

GLT contributed to the development of the methodology, performed the experiment, collected and analyzed the data, wrote the original draft, and reviewed and edited the manuscript. AJBA and HGR conceptualized the study, designed the methodology, provided some resources, supervised the research activity, assisted in the analysis of data, and reviewed and edited the manuscript.

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