Genotypic Profiling of Virulence and Antibiotic Resistance Patterns of *Staphylococci* and *Streptococci* Isolates from Dairy Cattle with Clinical Mastitis Infection in Region IV-A, Philippines

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Abstract

Staphylococci and Streptococci spp. play a significant role as primary causal agents of bovine mastitis. Farmers commonly employ the use of antibiotics as a widespread strategy for the management of mastitis infections. However, the frequent use of antibiotics is directly correlated to the increase in antimicrobial-resistant (AMR) bacterial strains resulting in some challenges in managing subsequent infections. In this current study, the virulence and AMR profiles of previously isolated Staphylococci and Streptococci bacterial strains from dairy cattle with clinical mastitis were evaluated to provide an overview of their pathogenic and resistant genetic characteristics. The incidence of virulence genes among Staphylococci isolates revealed low detection rates, whereas 42.2% of the Streptococci isolates were found to express the surface-anchored rib gene. The laminin-binding lmb and toxin β-hemolysin cylE genes were also frequently detected among Streptococci isolates. These observations suggest a strong association between Streptococci spp. and severe infection among the tested animals. Meanwhile, 28% of Staphylococci and 12.2% of Streptococci were observed to be resistant to three or more drug classes, thus considered multidrug-resistant. The most frequently detected AMR genes among Staphylococci isolates were aphA-1 (33%), mecA (25%), and blaSPM-1 (21%). Meanwhile, among Streptococci isolates, tetB (35%), blaIMP (31%) and ermA/TR (31%) were most prevalent. Although the phenotypic resistance of some isolates was comparable with its associated genotypic resistance, some isolates did not exhibit a consistent phenotype-genotype AMR. Nevertheless, the presence of these virulence and AMR genes in mastitis-associated pathogens is still critical because these genes can migrate across microbial populations, thus posing a serious threat to animal and public health.

***Keywords**:* antimicrobial resistance, bovine mastitis, staphylococcus, streptococcus, virulence

1. Introduction

Mastitis is regarded as the most common infectious disease affecting dairy animals. It is an inflammatory reaction of the udder tissue in the mammary gland caused by physical injury or microorganism infections (Cheng and Han, 2020). Symptoms of mastitis-infected cows include swelling, heat and soreness in the udder, abnormally colored milk, elevated body temperatures, lethargy and anorexia (Kibebew, 2017). Bovine mastitis manifests in three distinct clinical forms: subclinical, clinical and chronic, with varying degrees of severity ranging from mild to moderate or severe (Cheng and Han, 2020). Mastitis has a detrimental impact on milk production and quality placing a heavy financial burden on dairy farmers.

Mastitis is caused by a wide range of etiological agents. *Staphylococci* species are among the most significant and frequently identified bacteria causing mastitis. More than 50 species and subspecies of *Staphylococci* have been found to cause staphylococcal mastitis (Dabele *et al.*, 2021). They are classified as coagulase-positive *Staphylococcus* (CPS) or coagulase-negative *Staphylococcus* (CNS) based on their capacity to coagulate plasma (Simojoki *et al.*, 2011). *S. aureus*, a CPS, causes chronic mammary gland infections and is highly transmissible, extremely difficult to cure and prone to resurgence (Katsuda *et al.*, 2005; Hoque *et al.*, 2018). The classification of *S. aureus* as a priority II pathogen with significant global public health implications has been carried out by the World Health Organization (WHO) emphasizing its critical status (Centers for Disease Control and Prevention, 2013; WHO, 2017). On the other hand, CNS has long been thought to be minor pathogens since they do not appear capable of causing severe mastitis. However, it persists in the mammary gland and causes a slight increase in milk somatic cell count (Taponen and Pyörälä, 2009). Notably, the most prevalent CNS, such as *S. chromogenes, S. epidermidis* and *S. simulans,* have been identified to cause intra-mammary infections in dairy cattle (Thorberg *et al.*, 2009).

Streptococci is also one of the most important and commonly isolated mastitis-causing bacteria in dairy cattle. They could be classified as esculin-positive group D *Streptococci* and esculin-negative *S. agalactiae.* Several streptococcal species have been associated with mastitis infection. It could be contagious or environmental. *S. uberis* strains, an esculin-positive *Streptococci*, which are routinely isolated from the environment of dairy farms, have been shown to be host-adapted and to exhibit contagious characteristics, whereas other strains are not host-adapted and result in

transient intra-mammary infection of an environmental origin (Dego *et al.*, 2021). On the other hand, it has been observed that esculin-negative *S. agalactiae*, which is a bacterium causing mammary gland infections, can persist temporarily on hands, milking machine components and teat skin. This facilitates its transmission from one cow to another during the milking process. The rapid spread of infections is attributed to their high contagiousness and inconspicuous nature as noted by Lakew *et al.* (2019).

Both *Staphylococci* and *Streptococci* possess a diverse array of virulence factors that enhance their capacity to establish, endure and propagate within the host organism. These factors also contribute to the destruction of the host during infection. These factors include their capability to penetrate and adhere to host cells, invade the epithelium and synthesize toxins used by the pathogens to adapt to infection of the tissue in the mammary gland (Jain *et al.*, 2012; Hoque *et al.*, 2018).

Antibiotics are commonly used to treat mastitis and can be administered via intra-mammary, intramuscular, or intravenous injections. The best therapy should be long enough to cure mastitis infection and short enough to prevent antibiotic resistance. However, the dairy sector has seen certain issues as a result of the overuse and abuse of antibiotics in the treatment of bovine mastitis (Cheng and Han, 2020). Numerous antimicrobial-resistant (AMR) bacterial strains, notably multidrug-resistant (MDR) strains that lead to either subclinical or clinical mastitis, have risen and become a significant public health problem in recent years. Drug resistance develops over time, mainly as a result of genetic changes gained through mutation and selection (vertical gene transfer) and/or gene transfer across strains and species (horizontal gene transfer) (Biswas *et al.*, 2008). However, the inappropriate use of antimicrobials might also hasten the development of drug-resistant bacteria. AMR genes or MDR plasmids can be generated by administering low quantities of antimicrobial drugs to domestic animals resulting in the proliferation and dissemination of such genes across strains (Ter Kuile *et al.*, 2016). The usage of antimicrobials at sub-inhibitory doses has been found to promote the transmission of horizontal resistance genes (Jutkina *et al.*, 2016). Moreover, genetic factors associated with AMR, commonly located on movable genetic components, can be readily transferred among a wide range of hosts encompassing humans, animals and the environment (Biswas *et al.*, 2008; Ter Kuile *et al.*, 2016).

In the Philippines, the government is enhancing the local fresh milk supply to overcome the shortfall. However, attempts by the government to boost local milk output may be hampered by the prevalence of mastitis infection among dairy cows. In a recent study, different bacterial strains associated with mastitis infection from dairy cows with clinical mastitis in Region IV-A, Philippines were isolated and identified (Perez and Ancuelo, 2022; Ancuelo and Perez, 2023). Forty-eight and 26 isolates belong to *Staphylococci* and *Streptococci*, respectively. Thus, the present study aimed to investigate the presence of virulence and AMR genetic determinants in these previously isolated bacterial strains.

2. Methodology

2.1 Bacterial Strains and Culture Conditions

All previously isolated *Staphylococci* and *Streptococci* strains obtained from dairy cows manifesting signs of clinical mastitis infection were included in this study. All procedures in the previous research involving animal subjects were approved by the UPLB Animal Care and Use Committee (ACUC) (Assignment Protocol No. BIOTECH-2021-003) and the Bureau of Animal Industry (BAI), Department of Agriculture (DA) (Reference No. AR-2021-021). The strains were cultivated in a medium consisting of trypticase soy broth (TSBYE) (Titan Biotech Ltd., India) with 0.6% yeast extract. The incubation took place for a duration of 24-48 h at a temperature of 37 °C. For preservation, these cultures were stored using a 30% glycerol solution at a temperature of -80 °C. Prior to each utilization, a process of dual cultivation was employed to reactivate the cultures.

2.2 DNA Extraction of Bacteria Isolates

The genomic DNA of all specimens was obtained by employing the GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia) following the manufacturer's provided guidelines. The genomic DNA concentration was gauged using a NanoDrop spectrophotometer (Thermo Fisher Scientific, United States), and then it was adjusted to a uniform value of 10 ng/L using sterile ultrapure water. Subsequently, the DNA was stored at a temperature of -20 °C until its intended application.

2.3 Genotypic Detection of Virulence and Antimicrobial Resistance Genes

The presence of virulence and AMR genes in the samples was determined using the Polymerase Chain Reaction (PCR) technique. Specific primers, previously documented and listed in Tables 1 and 2, were used for this purpose. Each PCR reaction consisted of a 10 µL mixture containing 0.5 ng of DNA, 0.2 µM of each primer pair, and MyTaq™Mix from Meridian Life Science Inc., United States.

Table 1. Primers used for virulence-associated gene screening of *Staphylococci* and *Streptococci* isolates

Primer name	Description of the amplified gene	Primer sequence (5' – 3')	Amplicon size (bp)	Annealing temp. (°C)	Reference
Staphylococcus					
nuc	Extracellular thermostable nuclease	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	279	56	Hoque *et al.* (2018)
sea	Enterotoxin gene	F-GGTTATCAATGTGCGGGTGG R- CGGCACTTTTTTCTCTTCGG	102	55	Hoque *et al.* (2018)
seb	Enterotoxin gene	F- GTATGGTGGTGTAACTGAGC R- CCAAATAGTGACGAGTTAGG	164	51	Hoque *et al.* (2018)
sec	Enterotoxin gene	F-AGATGAAGTAGTTGATGTGTATGG R- CACACTTTTAGAATCAACCG	491	49	Hoque *et al.* (2018)
sed	Enterotoxin gene	F- CCAATAATAGGAGAAAATAAAAG R- ATTGGTATTTTTTTTCGTTC	495	44	Hoque *et al.* (2018)
see	Enterotoxin gene	F- AGGTTTTTTCACAGGTCATCC R- CTTTTTTTTCTTCGGTCAATC	430	48	Hoque *et al.* (2018)
pvl	Panton-valentine leukocidin gene	F-ATCATTAGGTAAAATGTCTGGACATGATCCA R-GCATCAAGTGTATTGGATAGCAAAAGC	433	57	Hoque *et al.* (2018)
tsst1	Toxic shock syndrome toxin 1 gene	F- ACCCCTGTTCCCTTATCATC R- TTTTCAGTATTTGTAACGCC	326	48	Hoque *et al.* (2018)
eta	Exfoliative toxin genes	F-ATATCAACGTGAGGGCTCTAGTAC R- ATGCAGTCAGCTTCTTACTGCTA	93	56	Hoque *et al.* (2018)
etb	Extracellular thermostable nuclease	F- CACACATTACGGATAATGCAAG R-TCAACCGAATAGAGTGAACTTATCT	226	52	Hoque *et al.* (2018)
Streptococcus					
bca	Bacterial penetration	F-TAACAGTTATGATACTTCACAGAC R- ACGACTTTCTTCCGTCCACTTAGG	535	50	Jain *et al.* (2012)
scpB	Binds fibronectin	F- ACAACGGAAGGCGCTACTGTTC R- ACCTGGTGTTTGACCTGAACTA	255	57	Jain *et al.* (2012)
rib	Present in invasive strains	F- CAGGAAGTGCTGTTACGTTAAAC R- CGTCCCATTTAGGGTTCTTCC	369	55	Jain *et al.* (2012)

Table 1 continued.

lmb	Invasion of damaged epithelium	F- ACCGTCTGAAATGATGTGG R- GATTGACGTTGTCTTCTGC	572	52	Jain *et al.* (2012)
cylE	Tissue injury and systemic spread of bacteria	F- TGACATTTACAAGTGACGAAG	248	51	Jain *et al.* (2012)

Table 2. Antimicrobial resistance primers used in this study

Antimicrobial drug class	Primer name	Primer sequence (5' → 3')	Amplicon size (bp)	Annealing temp. (℃)	References
Aminoglycosides	*aphA-1*	F-ATGGGCTCGCGATAATGTC R-CTCACCGAGGCAGTTCCAT	600	56	Sáenz *et al.* (2004)
	aphA-2	F-GAACAAGATGGATTGCACGC R-GCTCTTCAGCAATATCACGG	680	54	Sáenz *et al.* (2004)
	aphA-3	F-GGGGTACCTTTAAATACTGTAG R- TCTGGATCCTAAAACAATTCATCC	848	50	Poyart *et al.* (2003)
	aadA1/2	F-GCAGCGCAATGACATTCTTG R-ATCCTTCGGCGCGATTTTG	282	56	Sáenz *et al.* (2004)
	aad6	F-AGAAGATGTAATAATATAG R-CTGTAATCACTGTTCCCGCCT	978	36	Poyart *et al.* (2003)
β-Lactams	*blaIMP*	F-CTACCGCAGCAGAGTCTTTG R-AACCAGTTTTGCCTTACCAT	587	53	Sundsfjord *et al.* (2004)
	blaSHV	F-ATGCGTTATATTCGCCTGTG R-TTAGCGTTGCCAGTGCTCGA	860	54	Sundsfjord *et al.* (2004)
	blaSPM-1	F-CCTACAATCTAACGGCGACC R-TCGCCGTGTCCAGGTATAAC	649	56	Sundsfjord *et al.* (2004)
	blaTEM	F-ATGAGTATTCAACATTTTCGTG R-TTACCAATGCTTAATCAGTGAG	860	49	Sundsfjord *et al.* (2004)
	blaVIM	F-ATTCCGGTCGGAGAGGTCCG R-GAGCAAGTCTAGACCGCCCG	633	62	Sundsfjord *et al.* (2004)
	mecA	F-TGGCTATCGTGTCACAATCG R-CTGGAACTTGTTGAGCAGAG	310	54	Sundsfjord *et al.* (2004)
Macrolides	*ermA/TR*	F-TCAGGAAAAGGACATTTTACC R-ATACTTTTTGTAGTCCTTCTT	432	46	Sutcliffe *et al.* (1996)
	ermB	F-GAAAAGGTACTCAACCAAATA R- AGTAACGGTACTTAAATTGTTTAC	639	48	Sutcliffe *et al.* (1996)
	ermC	F-TCAAAACATAATATAGATAAA R-GCTAATATTGTTTAAATCGTCAA	642	38	Sutcliffe *et al.* (1996)
	mdfA/mefE	F-AGTATCATTAATCACTAGTGC R-TTCTTCTGGTACTAAAAGTGG	348	47	Sutcliffe *et al.* (1996)
Quinolones	*gyrA*	F-TTCTCCGATTTCCTCATG R-AGAAGGGTACGAATGTGG	458	49	de Toro *et al.* (2010)
	parC	F-TGGGTTGAAGCCGGTTCA R-CAAGACCGTTGGTTCTTTC	361	52	de Toro *et al.* (2010)
Streptomycin	*rpsL*	F-GGCCGACAAACAGAACGT R-GTTCACCAACTGGGTGAC	501	54	Sreevatsan *et al.* (1996)
	rrs	F-GAGAGTTTGATCCTGGCTCAG R-TGCACACAGGCCACAAGGGA	1042	56	Sreevatsan *et al.* (1996)
Sulfonamides	*sul1*	F-TGGTGACGGTGTTCGGCATTC R-GCGAGGGTTTCCGAGAAGGTG	789	62	Sáenz *et al.* (2004)

Table 2 continued.

	sul2	F-CGGCATCGTCAACATAACC R-GTGTGCGGATGAAGTCAG	722	54	Sáenz *et al.* (2004)
	sul3	F-CATTCTAGAAAACAGTCGTAGTTCG R-CATCTGCAGCTAACCTAGGGCTTTGGA	990	54	Sáenz *et al.* (2004)
Tetracyclines	*tetA*	F-GTAATTCTGAGCACTGTCGC R-CTGCCTGGACAACATTGCTT	937	54	Sáenz *et al.* (2004)
	tetB	F-CTCAGTATTCCAAGCCTTTG R-CTAAGCACTTGTCTCCTGTT	416	51	Sáenz *et al.* (2004)
	tetC	F-TCTAACAATGCGCTCATCGT R-GGTTGAAGGCTCTCAAGGGC	570	55	Sáenz *et al.* (2004)
	tetD	F-ATTACACTGCTGGACGCGAT R-CTGATCAGCAGACAGATTGC	1104	54	Sáenz *et al.* (2004)
	tetE	F-GTGATGATGGCACTGGTCAT R-CTCTGCTGTACATCGCTCTT	1179	55	Sáenz *et al.* (2004)
	tetK	F-GTAGGATCTGCTGCATTCCC R-CACTATTACCTATTGTCGC	552	48	Poyart *et al.* (2003)
	tetL	F-GGATCGATAGTAGCCATGGG R-GTATCCCACCAATGTAGCCG	516	56	Poyart *et al.* (2003)
	tetM	F-GTGGAGTACTACATTTACGAG R-GAAGCGGATCACTATCTGAG	359	50	Poyart *et al.* (2003)
	tetO	F-GCGGAACATTGCATTTGAGGG R-CTCTATGGACAACCCGACAGAAG	538	58	Poyart *et al.* (2003)
Lincosamides	*linB*	F-CCTACCTATTGTTTGTGGAA R-ATAACGTTACTCTCCTATTC	925	46	Bozdogan *et al.* (1999)
Vancomycin	*vanA*	F- GGAGTAGCTATCCCAGCATT R- TCTGCAATAGAGATAGCCGC	377	55	Lemcke and Bülte (2000)

The PCR process was conducted in a MultiGene Gradient Thermal Cycler (Labnet International, United States). The amplification protocol involved an initial cycle at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at temperatures specified in Tables 1 and 2 for 15 s, and elongation at 72 °C for 15 s. A final extension step was performed at 72 °C for 2 min.

To visualize the amplified DNA fragments, electrophoresis was performed using 1.5% (w/v) agarose gels in 0.5× TAE buffer containing ViSafe Red Gel Stain (Vivantis Technologies, Malaysia). To confirm the identity of the amplified genes, representative samples of each target DNA fragment were subjected to DNA sequencing.

2.4 Phenotypic Antimicrobial Susceptibility Profiling

The antibiotic sensitivity of the isolates was evaluated using the disc diffusion technique with commercial antimicrobial discs (Oxoid, United Kingdom). The antibiotic discs containing 10 µg penicillin (PEN), 25 µg amoxicillin (AML),

10µg tetracycline (TET), 15µg erythromycin (ERY), 10µg lincomycin (MY), 2µg, clindamycin (CLI), and 10µg streptomycin (STR) were used, as these were the frequently used antimicrobials in dairy farms. Data evaluation was performed through the 2021 Clinical and Laboratory Standards Institute (CLSI) guide provided by the manufacturer. The antibiotic resistance pattern of all isolates was categorized as susceptible, intermediate, or resistant.

3. Results and Discussion

3.1 Virulence Genotyping of Staphylococci Isolates

The presence of known *Staphylococci* virulence genes *nuc, sea, seb, sec, sed, see, pvl, tsst1, eta* and *etb* in 48 *Staphylococci* isolates from dairy cattle with mastitis was evaluated. Among the isolates examined, only two genes were present in three species, and the overall detection rate of the said genes, *nuc* and *see,* were only 2% (1 out of 48) and 4% (2 out of 48), respectively. The identity of the amplified genes was verified through DNA sequencing of representative samples from each target DNA fragment. The very low detection of the virulence genes among the *Staphylococci* isolates suggests that the severe infection observed among the sampled animals could be attributed to bacterial pathogens other than *Staphylococci* strains. This is consistent with the earlier reported findings on the unintended isolation of non-staphylococcal isolates from mastitic milk and swab samples, where other isolates were found to exhibit virulence traits such as hemolytic activity and biofilm-formation and MDR (Perez and Ancuelo, 2022; Ancuelo and Perez, 2023). Nevertheless, the detection of the virulence genes, *nuc* and *see,* in some *Staphylococci* isolates still suggests the possible involvement of these strains in the infection. The secreted nuclease, *nuc,* is one of the enzymes produced and released by *S. aureus* that contributes to its pathogenesis and ability to survive in the host (Olson *et al.,* 2013). In the current investigation, only one *S. aureus* isolate displayed a positive result for the *nuc* gene, whereas a higher occurrence of the identification of this gene has been documented elsewhere (Xu *et al*., 2015; Ewida and Al-Hosary, 2020). Hence, the relatively scarce presence of the *nuc* gene in our current study might be attributed to the overall lower prevalence of *S. aureus* or its relative scarcity within the isolates.

Another gene associated with the pathogenicity of *Staphylococci* strains is the *see* gene, a staphylococcal enterotoxin (SE). SEs are often associated with the most prevalent foodborne disease and staphylococcal food poisoning (SFP)

(Tang *et al.*, 2011). In this study, very low detection of the *see* gene was observed among the isolates. Solely one *S. epidermidis* isolate and one *S. simulans* isolate exhibited the existence of a *see* gene. Additionally, two *S. epidermidis* isolates from goat and sheep milk were also identified with the *see* gene (Rahmdel *et al*., 2018). However, this gene was absent in both *S. epidermidis* and *S. simulans* isolates from mastitic cow milk samples in India (Mahato *et al*., 2017).

3.2 Virulence Genotyping of Streptococci Isolates

The presence of known *Streptococci* virulence-associated genes *bca, scpB, rib, lmb* and *cylE* was investigated in 26 *Streptococci* isolates. Out of 26 isolates, results showed that 42.3% possess the *rib* gene (7 *S. agalactiae*, 1 *S. gallolyticus* and *3 S. uberis* isolates). The surface Rib protein, common among invasive *Streptococci* strains, is encoded by the *rib* gene (Jain *et al.*, 2012). The *lmb* gene was also present in 20% of the isolates (4 *S. agalactiae* and 1 *S. equinus* isolates). The *lmb* gene is responsible for laminin-binding protein (lmb), a surface protein that aids in invading damaged epithelium. The *cylE* gene was detected in 8 *S. agalactiae* (30.8%). The toxin β-hemolysin *cylE* gene inflicts tissue damage and bacterial systemic spread and promotes meningitis. Overall, the *rib* gene was the most frequent virulence gene (Figure 1).

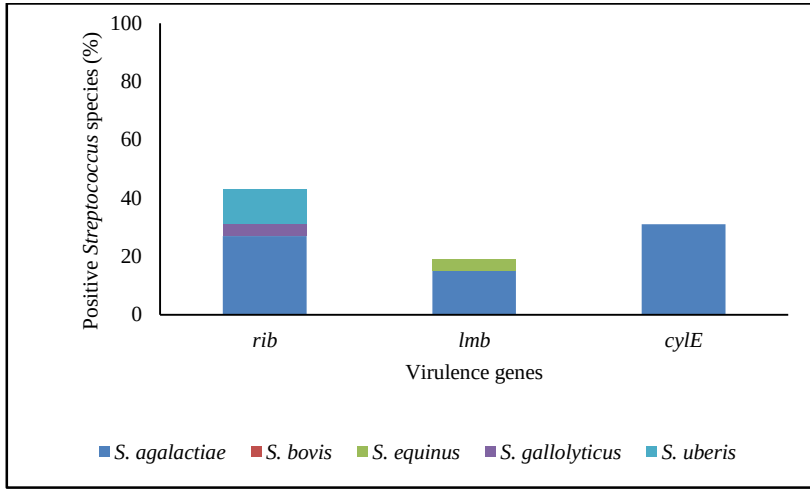


Figure 1. Percentage *Streptococci* spp. that tested positive for a specific virulence genetic determinant

Expectedly, the *S. agalactiae* isolates were found to carry the highest number of virulence genes. These results suggest that the *Streptococci* isolates, especially *S. agalactiae,* are a significant etiological agent to the severe infection manifested among the dairy animals tested. The authors of this present work earlier reported a study highlighting that MDR and virulent *Enterobacteriaceae* isolates were prevalent among dairy cattle with clinical mastitis infection (Ancuelo and Perez, 2023). These findings highlight the complex nature of mastitis infection caused by multiple etiological agents possibly having synergistic pathogenicity.

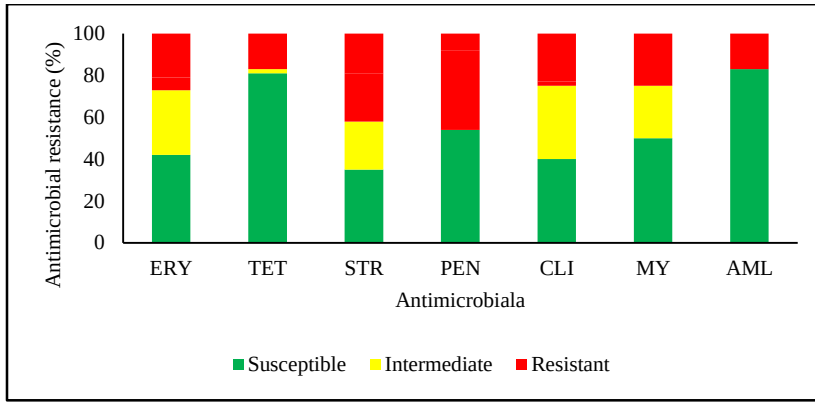
The detection of these virulence genes has also been reported in mastitis-causing *Streptococci* strains in other countries. Genes *rib* (33%) and *cylE* (78%) were detected in *S. agalactiae* strains isolated from clinical cases of bovine mastitis in Poland (Kaczorek *et al.*, 2017). In Egypt, *S. agalactiae* isolates originating from the milk of dairy cows afflicted with clinical mastitis were discovered to carry the *lmb* gene (Abd El-Aziz *et al.*, 2021). This finding underscores the extensive distribution of these genes within mastitis-associated strains across the globe.

Presently, the majority of *S. agalactiae* isolates were identified to possess the genes responsible for encoding surface-anchored proteins including Rib and lmb proteins (Figure 1). Protein C, which contains the surface protein Rib (*rib* gene), facilitates adhesion, colonization, and invasion to host cells and resistance to phagocytosis (Smith *et al.*, 2007; Bobadilla *et al.*, 2021). *S. agalactiae* adhesion is also greatly influenced by the surface-associated lipoprotein Lmb which is expressed by gene *lmb* (Jain *et al.*, 2012). *Lmb* mediates the binding of Group B *S. agalactiae* (GBS) to major glycoprotein laminin of the extracellular matrix of the host cell, which may be necessary for the bacterial colonization of damaged epithelium and bacterial translocation into the bloodstream (Al Safadi *et al.*, 2010; Bobadilla *et al.*, 2021). Thus, the high frequency of the *rib* and *lmb* gene may suggest that these genes are significant in the bovine mastitis infection development.

Hemolysis is a major virulence factor in *S. agalactiae*. Production of hemolysin is dependent on the cytolytic toxin *cylE* genes (Jain *et al.*, 2012). Gene *cylE* was frequently detected in *S. agalactiae* strains in the current study (Figure 1). This gene was contained in the GBS-specific *cyl* operon, which was necessary for hemolytic activity and pigment formation; both were identified as key determinants for GBS pathogenicity (Rosa-Fraile *et al.*, 2014).

3.3 Antimicrobial Resistance Genotyping

Antibiotic administration has been the common practice in managing mastitis infection. However, this practice has led to the emergence of AMR bacterial pathogens. In this study, *Staphylococci* isolates were phenotypically and genotypically profiled for their AMR. The summarized distribution of susceptible, intermediate and resistant staphylococcal isolates against each antimicrobial is shown in Figure 2.



Erythromycin: ERY, tetracycline: TET, streptomycin: STR, penicillin: PEN, clindamycin: CLI, lincomycin: MY and amoxicillin: AML

Figure 2. Antimicrobial resistance (%) of *Staphylococci* species (n = 48) obtained from dairy cattle displaying clinical mastitis categorized as susceptible, intermediate, or resistant based on the CLSI chart

The antibiotic with the highest susceptible isolates was amoxicillin, with a sensitivity rate of 83%. It was followed by tetracycline with 81% susceptibility. However, penicillin had the highest resistance percentage of 46%. Streptomycin was also with a high rate of resistant strains of 42%.

In total, 27% of all *Staphylococci* isolates were identified as MDR as these strains exhibited resistance to antibiotics from three antimicrobial drug classes. Specifically, 62.5% of *S. epidermis*, all S. *saprophyticus* and 33.4% of the *S. chromogenes* isolates were MDR (Table 3). The prevalence of these MDR strains associated with mastitis was reported globally. Field isolates of MDR *S. epidermidis* from the milk of dairy cattle with clinical mastitis were observed in China (Zhou *et al.*, 2015). An isolate of *S. saprophyticus* retrieved from cases of bovine mastitis on Tunisian dairy farms also demonstrated MDR (Dhaouadi *et al.*, 2020). Furthermore, South Africa reported a prevalence of

52% MDR *S. chromogenes* strains in composite milk samples from instances of subclinical mastitis (Phophi *et al*., 2019).

Table 3. Antimicrobial resistance (%) of *Staphylococci* and *Streptococci* spp. to a specific number of antimicrobial drug class

Species	No. of isolates	Resistance to number of antimicrobial drug class (%)						% MDR
		5	4	3	2	1	0	
Staphylococcus								
S. aureus	2	0	0	0	50	50	0	0
S. agnetis	11	0	0	0	0	90.9	9.1	0
S. arlettae	2	0	0	0	100	0	0	0
S. chromogenes	18	27.8	5.6	0	16.7	16.7	33.3	33.4
S. epidermidis	8	0	0	62.5	25	12.5	0	62.5
S. saprophyticus	2	0	0	100	0	0	0	100
S. simulans	5	0	0	0	20	20	60	0
Streptococcus								
S. agalactiae	9	0	11.1	0	0	88.9	0	11.1
S. bovis	2	0	50	0	0	50	0	50
S. equinus	3	0	0	0	66.7	33.3	0	0
S. gallolyticus	1	0	0	0	0	100	0	0
S. uberis	11	0	0	0	0	100	0	0

The prevalence of resistance genes for every *Staphylococci* isolate is shown in Table 4. Furthermore, the most frequently detected AMR gene among the *Staphylococci* isolates was *aphA-1* followed by *mecA* and *blaSPM-1*. The existence of three antimicrobial drug class genes was demonstrated in 8.3% of staphylococcal isolates, two in 37.5% and one in 47.9%. Three isolates (6.3%) did not harbor any AMR genes. Moreover, the species with a high percentage of the AMR gene were *S. chromogenes*, *S. agnetis* and *S. epidermidis*.

Gene *aphA-1* is the most common AMR gene in *Staphylococci* isolates in the current study. This gene is responsible for resistance to kanamycin, an aminoglycoside (Turutoglu *et al.*, 2009). AMR gene *mecA* was also frequently detected and phenotypic resistance to penicillin; a β-lactam was observed the most in staphylococcal isolates in this study. This resistance precludes treatment with any of the β-lactam antibiotics and may indicate resistance to other classes of antibiotics (Srednik *et al.*, 2017).

Table 4. Prevalence of antimicrobial resistance genes in *Staphylococci* and *Streptococci* spp. isolated from dairy cattle manifesting clinical mastitis

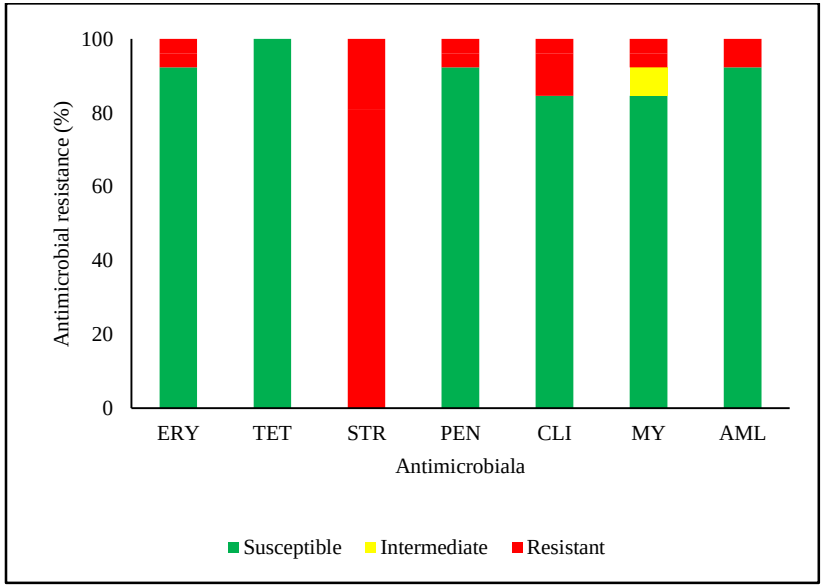
Species	No. of isolates	Aminoglycosides					β-Lactams		
		aphA-1	*aphA-2*	*aphA-3*	*aadA1/aadA2*	*aad-6*	*blaIMP*	*blaSPM-1*	*mecA*
Staphylococcus									
S. aureus	2	0	0	0	0	50	0	0	50
S. agnetis	11	0	54.5	0	0	18.2	0	90.9	0
S. arlettae	2	0	0	0	0	50	0	0	100
S. chromogenes	18	83.3	0	0	22.2	11.1	0	0	0
S. epidermidis	8	12.5	0	0	0	0	0	0	100
S. saprophyticus	2	0	0	0	0	0	0	0	50
S. simulans	5	0	0	0	0	0	0	0	0
Streptococcus									
S. agalactiae	9	0	0	0	0	0	77.8	0	0
S. bovis	2	0	0	0	0	0	0	0	0
S. equinus	3	0	0	0	0	66.7	0	0	0
S. gallolyticus	1	0	0	0	0	0	0	0	0
S. uberis	11	0	18.2	9.1	0	9.1	9.1	0	0

Table 4 continued.

Species	No. of isolates	Macrolides			Quinolones	Streptomycin		Tetracycline				Lincosamides
		ermA/TR	*ermB*	*mdfA/mefE*	*parC*	*rpsL*	*rrs*	*tetB*	*tetK*	*tetL*	*tetM*	*linB*
Staphylococcus												
S. aureus	2	0	0	0	0	0	0	0	0	0	0	100
S. agnetis	11	0	0	0	0	0	0	0	0	0	0	0
S. arlettae	2	0	0	0	0	0	0	0	0	0	0	0
S. chromogenes	18	0	0	0	0	0	5.6	0	5.6	33.3	0	27.8
S. epidermidis	8	0	0	0	0	0	0	0	25	0	0	12.5
S. saprophyticus	2	0	0	0	0	0	0	0	0	0	0	0
S. simulans	5	80	0	0	0	0	0	0	20	0	0	0
Streptococcus												
S. agalactiae	9	0	11.1	0	0	0	0	66.7	0	0	0	0
S. bovis	2	0	0	0	50	0	0	0	0	0	0	0
S. equinus	3	0	0	66.7	33.3	66.7	0	0	0	0	66.7	0
S. gallolyticus	1	0	0	0	100	0	0	0	0	0	0	0
S. uberis	11	72.7	27.3	0	0	0	0	27.3	0	0	0	9.1

On the other hand, *blaSPM-1*, a metallo-β-lactamase (MBL) gene, was also prevalent in the present work. MBLs are a class of enzymes capable of deactivating the majority of commonly used β-lactam-based antibiotics. The crystal structure in SPM-1 indicates that it could develop more potent activities through point mutation (Murphy *et al.*, 2006). In some studies, it has been shown that some MBL-encoding genes are linked to mobile genetic elements, which allow these genes to spread effectively between species (Zheng *et al*., 2015; Gudeta *et al*., 2016). As a result, the mentioned prevalent AMR genes may cause additional resistance to other antibiotic classes, the development of stronger actions, and the spread of these genes.

Moreover, 26 *Streptococci* isolates were phenotypically and genotypically characterized for their AMR. The distribution of streptococcal isolates that were susceptible, intermediate and resistant to each antibiotic is summarized in Figure 3. No resistance to tetracycline was detected in all isolates. A high sensitivity rate (85-100%) was recorded in all antibiotics except streptomycin, which had 100% resistance. Clindamycin was the second antimicrobial with the most resistant isolates, with a 16% rate.



Erythromycin: ERY, tetracycline: TET, streptomycin: STR, penicillin: PEN, clindamycin: CLI, lincomycin: MY and amoxicillin: AML

Figure 3. Antimicrobial resistance (%) of *Streptococci* species (n=26) obtained from dairy cattle displaying clinical mastitis categorized as susceptible, intermediate, or resistant based on the CLSI chart

Approximately, 7.7% of the *Streptococci* isolates were categorized as MDR. Among these, 11.1% of *S. agalactiae* isolates and 50% of *S. bovis* isolates demonstrated MDR characteristics (Table 3). Both *S. agalactiae* and *S. bovis* isolates showed resistance to almost all antibiotics tested except tetracycline. Nearly 67% of *S. equinus* was resistant to two antimicrobials, while the rest of the streptococcal strains were resistant to one antibiotic. In total, among the 26 streptococcal isolates, 12.2% exhibited a phenotype of resistance to three or more antimicrobials leading to their classification as MDR. These *Streptococci* strains from mastitis samples that were MDR were also being detected globally. An MDR serotype II strain *S. agalactiae* was recovered from a cow with mastitis in Argentina (Cadona *et al.*, 2021). *S. bovis* type II/1, currently termed *S. lutetiensis*, which was rarely associated with bovine mastitis, was also discovered MDR in 24% of the isolates in Beijing, China (Chen *et al.*, 2021).

The frequency of resistance genetic determinants for each *Streptococcus* species is also presented in Table 4. Thirty-five percent of the streptococcal isolates tested positive for the *tetB* gene making it the most prevalent gene among the isolates. With a 31% detection rate, *blaIMP* and *ermA/TR* were the second and third most frequent genes. Three antimicrobial drug class genes were found in 15.4%, two in 26.9% and one in 26.9% of *Streptococci* isolates. Only eight isolates (30.8%) did not harbor any genes for AMR. Additionally, *S. uberis* had the highest number of AMR genes present.

Although no resistance to tetracycline was observed in all *Streptococci* isolates, the tetracycline-resistance *tetB* gene was the most prevalent AMR gene detected in these strains. Meanwhile, β-Lactam resistance *blaIMP*, a metallo-β-lactamase (MBL) gene, was frequently detected in several isolates. MBLs are a group of enzymes capable of inactivating the majority of commonly used β-lactam antimicrobials. MBL-producing organisms are known to produce infections associated with high rates of mortality, morbidity and increasing medical expenses (Shanthi Amudhan *et al.*, 2012). Erythromycin-associated gene *ermA/TR* was also prevalent in *Streptococcus*. The two primary macrolide resistance phenotypes include the M phenotype, which is mediated by the *mef* genes and macrolide–lincosamide–streptogramin B (MLSB) phenotype, facilitated by the *erm* genes, which provides resistance to classes macrolides, lincosamides and Group B streptogramin (Montes *et al.*, 2006).

The phenotypic and genotypic antimicrobial resistance profiles of the *Staphylococci* isolates were observed and compared in this study. Seven *Staphylococci* isolates were found phenotypically and genotypically resistant to clindamycin and lincomycin, but a few isolates did not show consistent phenotypic and genotypic resistance. Only one exhibited the presence of *rrs*, while the majority of the samples were resistant to streptomycin. Eleven isolates were positive for *mecA* and resistant to penicillin. However, the samples that exhibited *blaSPM-1* were all susceptible to beta-lactamases.

Meanwhile, *Streptococci* isolates that were positive for macrolide and tetracycline-related AMR genes were phenotypically sensitive to erythromycin and tetracycline. While all strains were resistant to streptomycin, only two exhibited *rpsL*. Most of the isolates showed susceptibility to penicillin, amoxicillin, clindamycin and lincomycin, and as a result, tested negative for the presence of AMR genes. However, a few isolates tested positive for a resistance gene but did not exhibit phenotypic resistance to the corresponding antibiotic, and vice versa.

The findings of the present study revealed resistance of staphylococcal and streptococcal isolates to several antimicrobials and the existence of its corresponding AMR genes and its sensitivity to antibiotics and the absence of AMR gene. However, some isolates exhibited phenotypic resistance to an antibiotic but did not harbor the designated resistance genetic determinants. Resistance phenotypes can be stimulated by various genetic determinants not examined in this study; each of which may have a distinct epidemiological character that could account for the inconsistent genotype-phenotype correlation of AMR (Van *et al.*, 2020). Conversely, results revealed that some isolates harbored antimicrobial resistance genes but were not phenotypically resistant to the corresponding antimicrobial. Previous studies have corroborated these findings by establishing connections between resistance phenotypes and resistance genes in other bacterial strains. Acquisition of resistance genes may not be enough to provide corresponding antibiotic resistance (Urmi *et al.*, 2020). Some antibiotic resistance genes develop dysfunctional resistance genes (resistance pseudogenes) that do not display anticipated resistance phenotypes as a result of accumulating numerous random mutations in their gene sequences over time (Davis *et al.*, 2011). The antibiotic itself could also modify the antimicrobial resistance genes to have low *in vitro* expression (Depardieu *et al.*, 2007). Another possibility raised the prospect of other mechanisms, such as target site changes, efflux pump overexpression, or mutations (Shivakumaraswamy *et al.*, 2019).

4. Conclusion and Recommendation

The present study evaluated the virulence and AMR profiles of previously obtained *Staphylococci* and *Streptococci* isolates obtained from dairy cows with clinical mastitis from Region IV-A, Philippines. The findings revealed the *Streptococci* isolates were likely to be involved in the infection of the tested animals due to the high detection rate of the virulence genes. Although the virulence genes among the *Staphylococci* isolates were less prevalent, which suggests their lesser involvement in the infection, their pathogenicity still cannot be discounted. Meanwhile, the high prevalence of MDR among *Staphylococci* and *Streptococci* isolates is worrisome as it threatens the future management of mastitis infection in the dairy industry. Furthermore, the high detection rate of AMR genes among *Staphylococci* and *Streptococci* is alarming. This poses a significant risk of the possible transfer of these genetic elements to other organisms facilitating the further spread of antibiotic resistance that threatens both animal and human health.

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