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Original Article

Identification of *Streptococcus* spp. isolated from bovine milk and characterization of their antimicrobial susceptibility profiles in Taiwan

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Abstract

Bovine mastitis has had a great impact on the economic losses of the dairy industry. Nowadays, environmental pathogens cause the main types of bovine mastitis, especially the streptococci. The aim of the study was to identify the major *Streptococcus* species causing bovine mastitis in Taiwan as well as to reveal their antimicrobial susceptibility profiles. By colony morphology identifying, Gram staining, and subculturing on Edwards agar, the preliminary identified streptococci were subjected to biochemical identification, latex agglutination tests and dick diffusion tests for susceptibility tests of 16 antimicrobial agents. Among a total of 157 strains, *Streptococcus uberis* was the most dominant species found, followed by *S. bovis*, *S. dysgalactiae*, *S. agalactiae*, *S. equinus* and *S. mitis*. Most of them were sensitive to all the β-lactams we selected in the study, including amoxicillin, cefuroxime, cefazolin, bacitracin, ceftiofur, cloxacillin, penicillin and ampicicllin. However, high resistant rates were observed to aminoglycosides, tetracyclines and lincomycin. *S. uberis* was the main environmental mastitis causative pathogens in the present study. Beta-lactams were the effective antimicrobials agents against them.

Keywords: Antimicrobial susceptibility, Dairy cow, Mastitis, *Streptococcus*, Taiwan

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Introduction

Mastitis causes damage to mammary glands and results in a reduced yield and poor quality of milk. Moreover, bovine mastitis incurs costs to treat or even cull cattle and may result in great economic loss to the dairy industry (Shaheen et al., 2016). The microbial causative agents of mastitis are mainly divided into contagious and environmental pathogens based on the primary reservoir and mode of transmission. On the past decades, a five-point plan has been implemented to prevent and control *Staphylococcus aureus* and *Streptococcus agalactiae* (Bradley, 2002; Ruegg, 2012). Thus, environmental microorganisms, particularly coliforms and streptococci, are now the major global causes of bovine mastitis (Bradley, 2002; Makovec and Ruegg, 2003; Hogan and Smith, 2012). In most cases, infections caused by environmental streptococci lead to minor to moderate mastitis manifested as local inflammations in the mammary glands, with or without swollenness and abnormal milk production (Roberson, 2012). However, streptococci may resist the phagocytosis by leukocytes and develop chronic infections in the mammary glands (Bradley, 2002; Denis et al., 2006). Moreover, quarters that neighbour those infected with *Streptococcus* spp. or previously recovered from streptococcal infection are highly likely to contract new infections (Guélat-Brechbuehl et al., 2010). Cows suffering from mastitis are often treated with antibiotics; however, the presence of antimicrobial-resistant bacteria is one of the reasons for treatment failure. Until now, there have been no studies on bovine streptococcal mastitis in Taiwan. Therefore, the objective of the present study was to identify the causative agents of mastitis and reveal the antimicrobial susceptibility patterns of streptococcal mastitis in Taiwan.

Materials and Methods

***Collection, culture, and presumptive identification*:** There were a total of 4,810 raw milk samples from 53 dairy farms in Taiwan collected for mastitis diagnosis from August 2012 to July 2013. The bovine raw milk samples were collected by National Mastitis Council recommended procedures. All milk samples were collected from cows with clinical mastitis together with samples without clinical signs. Milk samples were collected promptly into sterile tubes, and then the samples were stored on ice and immediately transported to our laboratory for analysis within 6 hours of sampling.

From each milk sample about 10 µl was streaked onto tryptone soy agar with 5% sheep blood agar (Blood agar plate, Creactive Life Science, Taiwan) and incubated aerobically at 35–37°C for 18–24 hours. After incubation, bacteria were recognized as streptococci based on colony morphology and Gram staining. Then we randomly selected streptococci and subcultured then on Edwards agar (Oxoid™, England) with 5% washed cattle erythrocytes, which is a streptococcal selective medium containing esculin, at 35–37°C for 18–24 hours (Sawant et al., 2002). The isolates were deposited at the GermBank (Creactive Life Science, Taiwan) and stored at -70°C for further analysis.

***Biochemical identification and serological grouping*:** The streptococci were identified using the API® 20 Strep system (bioMérieux, France) following the manufacturer's instructions. The obtained API code were interpreted by the online apiweb™ software (bioMérieux, France). Conversely, the streptococci showing β-hemolysis on primary culture were subjected to the Christie Atkins Munch-Peterson (CAMP) test and the results recorded (Facklam, 2002; Guélat-Brechbuehl et al., 2010). The isolates were subjected to Streptococcal Grouping Kit (Oxoid™, England) for serological grouping, which was used to identify specific carbohydrate antigens present in the cell wall of the majority of streptococci. The procedures followed the manufacturer's instructions. The bacteria were emulsified with the Oxoid Streptococcus Extraction Enzyme and incubated for 10 min at 37°C in a water bath. The six latex reagents were added separately and mixed with the bacteria–enzyme suspension. Visible agglutination observed after mixing was considered to be a positive result.

***Antimicrobial susceptibility testing*:** Antimicrobial susceptibility was assessed using an agar disk diffusion test as per the guidelines of the Clinical and Laboratory Standards Institute (2015). The chosen antimicrobial agents included β-lactam antibiotics (penicillin, cloxacillin, ampicillin, amoxicillin, cefazolin, cefuroxime and ceftiofur), tetracyclines (tetracycline and oxytetracycline), aminoglycosides (amikacin, kanamycin, neomycin and streptomycin), a polypeptide (bacitracin), chloramphenicol and lincomycin. Colonies were aseptically picked up with a loop and placed into sterile saline to obtain 0.5 McFarland turbidity suspensions. The suspensions were inoculated onto Mueller–Hinton agar with 5% sheep blood (BBL™) and incubated at 35°C ± 2°C for 16–18 h with antimicrobial disks. Then, the diameters of inhibition zones were measured.

***Statistical analysis*:** All data was analyzed using IBM SPSS Statistics v. 20 (IBM Corporation, USA). The Fisher's exact test was used to assess the antimicrobial susceptibility of the isolates. A probability value less than 0.05 was considered statistically significant.

Results

Biochemical identification and serological grouping: Based on colony morphology and Gram staining among 2,110 culture-positive results, 1,047 were identified as streptococci presumptively. Two hundred selected isolates were subcultured on Edwards agar and then 185 esculin-positive strains from 30 dairy farms were further assessed by biochemical identification and streptococcal grouping tests. Among the 157 isolated strains identified as *Streptococcus* spp., the predominant species was *S. uberis* (n=116), followed by *S. bovis* (n=16), *S. dysgalactiae* ssp. *dysgalactiae* (n=16), *S. agalactiae* (n=6), *S. equinus* (n=2), and *S. mitis* (n=1) (Table 1). All *S. agalactiae* isolates were obtained from one farm and were positive to CAMP test (Fig 1). *S. dysgalactiae* and *S. uberis* were isolated from 10 and 27 farms, respectively. Additionally, *S. bovis* were identified as biotype II/4

except that one was biotype II/1. Other nonstreptococcal strains were identified as *Aerococcus viridans* (n=22), *A. urinae* (n=1), *Enterococcus durans* (n=2), *E. faecium* (n=1), *E. faecalis* (n=1), and *Lactococcus lactis* (n=1).

All *S. agalactiae* and *S. dysgalactiae* were defined as group B and C, respectively. Sixteen strains were defined as group D and were *S. bovis* (n=13), *S. equinus* (n=2), and *E. faecalis* (n=1). There were 2 strains classified as group G, one *S. bovis* and one *E. faecalis*. No *S. uberis* had an agglutination reaction, indicating that they did not belong to groups A, B, C, D, F and G. in addition, 2 *S. bovis* (one of the two was biotype II/1), 1 *S. mitis*, 23 *Aerococcus* spp., and 2 *E. durans* were also non-grouped. Based on specific carbohydrate antigens, none of the streptococci in the present study was in groups A and F (Table 1).

Table 1 Bacterial identification and serological grouping of 185 Streptococcal isolates from bovine raw milk

Species	Streptococcal group						Non-A, B, C, D, F, G	Total
	A	B	C	D	F	G		
S. agalactiae	0	6	0	0	0	0	0	6
S. bovis	0	0	0	13	0	1	2	16
S. dysgalactiae	0	0	16	0	0	0	0	16
S. equinus	0	0	0	2	0	0	0	2
S. mitis	0	0	0	0	0	0	1	1
S. uberis	0	0	0	0	0	0	116	116
Aerococcus spp.	0	0	0	0	0	0	23	23
E. durans	0	0	0	0	0	0	2	2
E. faecium	0	0	0	0	0	1	0	1
E. faecalis	0	0	0	1	0	0	0	1
Lactococcus sp.	NT[a]	NT	NT	NT	NT	NT	NT	1
Total	0	6	16	16	0	2	144	185

[a]: NT, not tested.

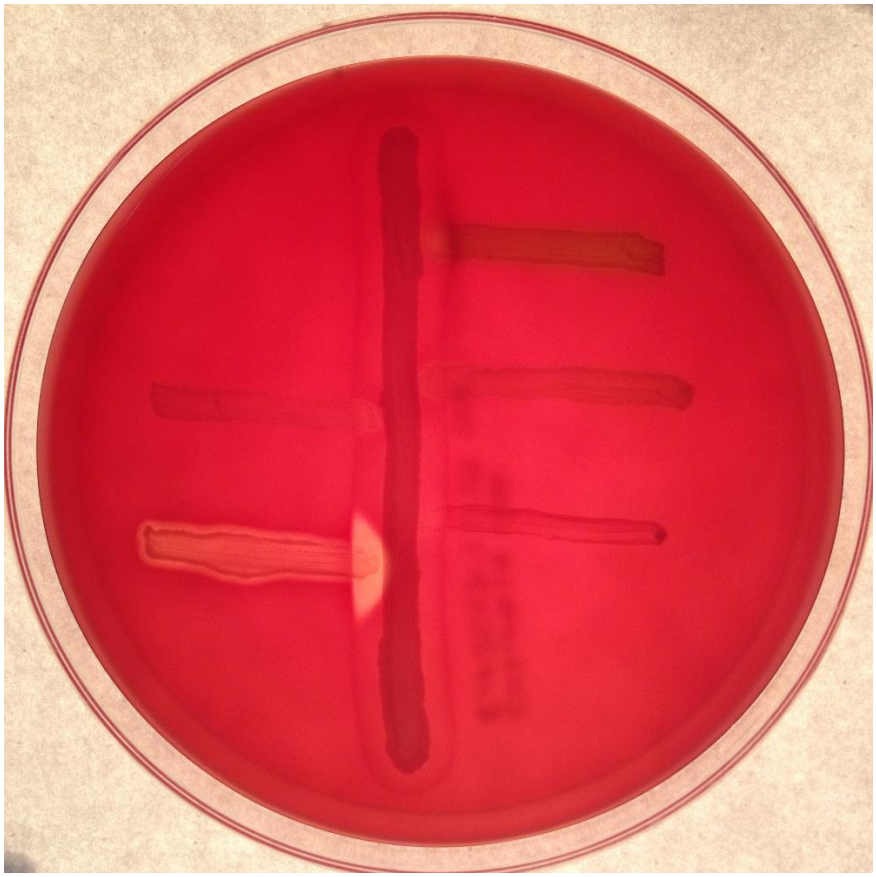

Figure 1 Result of CAMP test. *Staphylococcus aureus* (BCRC 12654) with incomplete-hemolysis was streaked on the center of the Blood agar plate. Other *Streptococcus* (from top to bottom, left to right: *S. bovis*, *S. agalactiae*, *S. dysgalactiae*, *S. equinus* and *S. uberis*) were streaked perpendicular to and within 3 to 5 mm of the line of *Staphylococcus aureus* inoculation on agar plate. The increased hemolytic area between the staphylococcal and streptococcal lines indicated a positive result of CAMP test which was the specific characteristic of *S. agalactiae*.

Antimicrobial susceptibility tests: In general, the 157 identified streptococci had similar susceptibility patterns (Table 2). Among the 16 antimicrobial agents, the isolates showed the highest resistance rate to amikacin (98.7%), followed by kanamycin (98.1%), neomycin (98.1%), streptomycin (98.1%), tetracycline (86.0%) and oxytetracycline (85.4%) and lincomycin (74.5%). In contrast, the bacteria were susceptible to amoxicillin (98.7%), cefuroxime (96.8%), cefazolin (96.8%), bacitracin (96.8%), ceftiofur (96.2%), cloxacillin (93.0%) and penicillin (80.9%). Only 2 out of the 157 isolates, both identified as *S. agalactiae* and were sensitive to all antimicrobial agents, except tetracycline and oxytetracycline. The other isolates were resistant to more than three antimicrobial agents. However, 9 of the isolates were sensitive to all the antimicrobial agents when aminoglycosides were excluded.

Based on the classification of the antimicrobial agents, the streptococci were sensitive to β-lactams, chloramphenicol and bacitracin. Tetracyclines, lincomycin and aminoglycosides showed up with a high proportion of resistance. There were 111 (71.3%) strains sensitive to the β-lactam antimicrobials; however, one *S. uberis* was resistant to all β-lactams except for amoxicillin. Penicillin and ampicillin showed significantly higher resistance rates than amoxicillin and cloxacillin ($P < 0.05$). However, there were no statistically significant differences between penicillin and ampicillin or amoxicillin and cloxacillin. Among seven cloxacillin-resistant streptococci, four strains were also resistant to ampicillin. There were 73.2% of *Streptococcus* sensitive to chloramphenicol; however, 22.9% displayed intermediate susceptibility.

Table 2 Antimicrobial susceptibility profiles of *Streptococcus* isolates in this study

	Number of susceptible strains						
Agents[a]	***S. agalactiae*** **(n=6)**	***S. bovis*** **(n=16)**	***S. dysgalactiae*** **(n=16)**	***S. equinus*** **(n=2)**	***S. mitis*** **(n=1)**	***S. uberis*** **(n=116)**	**Total (*n* = 157)**
β-lactams							
AML	6	16	16	2	1	114	98.7%
AMP	6	14	16	2	1	82	77.1%
OB	6	16	15	2	1	106	93.0%
P	6	15	16	2	1	87	80.9%
KZ	6	16	16	2	1	111	96.8%
CXM	6	16	16	2	1	111	96.8%
EFT	6	16	16	2	1	110	96.2%
Tetracyclines							
OT	0	0	0	0	1	3	2.5%
TE	0	0	0	0	1	2	1.9%
Aminoglycosides							
AK	2	0	0	0	0	0	1.3%
KA	1	0	0	0	0	0	0.6%
N	2	0	0	0	0	0	1.3%
S	2	0	0	0	0	1	1.9%
Others							
B	6	16	15	2	1	112	96.8%
C	6	14	13	2	1	79	73.2%
MY	6	5	2	0	1	8	14.0%

[a] AML, amoxicillin; AMP, ampicillin; OB, cloxacillin; P, penicillin; KZ, cefazolin; CXM, cefuroxime; EFT, ceftiofur; OT, oxytetracycline; TE, tetracycline; AK, amikacin; KA, kanamycin; N, neomycin; S, streptomycin; B, bacitracin; C, chloramphenicol; MY, lincomycin

Overall, there were 32 distinct drug resistance patterns to 16 selected antimicrobials among the isolates. The highest incidence of resistance (43.3%) was demonstrated by isolates resistant to seven antimicrobials, including kanamycin, amikacin, neomycin, streptomycin, tetracycline, oxytetracycline, and lincomycin. A high proportion of the isolates with this resistance pattern (76.5%) was *S. uberis*, and 16.2% were *S. dysgalactiae* ssp. *dysgalactiae*. Of note, four *S. uberis* isolates were resistant to 11 different antimicrobials although the resistance patterns were all different. Table 3 lists all the resistant patterns to the antimicrobial agent, excluding aminoglycosides. 43.9% of *Streptococcus*, including 53 of *S. uberis*, 11 of *S. dysgalactiae* ssp. *dysgalactiae*, four of *S. bovis*, and one of *S. equinus*, were coincidently resistant to tetracycline, oxytetracycline, and lincomycin.

Discussion

Contagious mastitis agents, including Staphylococcus aureus and Streptococcus agalactiae, were major causative pathogens in the past (Bradley, 2002). Owing to enhanced milking hygiene and practical management, the dominant causative agents of mastitis are now changing to environmental bacteria (Olde Riekerink et al., 2008). Unlike coliforms with an inflammatory response, however, infection of

Streptococcus spp. is asymptomatic or displays slight elevated somatic cell counts, leading to difficulty in figuring out infection and the need for treating mastitis in the field. Therefore, *Streptococcus* spp. has been frequently isolated from clinical and subclinical milk samples in recent years. To our knowledge, this is the first study in Taiwan focused on streptococcal mastitis. Based on our results, almost half of the culture-positive were morphologically identified as *Streptococcus* spp. Among them, *S. uberis* is the most prevalent streptococcal mastitis pathogen in Taiwan. *S. uberis* can infect and replicate inside the macrophages from the mammary glands *in vitro*, rendering latent, persistent, or chronic mastitis (Denis et al., 2006). In addition, it is likely to increase the chance of infection since its neighbors had been suffering from *S. uberis* (Guélat-Brechbuehl et al., 2010). The species was the most dominant *Streptococcus* isolated from bovine milk in many places, including America (Hogan and Smith, 2012), Canada (Olde Riekerink et al., 2008), England and Wales (Bradley et al., 2007), Germany (Minst et al., 2012), Switzerland (Guélat-Brechbuehl et al., 2010), Australia (Shum et al., 2009), New Zealand (Petrovski et al., 2011) and Korea (Nam et al., 2009). *S. dysgalactiae*, on the other hand, was the major agent of mastitis in central California (Rossitto et al., 2002). Since non-*agalactiae* streptococci are presented in the environment, cows are easily exposed to the pathogens between milking. Yet, streptococci have not been reported as dominant causative pathogens in Norway (Østerås et al., 2006) or Belgium (Piepers et al., 2007). The differences of environment between dairy farms, for instance, temperature, humidity, and even the design of barns, results in the difference of the dominant agents that cause mastitis. Moreover, several previous studies have determined numerous risk factors of streptococcal mastitis, such as high yields, long days in milk, multiparous cows, concave teat ends, poor sanitation, liner slips during milking and the use of straw or manure solids as bedding materials (Guélat-Brechbuehl et al., 2010; Hogan and Smith, 2012). Despite using streptococcal selective agar, 28 non-streptococcal isolates could not ensure preliminary exclusion. It was difficult to differentiate those bacteria from *Streptococcus* by basic laboratory tests and API microbiological tests (Wyder et al., 2011).

Table 3 The resistance patterns of streptococcal isolates to the antimicrobial agents except for aminoglycosides.

Resistant patterns[a]	No. of isolates (n=148)
TE-OT-MY	69
TE-OT	23
TE-OT-MY-AMP-P	11
TE-OT-MY-AMP	10
MY	6
TE-OT-MY-C-AMP-P	4
TE	2
TE-OT-MY-OB	2
TE-OT-MY-P	2
P	1
TE-MY	1
OT-MY	1
MY-P	1
AMP-P	1
TE-OT-B	1
TE-OT-AMP	1
OT-AMP-AML	1
OT-MY-AMP-P	1
TE-OT-MY-OB-B	1
TE-MY-C-AMP-P	1
TE-OT-MY-AMP-OB-EFT	1
TE-OT-MY-AMP-P-AML	1
TE-OT-MY-B-P-KZ	1
AMP-OB-P-KZ-CXM-EFT	1
TE-OT-MY-C-P-B-KZ	1
TE-OT-MY-AMP-P-OB-EFT	1
TE-OT-MY-AMP-P-OB-AML	1
TE-OT-MY-AMP-P-EFT-B	1

[a] AML, amoxicillin; AMP, ampicillin; OB, cloxacillin; P, penicillin; KZ, cefazolin; CXM, cefuroxime; EFT, ceftiofur; OT, oxytetracycline; TE, tetracycline; AK, amikacin; KA, kanamycin; N, neomycin; S, streptomycin; B, bacitracin; C, chloramphenicol; MY, lincomycin

The Lancefield grouping system is a rapid and practical tool for differentiating some pathogenic streptococci, especially for β-hemolytic streptococci. All of *S. agalactiae* and *S. dysgalactiae* ssp. *dysgalactiae* resulted in groups B and C. However, *S. uberis* were not categorized in any of the groups A, B, C, D, F and G. This might have been due to the heterogeneous serological classification of *S. uberis* (Leigh, 1999). In general, *S. bovis* and *S. equinus* are considered group D streptococci. This was consistent in the present study except for 3 *S. bovis*. Moreover, there were two biotypes identified by API. The classification and identification of *S. bovis* is still argued when comparing phenotypic and genotypic methods. Although it needed more strains and alternative methods for precise taxonomy of *S. bovis*, the biochemical tests were considered the preferred method for the final interpretation (Facklam, 2002; Dekker and Lau, 2016).

In general, streptococci in the present study were susceptible to penicillins, cephalosporins, and

bacitracin while being highly resistant to tetracyclines, lincomycin and aminoglycosides. This is similar to recent researches (Nam et al., 2009; Petrovski et al., 2011; Minst et al., 2012; Thomas et al., 2015). Among penicillins, which are the most frequently used drugs against gram-positive bacterial infections, strains isolated in our study showed higher susceptibility to amoxicillin followed by penicillin and ampicillin, as had also been previously found in other countries (Nam et al., 2009; Petrovski et al., 2011; Minst et al., 2012; Thomas et al., 2015). This may be due to different antimicrobial agents being conventionally used to treat dairy animals in different countries. Ampicillin is one of the ingredients of intrammamary ointments that are frequently available for mastitis treatment in Taiwan. Under an antimicrobial selective environment, the bacteria might shift to resistance and lead to treatment failure (Haenni et al., 2010; Pyörälä et al., 2014). Aminoglycosides are more effective against gram-negative bacteria because of their poor ability to penetrate the cell walls of gram-positive bacteria and therefore, they were mostly ineffective against the streptococci examined in this study. Our *Streptococcus* isolates were susceptible to chloramphenicol, which agrees with the findings of a previous study (Ma et al., 2006). Nevertheless, this antimicrobial was banned in 2002 from use in livestock animals bred for human consumption in Taiwan because of the occurrence of aplastic anemia. Tetracyclines had extremely high resistance in this study, which was owed to their frequent administration parenterally or intramammarily on Taiwanese dairy farms during the past decades because of broad spectrum activity. According to our susceptibility tests, most of the *S. agalactiae* isolates were sensitive to the antimicrobial agents except for tetracyclines and aminoglycosides, similar to the findings in Europe (Malinowski et al., 2008; Idriss et al., 2014). These *S. agalactiae* were collected from one farm that was suffering from re-occurrence mastitis. After being prescribed treatment and prevention, there was no more positive culture of *S. agalactiae*.

In conclusion, *S. uberis* was found to be the major species of streptococcal mastitis in Taiwan. Penicillins and cephalosporins remain the effective drugs chosen for treating them. For the sake of food sanitation and safety, it is necessary to update the population and resistant patterns of mastitis pathogens routinely.

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