Prevalence of *Streptococci* spp. and Unexpected Non-*Streptococci* Strains Associated with Bovine Mastitis Infection in Dairy Cattle in Region IV-A, Philippines

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Abstract

Bovine mastitis is an inflammatory response of the udder tissue in the mammary gland caused by microbial infections. Streptococcus spp. is among the most prevalent mastitis-inducing etiological agents. Thus, this study intended to isolate and evaluate the prevalence of Streptococci in dairy cattle infected with clinical mastitis in Region IV-A, Philippines. Edward Agar medium with 6% defibrinated sheep blood was employed as a selective medium. The bacterial isolates were phenotypically and genotypically characterized. Remarkably, out of 98 isolates, only 26.5% belonged to the genus Streptococcus despite the use of a Streptococci-specific medium. Five Streptococci species and 22 non-Streptococci species were identified. The most prevalent species were S. uberis (prevalence rate: 11.2%). The antimicrobial resistance profiling also revealed that S. agalactiae exhibited resistance to all antimicrobials used, while S. bovis showed hyper-resistance to five out of seven antibiotics. Surprisingly, most of the non-streptococcal isolates exhibited hyper-resistance to multiple antibiotics. For instance, Klebsiella pneumoniae isolates showed high resistance against all antimicrobials. Proteus and Providencia isolates exhibited resistance against six out of seven antibiotics. Strong hemolytic activity was also observed in Bacillus subtilis. The detection of diverse species of microorganisms causing mastitis is significant to the dairy industry as distinct pathogens may entail different risks and necessitate specific treatments, primarily in terms of the antimicrobials that will be utilized to cure the infection. Application of inappropriate antibiotics might unduly expose the udder microbial flora to antimicrobials, increasing the establishment of multidrug-resistant bacteria, which is a severe hazard to animal and human health.

Keywords*:* antimicrobial resistance, Bovine mastitis, *Streptococcus*, *Streptococcus agalactiae*

1. Introduction

Bovine mastitis is an inflammatory disease that damages the mammary glands in dairy cattle. It occurs in two different clinical manifestations: subclinical and clinical mastitis, which can be mild, moderate to severe. Reduced milk supply and quality, rejected milk, medication and veterinary expenditures, and forced culling are all regarded as important causes of economic losses in the dairy industry caused by mastitis (Azooz *et al*., 2020). *Streptococci* spp. are among the most common etiological agents that induce mastitis in dairy cattle. Main *streptococcal* species linked with clinical and subclinical mastitis include *S. agalactiae, S. canis, S. dysgalactiae* and *S. uberis* (Lundberg *et al*., 2014; Richards *et al*., 2014). Other minor mastitis-causing pathogens have also been identified, particularly *S. gallolyticus* and *S. parauberis* (Park *et al.*, 2013; Dumke *et al*., 2015). *Streptococcus* pathogenicity is determined by its capacity to produce several virulence factors. Neuraminidase, pyrogenic exotoxin, M protein, lipoteichoic acid, capsular polysaccharide antigen, Christie-Atkins-Munch-Peterson (CAMP) factor and hemolysin are among the virulence factors which have an important role in *streptococcal* pathogenicity, anti-phagocytic activity, and high adsorption on surfaces and cells (González-Outeiriño *et al*., 2005; Tian *et al*., 2019).

Mastitis-causing *Streptococci* pathogens can be split into those that transmit through a contagious route such as *S. agalactiae,* and those that infect the udder often from an environmental reservoir such as *S. uberis* (Abebe *et al*., 2016). The environment of the dairy cow is the principal reservoir of environmental pathogens and uninfected quarters can be exposed to these microorganisms at any moment during the life of a cow. The cow, on the other hand, is the principal reservoir for contagious mastitis, and uninfected mammary quarters are only exposed to contagious bacteria during the milking process (Abebe *et al*., 2016).

S. agalactiae, commonly known as Group B *Streptococcus* (GBS), is a highly contagious pathogen that is regarded as one of the most important causal factors of bovine mastitis (Shang *et al*., 2020). Unlike *Staphylococcus aureus*, *S. agalactiae* only grows and reproduces in the udder. It may, however, persist for brief periods on hands, milking machine parts and teat skin, which allows it to transfer from cow to cow during milking. The disease can spread rapidly due to the silent nature of infection from *S. agalactiae* and the fact that they are very contagious (Lakew *et al*., 2019). On the other hand, *S. uberis* is also responsible for a significant number of clinical and subclinical intramammary infections (Kromker *et al*., 2014). The disease induced by *S. uberis* can be

transient, recurring, or chronic (Samson *et al*., 2016). *S. uberis* can be located in many areas in the environment of dairy cattle, including bedding materials, milking equipment, and other objects in the quarters of the animal (Käppeli *et al*., 2019). However, *S. uberis* has also been linked to cow-to-cow transmission indicating that it is a contagious pathogen (Zadoks *et al*., 2003; Pullinger *et al*., 2007; Leelahapongsathon *et al*., 2020); thus, demonstrating the complexities of the bacterium, environment and dairy cow relationship.

Although effective milking hygiene techniques reduce the prevalence of *streptococcal* bovine mastitis, complete eradication is unachievable (Zigo *et al*., 2019). As a result, antibiotics remain the primary prevention and treatment agent for bovine mastitis (Garcia *et al*., 2019). However, bacteria have become increasingly resistant as a consequence of long-term, widespread and inappropriate antimicrobial usage (Negri *et al*., 2014). Antimicrobial resistance (AMR) in mastitis pathogens became a crucial concern in the dairy industry as it hampers mastitis infection treatment. If resistant bacteria are present in contaminated food or food products, transfer of resistance genes to the gut microbiota in humans is possible (Verraes *et al*., 2013). Moreover, antimicrobial residues in milk pose a serious concern in public health because they can trigger allergic responses in individuals who are allergic to antimicrobials (Rahman *et al*., 2021).

The prevalence of mastitis in the Philippines could hamper the efforts of the government to increase local milk production. Identification of the mastitis-causing agents and their characteristics, such as AMR, is crucial for the dairy industry in the country as this will serve as baseline information in future applications. Thus, the aim of the present work was to isolate and identify *Streptococci* spp. from dairy cattle infected with mastitis in Region IV-A, Philippines.

2. Methodology

2.1 Ethical Approval and Informed Consent

Only voluntarily participating farm owners took part in the study, and swab and milk samples were collected. All the practices used in this study were approved by the University of the Philippines Los Baños Animal Care and Use Committee (Assignment Protocol No. BIOTECH-2021-003). The Bureau of Animal Industry, Department of Agriculture (Ref No. AR-2021-021)

authorized all the practices used in this study that involved using animals as test subjects.

2.2 Animal Subject

Fifteen dairy cattle exhibiting symptoms of clinical mastitis infection were involved in the present study. From June to November 2021, the samples from dairy cows were collected from several farms in Region IVA-CALABARZON, Philippines.

2.3 Sample Collection

The sample collection was done following the previously reported method (Perez and Ancuelo, 2022). Briefly, teat swabbing and milk sampling were both used to acquire samples. Before aseptically swabbing the infected quarters with a sterile cotton swab that had already been dipped in peptone water saline, the infected quarters were cleaned with water to remove any adhering dirt or debris. The cotton swabs were then placed in peptone water saline and then sealed within the sample collecting tube. Fifteen to 20 mL of milk from the same contaminated quarters were aseptically obtained by hand-stripping and kept in a collection tube. Following collection, samples were put in an icebox and brought right away to the Molecular Genetics Laboratory, BIOTECH-UPLB (Philippines) for further processing and microbiological testing.

2.4 Targeted Isolation of Streptococci Strains

To concentrate the bacterial cells in the sediment, an aliquot of the freshly obtained milk and swab samples was centrifuged at 13,500 rpm for 15 min. The sediments were then retrieved and inoculated into fresh 2.5-mL trypticase soy broth (Titan Biotech Ltd., India) with 0.6% yeast extract (Titan Biotech Ltd., India) (TSBYE) and incubated at 37 °C for 24-48 h. After that, they were serially diluted and plated in Edwards Agar medium (peptone – 10 g/L; beef extract – 10 g/L; esculin – 1 g/L; NaCl – 5 g/L; crystal violet – 0.0013 g/L; thallous sulfate – 0.33 g/L; agar – 15 g/L) supplemented with 6% defibrinated sheep blood.

For 24-48 h, the plated samples were incubated at 37 °C. The colony shape and other characteristics of bacterial colonies from the incubated plates were observed. Then, isolated colonies that had a black appearance (esculin-positive group D *Streptococci*) and a blue to colorless appearance (esculin-

negative *S. agalactiae*) were selected, inoculated in TSBYE and incubated at 37 °C for 24-48 h. Each bacterial isolate was streak plated in trypticase soy agar with 0.6% yeast extract (TSAYE) and a single colony was picked to ensure the purity of each isolate. The purity of the isolates was validated by microscopic analysis, and pure isolates were stored in 30% glycerol and preserved at -80 °C.

2.5 Phenotypic Characterization of Isolates

All the data from the samples were taken from 2021 to 2022. The colony and cellular shape, Gram reaction, catalase activity and hemolysis of the presumed *Streptococci* strains were observed. The bacterial culture was streaked into a nutrient agar enriched with 6% defibrinated sheep blood to evaluate the hemolytic activity.

2.6 Genotypic Characterization of Isolates

Using the GF-1 Bacterial Deoxyribonucleic Acid (DNA) Extraction Kit (Vivantis Technologies, Malaysia), the genomic DNA of the isolates was extracted by following the instructions of the manufacturer. NanoDrop spectrophotometer (Thermo Fisher Scientific, United States) was used to quantify the genomic DNA concentration, which was then standardized to 10 ng/L using sterile, ultrapure water and kept at -20 °C.

Amplifications using polymerase chain reaction (PCR) were carried out in a 40 µL reaction solution using MyTaq™Mix (Meridian Life Science Inc., USA), with 0.5 ng of DNA and 0.2 µM of each primer. The polymerase chain reaction was performed using MultiGene Gradient Thermal Cycler (Labnet International, United States). The amplification processes were set with the following conditions: 95 °C for 1 min for an initial cycle, 30 cycles of denaturation at 95 °C for 15 s, annealing at 53 °C for 15 s, elongation at 72 °C for 15 s and final extension step at 72 °C for 2 min. Electrophoresis in 1.5% (w/v) agarose gels in 0.5x Tris-acetate EDTA (TAE) buffer with ViSafe Red Gel Stain (Vivantis Technologies, Malaysia) was used to separate the amplified products. In this investigation, the primers 27F AGAGTTTGATCMTGGCTCAG and U1492 R GGTTACCTTGTTACGAC TT were utilized.

The sequencing of all PCR products was done at Macrogen, Inc. (South Korea). The Basic Local Alignment Search Tool (BLAST) method (Altschul

et al., 1990), which can be accessed on the website of the National Center for Biotechnology Information (n.d.), was used to compare the data from the produced DNA sequences to data from the GenBank database.

2.7 Antimicrobial Susceptibility Profiling

The Kirby-Bauer disc diffusion technique (Bauer *et al*., 1996) was used to determine the sensitivity of the strains to antibiotics. All isolates were tested using seven different antibiotic discs (Oxoid, United Kingdom) to evaluate their susceptibility and/or resistance profile. The antibiotic discs that were utilized were penicillin (10 μg); amoxicillin (25 μg); tetracycline (10μg); erythromycin (15μg); lincomycin (10 μg); clindamycin (2 μg); and streptomycin (10 μg). The width of the inhibition zone surrounding each antibiotic disc was used to assess the antimicrobial susceptibility profile of the isolates, which was then interpreted using the manufacturer-supplied 2021 Clinical and Laboratory Standards Institute chart. The antimicrobial sensitivity of the isolates was classified as susceptible, intermediate and resistant.

3. Results and Discussion

3.1 Prevalence of Streptococci Strains and their Phenotypic Characteristics

Ninety-eight bacterial isolates were isolated from mastitic dairy cattle, 54.1% of which were from swabs, and 45.9% were from milk. *Streptococci* isolates were 19.2% from swab and 80.8% from milk samples. Non-*Streptococci*, on the other hand, were 66.7% swab and 33.3% milk (Table 1).

Table 1. Distribution (%) of *Streptococci* and non-*Streptococci* species in swab and milk samples

Bacterial species	Swab (%)	Milk (%)
Streptococci species (*n* = 26)	19.2	80.8
Non-*Streptococci* species (*n* = 72)	66.7	33.3

Colonies that appear black and grayish to colorless were picked (Figure 1a-1f). According to the microscopic analysis, most of the isolates were cocci in shape which appears in singles, pairs, clusters, and/or chains. The majority of

pathogens isolated were Gram and catalase-negative. Testing for hemolysis found that 84.7% of the isolates had hemolytic activity.

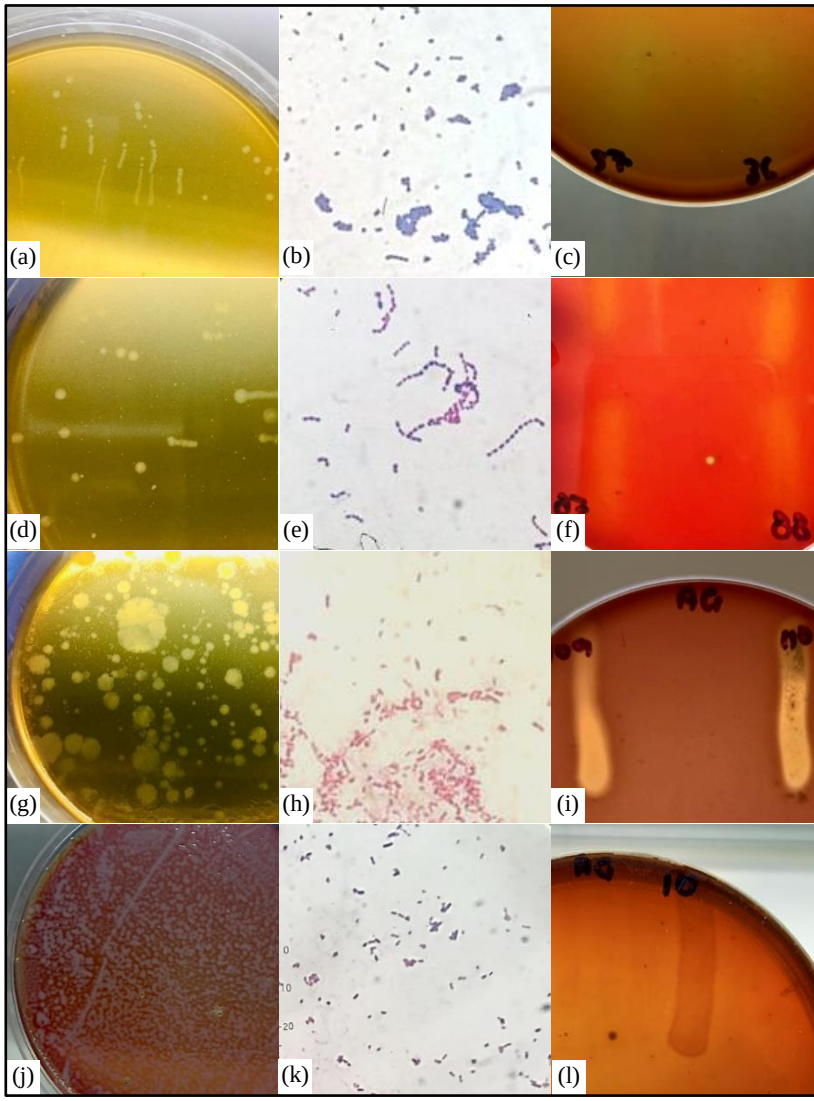

Figure 1. Colony morphology of a representative bacterial isolate from mastitic milk; *S. agalactiae* (a), *S. uberis* (d), *B. subtilis* (g) and *L. lactis* (j) isolates grown on Edward Agar Medium supplemented with 6% defibrinated sheep blood, its Gram-reaction and cellular morphology (b, e, h and k, respectively) and hemolytic activity (c, f, and i and l, respectively)

Multiplex PCR was initially used to perform a PCR-based preliminary identification of the isolates, as described by Shome *et al*. (2011). The primers used were *StrepAga* F-GCTAATACCGCATAAGAGTAATTAAC and R-GGTAGATTTTCCACTCCTACCAA, *StrepDysga* F-GGGAGTGGAAAA TCCACCAT and R-AAGGGAAAGCCTATCTCTAGACC, and *StrepUber* F-TCGCGGTATTGAAAAAGCAACAT and R-TGCAATAATGAGAAGG GGACGAC for the identification of *S. agalactiae*, *S. dysgalactiae and S. uberis*, respectively. This methodology has previously been found to have high accuracy and throughput when it comes to detecting bovine mastitis-related pathogens such as *Streptococci* strains. Unfortunately, despite several PCR optimization trials, the specificity of the said protocol for the identification of bacterial strains was not as accurate as they have stated. Multiple positive PCR amplicons in presumably species-specific PCR primers were found in a number of isolates (Figure 2).

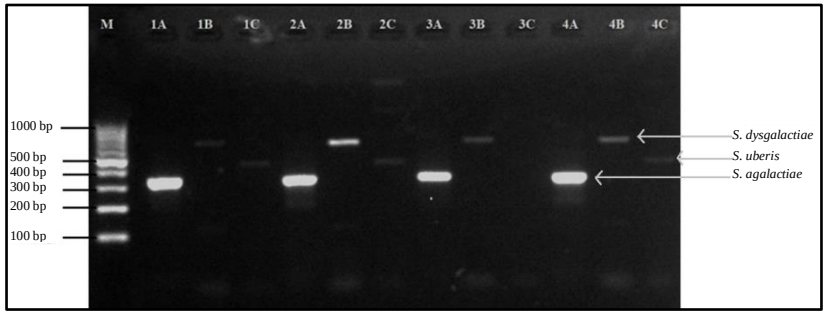

Figure 2. Uniplex PCR reactions using genomic DNA of isolate 1-4 using A*: StrepAga* (317 bp), B: *StrepDysga* (572 bp), and C: *StrepUber* (400 bp) primers for the identification of *S. agalactiae*, *S. dysgalactiae* and *S. uberis*, respectively

Despite the use of a *Streptococci*-specific selective medium, the overall isolation rate of *Streptococci* species was only 26.5%. The prevalence of esculin-negative *S. agalactiae* was 9.18% and esculin-positive *S. bovis*, *S. equinus*, *S. gallolyticus* and *S. uberis* were 2.04, 3.06, 1.02 and 11.2%, respectively (Table 2). Among the *streptococcal* bacterial isolates, *S. uberis* was the most prevalent pathogen.

The 9.18% prevalence of *S. agalactiae* in the present study was somehow comparable to that of Lakew *et al.* (2019) which isolated 10.3% from milk samples. However, a higher occurrence of 67% *S. agalactiae* was reported by Mesquita *et al.* (2019), who also used a modified Edwards Agar medium enriched with 5% defibrinated sheep blood. *S. agalactiae* is an extremely contagious obligate parasite of the cow mammary gland that usually creates a

low-grade, long-lasting infection with a low self-cure rate. It is linked to an increase in somatic cell count and total bacteria count, as well as a reduction in the amount and quality of milk produced (Keefe, 2012).

Table 2. Prevalence (%) of *Streptococci* and non-streptococci isolated from mastitis-infected dairy cattle

Classification		Prevalence (%)
Streptococci		
S. agalactiae		9.18
S. bovis		2.04
S. equinus		3.06
S. gallolyticus		1.02
S. uberis		11.2
Non-*Streptococci* species		
Family	Species	
Aeromonadaceae	*Aeromonas aquariorum*	1.02
Bacillaceae	*Bacillus subtilis*	3.06
Enterobacteriaceae	*Escherichia cloacae*	1.02
	Escherichia coli	7.14
	Klebsiella pneumoniae	10.2
	Proteus mirabilis	10.2
	Proteus penneri	1.02
	Providencia alcalifaciens	2.04
	Providencia rettgeri	1.02
	Providencia stuartii	4.08
Enterococcaceae	*Enterococcus casseliflavus*	1.02
	Enterococcus durans	3.06
	Enterococcus faecalis	7.14
	Enterococcus faecium	4.08
	Enterococcus hirae	6.12
Morganellaceae	*Morganella morganii*	2.04
Pseudomonadaceae	*Pseudomonas mendocina*	1.02
Staphylococcaceae	*Staphylococcus haemolyticus*	1.02
	Staphylococcus saprophyticus	1.02
Streptococcaceae	*Lactococcus formosensis*	1.02
	Lactococcus garvieae	3.06
	Lactococcus lactis	2.04

Esculin-positive *Streptococci*, on the other hand, was more predominant than *S. agalactiae*. The majority of these were isolated from milk samples (Table 3). *S. bovis* and *S. uberis* were the only esculin-positive *Streptococcus* source of swab isolates. All *S. equinus* and *S. gallolyticus* samples were from milk, and 90.9% of *S. uberis* was from milk as well. Among the said species, *S. uberis* was the most prevalent pathogen. It has also been reported as the most dominant causative agent of mastitis in dairy cattle in many countries. In New Zealand, *S. uberis* with an isolation percentage of 23.3% was one of the most common isolates (Petrovski *et al.*, 2009). Additionally, it accounted for 18.2% of cases and was the most commonly reported bacteria in Flemish dairy herds (Verbeke *et al*., 2014). *S. uberis* is considered an environmental pathogen, which is defined as opportunistic mammary gland pathogen that might be transmitted from a contaminated environment to the cow mammary gland during the milking process (El-Aziz *et al*., 2021). Studies also described *S. uberis* as a pathogen that can resist phagocytosis (Cremonesi *et al*., 2022) and intracellular killing by leukocytes (Leigh *et al*., 1990).

Table 3. Distribution (%) of *Streptococci* species in swab and milk samples

Bacterial species	Swab (%)	Milk (%)
S. agalactiae	33.3	66.7
S. bovis	50	50
S. equinus	0	100
S. gallolyticus	0	100
S. uberis	9.09	90.9

3.2 Unintended Isolation of Non-Streptococci Bacterial Pathogens

Remarkably, swab and milk samples from mastitic dairy cattle yielded a number of non-*Streptococci* strains (Table 2). The colony and cellular morphology, as well as the hemolytic activity of a representative isolate, were presented in Figure 1g-1l. Crystal violet and thallium salts are the most commonly used selective agents to isolate *Streptococci*. However, regardless of the presence of these selective agents in the *Streptococcus*-specific growth medium, non-*Streptococci* strains were still identified at a high rate indicating their dominance in the samples. All of the grayish-to-black colonies picked from the medium that was supposedly esculin-positive group D *Streptococci* were discovered to be non-*Streptococci* species. It is also worth noting that the animals from whom all of the samples were taken had severe mastitis infections. Therefore, it is very likely that these non-*streptococcal* isolates were the prevailing mastitis strains among the animal subjects and were suspected to be the cause of the severity of the infections.

In total, 22 non-*streptococcal* species were detected in this study. Among these, the isolates with the highest prevalence of 10.2% were *Klebsiella pneumoniae* and *Proteus mirabilis*. Both belong to the *Enterobacteriaceae* family which accounts for 36.7% of the total mastitis bacterial isolates. *K. pneumoniae* is a mastitis-causing opportunistic and environmental pathogen with the propensity for contagious transmission. It is often isolated from different environmental sources on dairy farms such as bedding, alleyways and holding pens (Verbist *et al*., 2011; Zadoks *et al*., 2011). *Klebsiella* mastitis is frequently severe and does not react well to antimicrobial therapy resulting in a sustained intramammary infection and significant milk output loss (Schukken *et al*., 2012).

P. mirabilis was one of the most prevalent non-*streptococcal* species in this study as well. Although less common in other mastitis research, *P. mirabilis* was shown to be present in several bovine and caprine mastitis cases. It was detected in dairy cows with subclinical mastitis (Jas, 2017; Younis *et al*., 2017). It was also detected in the milk of infected lactating sheep and goats (Housawi *et al*., 2008). *P. mirabilis* often invades the mammary gland as opportunistic microorganisms when defense systems are weak or when they are mistakenly transported into the gland (Phiri *et al*., 2010). It has also been associated with gangrenous mastitis – a severe clinical condition in the mammary glands of the dairy animal. This occurs seldom, but once it does, afflicted cows have a high mortality rate. When medicinal treatment fails, veterinarians resort to partial mastectomy or drying off the affected quarters and allowing them to slough off (Phiri *et al*., 2010).

Bacillus subtilis has been found to be present in bovine and caprine mastitis (Reem and Basit, 2011; Pirzada *et al.*, 2016). In the current study, *B. subtilis* was 3.06% prevalent among the isolates. It also exhibited strong beta-hemolytic activity, wherein the red blood cells in the media around and under the colonies were completely lysed (Figure 1i). In many bacteria, hemolytic activity is a virulence factor and has been linked to pathogenicity (Pan *et al.*, 2014). *B. subtilis* strains were found to be β-hemolytic and γ-hemolytic. Hemolysis indicates the presence of cytotoxic phospholipases in bacteria, and the hemolytic factor reduces the amount of hemoglobin available to the host as an iron source (Dabiré *et al*., 2022). Several biologically active lipopeptide produced by *B. subtilis*, such as surfactins, fengycins, iturins, lichenysin, were also shown to cause hemolysis (Dey *et al.*, 2015; Coronel *et al.*, 2016; Zakharova et al., 2019; Fei *et al.*, 2020).

3.3 Antimicrobial Susceptibility Profile of Streptococcal Isolates

Distinct resistance patterns against the seven antibiotics were exhibited by different *Streptococci* species (Table 4). The majority of *S. agalactiae* showed 33.3-88.9% susceptibility to all antimicrobials, but a few portions exhibited resistance. About 70% of *S. agalactiae* isolates were resistant to streptomycin and 22% against tetracycline. This is in agreement with Effendi *et al.* (2018) who detected nine out of 36 *S. agalactiae* samples (25%) resistant to tetracycline from milk with subclinical mastitis. However, Ariffin *et al.* (2019) revealed 100% sensitivity of *S. agalactiae* against tetracycline. It also reported 66.7% susceptibility to penicillin, whereas, in the current study, it showed 88.9% sensitivity against the said antimicrobial. The highest resistance of *S. agalactiae* (85.1%) was also found for streptomycin as observed by Jain *et al*. (2012).

Susceptibility to tetracycline and resistance to hyper-resistance against the remaining antibiotics tested were demonstrated by *S. bovis*. *S. equinus* was also susceptible to all antimicrobials, except streptomycin which exhibited 100% hyper-resistance. The same is true with *S. gallolyticus* and *S. uberis*, which exhibited absolute susceptibility to all antibiotics, except streptomycin wherein 100% of the former was resistant and 100% of the latter was hyper-resistant. Following the antibiotic resistance trend in the *Streptococci* isolates, it is evident that resistance was extremely frequent in streptomycin – an aminoglycoside antimicrobial. Clinical isolate resistance to aminoglycoside antibiotics varies depending on the drug, the microorganism, the resistance mechanism, the geographic location and a variety of other factors (Vakulenko and Mobashery, 2003). In general, resistance to aminoglycosides is linked to antibiotic enzymatic modification. Three kinds of enzymes, bifunctional AAC(6')-I-APH(2"), ANT(4')-I, and APH(3')-III, are particularly essential because of their ability to create resistance to therapeutically significant aminoglycosides. In *Mycobacterium tuberculosis*, the primary mechanism of aminoglycoside resistance is through mutational modification of the ribosomal target. The epidemiology of resistance to aminoglycoside in gram-positive bacteria such as *Staphylococci* and *Streptococci* has also been researched but to a lesser extent (Vakulenko and Mobashery, 2003). Moreover, because of their synergistic impact, aminoglycosides are typically used in conjunction with a β-lactam antibiotic, although high resistance levels to aminoglycosides in target bacteria typically lead to the lack of synergism resulting in therapeutic failure (Chow, 2000).

Table 4. Percentage of *Streptococci* strains obtained from dairy cattle with clinical mastitis that were susceptible (S), intermediate (I), resistant (R) to different antimicrobials

Antimicrobial[a]	Antimicrobial resistance pattern														
	S. agalactiae *n* = 9			*S. bovis* *n* = 2			*S. equinus* *n* = 3			*S. gallolyticus* *n* = 1			*S. uberis* *n* = 11		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
ERY	88.9	0	1.11	50	0	50*	100	0	0	100	0	0	100	0	0
TET	77.8	0	22.2	100	0	0	100	0	0	100	0	0	100	0	0
STR	33.3	0	66.7	0	0	100 (50*)	0	0	100*	0	0	100*	0	0	100
PEN	88.9	0	1.11	50	0	50*	100	0	0	100	0	0	100	0	0
CLI	88.9	0	1.11	50	0	50*	33.3	0	66.7	100	0	0	100	0	0
MY	88.9	0	1.11	50	0	50*	33.3	66.7	0	100	0	0	100	0	0
AML	88.9	0	1.11	50	0	50	100	0	0	100	0	0	100	0	0

[a] ERY – erythromycin; TET – tetracycline; STR – streptomycin; PEN – penicillin; CLI – clindamycin; MY – lincomycin; AML – amoxicillin
* Isolates did not show any inhibition zone around the antibiotic disc referred here as hyper resistant.

3.4 Non-streptococcal Isolates Exhibited Strong Antibiotic Resistance

Sensitivity profiles of non-*streptococcal* bacterial isolates against different antimicrobials tested vary from species to species (Table 5).

Table 5. Percentage of non-*Streptococci* strains obtained from dairy cattle with clinical mastitis that were susceptible (S), intermediate (I) and resistant (R) to different antimicrobials

Antimicrobial[a]		ERY	TET	STR	PEN	CLI	MY	AML
A. aquariorum *n* = 1	S	0	0	0	0	0	0	0
	I	0	0	0	0	0	0	0
	R	100*	100	100*	100*	100*	100*	100*
B. subtilis *n* = 3	S	33.3	66.7	0	0	66.7	0	100
	I	66.7	33.3	66.7	0	33.3	66.7	0
	R	0	0	33.3*	100	0	33.3	0
E. cloaceae *n* = 1	S	0	0	0	0	0	0	0
	I	0	0	0	0	0	0	0
	R	100*	100	100	100*	100*	100*	100
E. coli *n* = 7	S	0	85.7	0	0	0	0	57.1
	I	0	0	0	0	0	0	0
	R	100*	14.3	100 (14.3*)	100 (42.9*)	100 (71.4*)	100 (71.4*)	42.9 (14.3*)
K. pneumoniae *n* = 10	S	0	70	20	0	0	0	10
	I	0	0	0	0	0	0	0
	R	100*	30	80 (30*)	100 (90*)	100*	100*	90 (80*)
P. mirabilis *n* = 10	S	0	0	0	0	0	0	100
	I	0	0	0	0	0	0	0
	R	100*	100 (70*)	100 (50*)	100	100*	100*	0
P. penneri *n* = 1	S	0	0	0	0	0	0	100
	I	0	0	0	0	0	0	0
	R	100*	100*	100	100	100*	100*	0
P. alcalifaciens *n* = 2	S	0	0	0	0	0	0	100
	I	0	0	0	0	0	0	0
	R	100*	100*	100	100	100*	100*	0
P. rettgeri *n* = 1	S	0	0	100	0	0	0	100
	I	0	0	0	0	0	0	0
	R	100*	100*	0	100	100*	100*	0

Table 5 continued.

	S	0	0	0	0	0	0	75
P. stuartii *n* = 4	I	0	0	0	0	0	0	0
	R	100*	100	100	100	100*	100*	25*
	S	100	0	0	0	0	0	100
E. casseliflavus *n* = 1	I	0	0	0	0	0	0	0
	R	0	100	100	100	100	100	0
	S	33.3	100	66.7	100	0	0	100
E. durans *n* = 3	I	0	0	0	0	0	0	0
	R	66.7*	0	33.3*	0	100*	100 (66.7*)	0
	S	0	100	0	0	0	0	100
E. faecalis *n* = 7	I	100	0	0	0	0	0	0
	R	0	0	100*	100	100*	100*	0
	S	25	50	25	0	0	0	75
E. faecium *n* = 4	I	25	0	0	0	0	0	
	R	50*	50 (25*)	75 (50*)	100 (25*)	100 (50*)	100 (50*)	25*
	S	66.7	83.3	0	66.7	0	0	83.3
E. hirae *n* = 6	I	0	0	0	0	0	0	0
	R	33.3*	16.7	100 (83.3*)	33.3 (16.7*)	100*	100 (33.3*)	16.7
	S	0	0	0	0	0	0	0
M. morganii *n* = 1	I	0	0	0	0	0	0	0
	R	100*	100	100*	100*	100*	100*	100*
	S	0	100	0	0	0	0	0
P. mendocina *n* = 1	I	100	0	0	0	0	0	0
	R	0	0	100*	100*	100*	100*	100
	S	100	100	100	0	100	100	100
S. haemolyticus *n* = 1	I	0	0	0	0	0	0	0
	R	0	0	0	100	0	0	0
	S	100	100	100	0	100	100	100
S. saprophyticus *n* = 1	I	0	0	0	0	0	0	0
	R	0	0	0	100	0	0	0
	S	0	0	0	0	0	0	100
L. formosensis *n* = 1	I	0	0	0	0	0	0	0
	R	100*	100	100*	100	100*	100*	0
	S	0	0	0	100	0	0	100
L. garvieae *n* = 3	I	0	0	0	0	0	0	0

Table 5 continued.

	R	100*	100 (33.3*)	100*	0	100*	100*	0
	S	50	50	0	50	50	50	100
L. lactis $n = 2$	I	0	0	0	0	0	0	0
	R	50*	50*	100 (50*)	50	50*	50*	0

[a] ERY – erythromycin; TET – tetracycline; STR – streptomycin; PEN – penicillin; CLI – clindamycin; MY – lincomycin; AML – amoxicillin

* Isolates did not show any inhibition zone around the antibiotic disc referred here as hyper resistant.

In the present work, *K. pneumoniae* was observed to be resistant to highly resistant to all antimicrobials. Multidrug resistance of *K. pneumoniae* was also reported in several studies. Yang *et al.* (2021) detected 21.2, 13.6 and 12.1% resistance to tetracycline, chloramphenicol and aminoglycosides, respectively. It also reported resistance to ampicillin and amoxicillin in all isolates. Coliform bacteria such as *E. coli* and *K. pneumoniae* also showed resistance to the majority of the antimicrobials used by Haftu *et al*. (2012). In many bacteria, multidrug efflux pumps are responsible for multidrug resistance. Resistance to β-lactams has been attributed to extended-spectrum β-lactamase (ESBL) in clinical isolates of *K. pneumoniae* (Pagani *et al*., 2000; Sękowska *et al*., 2002). In addition, strains of *K. pneumoniae* that generate ESBL have been found to be resistant to a variety of antibiotics (Sękowska *et al*., 2002; Li and Nikaido, 2004; Carvalho *et al*., 2021). Despite the fact that fluoroquinolones target DNA gyrase and topoisomerase IV, fluoroquinolone-resistant *K. pneumoniae* strains without mutations in these genes have been described in several studies (Chen *et al*., 2003; Fendukly *et al*., 2003). These findings imply that multidrug efflux pumps are responsible for clinical *K. pneumoniae* isolates resistance to multiple antimicrobial drugs (Ogawa *et al*., 2005).

Hyper-resistance against a number of antimicrobials was observed in *Proteus* isolates as well. This was in agreement with the findings of Olivares-Pérez *et al*. (2015), wherein *P. mirabilis* and *P. vulgaris* from subclinical mastitic milk showed no susceptibility to the tested active agents. Hawari and Al-Dabbas (2008), on the other hand, reported 75, 50 and 0% resistance of *Proteus* spp. isolated from milking cows against tetracycline, erythromycin and penicillin G, respectively. All *Proteus* spp. bacterial isolates from milk were multidrug-resistant to ciprofloxacin and ampicillin (Reta *et al.*, 2016).

The fact that these antibiotic-resistant microorganisms are members of a bacterial family that is often present in the human gut microbiota may be a real cause for concern. The *Enterobacteriaceae* family, for example, where

Proteus spp., *Providencia* spp. *Escherichia* spp. and *K. pneumoniae* detected in this study belong, is one of the most frequent bacterial groups identified in the human gastrointestinal tract. Regardless of the fact that these strains are naturally present in the human gut microflora, they are still classified as opportunistic pathogens. The existence of these strains in milk represents a substantial threat to the probability of the transmission of multi-drug resistance genetic elements to actual strains in the human intestinal microbiota through milk and milk products.

4. Conclusion and Recommendation

This study reports the prevalence of different *Streptococci* and non-*Streptococci* species isolated from dairy cattle infected with clinical mastitis in Region IV-A, Philippines. Despite the use of a *Streptococcus*-specific medium, only 26.5% of the isolates were identified *Streptococci*, and 73.5% were non-*Streptococci*. Five species of *Streptococci* were identified as *S. agalactiae, S. bovis, S. equinis, S. gallolyticus* and *S. uberis*, while 22 non-*Streptococci* species were detected. *S. uberis* was the most prevalent strain among all of the isolates. Sensitivity profiling of the isolates to different antibiotics revealed multiple antimicrobial resistance. *K. pneumoniae*, *Proteus* spp., *Providencia* spp. and other non-*Streptococci* strains exhibited resistance to multiple antimicrobials tested. To the best of the authors' knowledge, this is the first report on the multi-drug hyper-resistant strains isolated from bovine mastitis cases in the Philippines. The result of this study is a preliminary step toward further research into the relationship between certain symptoms and the causal factor.

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