PHENOTYPIC CHARACTERIZATION AND ANTIBIOGRAM OF *STREPTOCOCCI* ISOLATED FROM BOVINE INTRAMAMMARY INFECTIONS

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SUMMARY

The biochemical characterization and determination of antibiogram of 22 *Streptococcus agalactiae*, 20 *Streptococcus dysgalactiae* and 26 *Streptococcus uberis* strains from bovine intramammary infections were carried out. A simple test scheme based on seven biochemical tests allowed the identification and differentiation of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* isolates from bovine mastitis. All *S. agalactiae* strains were b-haemolytic, CAMP positive, utilized hippurate, salicin and raffinose but failed to utilize esculin, inulin and mannitol. The *S. dysgalactiae* cultures were a-haemolytic and only fermented trehalose and raffionose. All *S. uberis* strains hydrolysed esculin and fermented inulin and mannitol. The API 20 Strep System characterized accurately 100% isolates of *S. agalactiae* and *S. dysgalactiae* and 96.1% isolates of *S. uberis*. The determination of antimicrobial susceptibility patterns revealed that most of the isolates were susceptible to all the antimicrobials tested. However, more than 50% of *S. agalactiae* and *S. dysgalactiae* isolates were resistant to kanamycin and more than 50% of *S. dysgalactiae* were resistant to cephalexin. A high level of susceptibility to nitrofurantoin and oxacillin was common among all the isolates. These data allowed individual characterization of *Streptococci* of bovine origin and may augment further epidemiological studies.

Key words: antibiogram, biochemical profile, bovine, mastitis, *Streptococci*

INTRODUCTION

Mastitis remains the most economically important disease in dairy milk production worldwide and is usually caused by bacterial infections (Aarestrup *et al.* 1996a). Three species of *Streptoccoci* reported to be frequent causes of clinical and subclinical intramammary infections (IMI) are *Streptoccocus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* (Lammler, 1991). The widespread use of antibiotics and strict hygienic measures has greatly reduced the incidence of mastitis caused by contagious pathogens but infections by environmental bacteria have increased markedly during recent years (Jayarao *et al.* 1991b). *Streptococcus agalactiae* is a highly contagious obligate parasite of bovine mammary glands and causes low grade persistent infection (Keefe *et al.* 1997). *Streptococcus dysgalactiae*, contagious as well as environmental in nature, is responsible for about 8% of IMI in the UK (Hillerton *et al.* 1993). *Streptococcus uberis* is strictly an environmental pathogen and had accounted for 33% of all clinical cases of bovine mastitis in the UK (Hillerton *et al.* 1993). This organism can also be isolated from other sites of the body of the cow, the cows' environment and from nonlactating mammary glands (King, 1981).

Species identification of mastitis pathogens in most diagnostic laboratories is currently based on phenotypic characteristics of the bacteria using a number of biochemical and enzymatic profiles, serology and antibiotic resistant patterns (Jayarao *et al.* 1991b). The genus *Streptococcus* comprises a large group of heterogeneous bacteria and their identification by conventional methods require a battery of tests (generally between 20-25), 3 to 7 days and are labour intensive (Jayarao *et al* 1991a). A number of test schemes have been proposed for the identification and differentiation of many species of *Streptococci*. Various commercial kits are also presently available for the identification of *Streptococci* and the most widely used is perhaps the API 20 Strep System. Patterns of susceptibility to antimicrobial agents are also used for typing because they are readily available, easy to determine and relatively inexpensive. The present study was design to formulate an accurate, time saving and cost effective biochemical identification scheme and to determine the antibiotic susceptibility patterns of the pathogenic *Streptococci* frequently isolated from bovine IMI.

MATERIALS AND METHODS

Bacterial isolates

A total of 68 isolates comprising 22 S. agalactiae, 20 S. dysgalactiae and 26 S. uberis recovered from clinical and sub-clinical IMI in cows and reference strains, S. agalactiae 0250 and 0247a, S. dysgalactiae A15 and A17 and S. uberis 0140J and EF20 (all kindly provided by Dr. James A Leigh, Compton UK), were used in this study. Preliminary identification of isolates was done on the basis of Gram-stain, catalase test, haemolysis on blood agar and CAMP reaction on 5% sheep blood agar plate. The cultures were maintained on brain heart infusion agar slants and stored in Todd Hewitt broth containing 25% glycerol at -20°C.

Conventional biochemical test

A simple test scheme was designed based on the methods of McDonald and McDonald (1976) and Watts

(1988) consisting of hydrolysis of esculin and hippurate and fermentation of inulin, mannitol, trehalose, salicin and raffinose to differentiate the species of major streptococci causing mastitis in bovines. Hydrolysis of 0.1% esculin and 1% sodium hippurate was carried out in brain heart infusion broth and utilization of carbohydrates were determined in phenol red broth at 1% final concentration. Hydrolysis of esculin was determined from the dark brown discoloration of the broth after addition of ferric citrate solution and that of hippurate by the addition of ferric chloride reagent to the culture supernatant (persistence of reddish-brown precipitate).

API 20 Strep System

The API 20 Strep System (bioMerieux SA, France) was used following the manufacturer's instructions. The organism was grown on sheep blood agar plate and incubated at 37°C for 24 hours. Growth was removed and suspended in 2ml sterile distilled water (turbidity>4 McFarland standard). Each microcupule was filled with 100ml suspension and the strips were incubated at 37°C. After 4h incubation, reagents were added for reading enzymatic activities and a seven-digit profile number was generated. Strips were incubated again for 20h. At the end of 24h incubation, a new profile number was constructed. The level of identification was based on the similarity of the profile and the taxa that constitute the computer stored database.

Antibiogram

The determination of antimicrobial susceptibility was performed according to the method described by Sippel *et al.* (1995). Four to five colonies of the bacteria was incubated in Todd Hewitt broth (THB) for 2h at 37°C. Approximately 0.1ml of this suspension was spread on to Muller-Hinton agar (Oxoid) containing 5% sheep blood. The antibiotic impregnated paper discs used were: ampicillin (10mg), chloramphenicol (30mg), cephalexin (30mg), doxycycline (30mg), kanamycin (30mg), gentamycin (10mg), methicillin (30mg), nitrofurantoin (300mg), oxacillin (10mg), penicillin G (10 IU), tetracycline (30mg) and vancomycin (30mg) (Becton-Dickinson, UK), After 24h at 37°C, zones of inhibition were measured and the results were interpreted according to recommended ranges.

RESULTS

The biochemical properties of 26 *S. uberis*, 22 *S. agalactiae* and 20 *S. dysgalactiae* strains used in this study are summarised in Tables 1 and 2. The biochemical profiles of the isolates within each species appeared to be almost identical but could be clearly distinguished between species.

Table 1. Conventional biochemical test scheme for characterization of *S. galactiae, S. dysgalactiae* and S. uberis isolates from bovine IMI

Tests	*S. agalactiae (n=22)*	*S. dysgalactiae (n=20)*	*S. uberis (n=26)*
Haemolysis on Blood Agar	β–100%	α–100%	NH–92.3%
CAMP reactivity	100%	0	7.6%
Hydrolysis of:			
Hippurate	100%	0	96.1%
Esculin	0	0	100%
Fermentation of:			
Inulin	0	0	100%
Mannitol	0	0	100%
Salicin	100%	0	100%
Trehalose	100%*	100%	100%
Raffinose	100%	100%	0

* Delayed reaction; NH – non-haemolytic

Table 2. Biochemical characteristics (% positive) of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* utilizing the API 20 Strep System

Tests	*S. agalactiae* (n=22)	*S. dysgalactiae* (n=20)	*S. uberis* (n = 26)
VP	100	0	96.1
HIP	100	0	92.3
ESC	0	0	100
PYRA	0	0	30.7
AGAL	0	0	7.6
BGUR	81.8	100	84.6
BGAL	0	0	0
PAL	100	100	26.9
LAP	100	100	100
ADH	100	100	100
RIB	100	100	100
ARA	0	0	0
MAN	0	0	100
SOR	0	20	100
LAC	36.3	90	100
TRE	100	100	100
INU	0	0	100
RAF	100	100	0
AMD	36.3	100	46.1
GLYG	0	23	0

All *S. agalactiae* but none of the *S. dysgalactiae* were CAMP positive and only two (7.14%) *S. uberis* isolates demonstrated CAMP like synergistic haemolytic activities on sheep blood agar. *Streptococcus dysgalactiae* isolates fermented raffinose and trehalose but not inulin, mannitol and salicin and did not hydrolyse hippurate and esculin, All *S. uberis* isolates were non haemolytic except 2 strains which were weakly a–haemolytic, hydrolysed esculin and fermented inulin.

Table 3. Antimicrobial susceptibility patterns of Streptococci isolated from bovine intramammary infections

Antimicrobials	*S. agalactiae* (n=22)	*S. dysgalactiae* (n=20)	*S. uberis* (n = 26)
Ampicillin (10µg)	22 (100%)	20 (100%)	22 (84.6%)
Chloramphenicol (30µg)	22 (100%)	18 (100%)	24 (100%)
Cephalexin (30µg)	14 (63.6%)	9 (45.0%)	22 (84.6%)
Doxycycline (30µg)	19 (86.3%)	17 (85.0%)	23 (88.4%)
Nitrofurantoin (300µg)	22 (100%)	20 (100%)	26 (100%)
Gentamycin (10µg)	20 (90.9%)	18 (90.0%)	26 (100%)
Kanamycin (30µg)	9 (40.9%)	4 (20.0%)	23 (88.4%)
Methicillin (30µg)	22 (100%)	18 (90.0%)	26 (100%)
Oxacillin (10µg)	22 (100%)	20 (100%)	26 (100%)
Penicillin G (10 IU)	22 (100%)	18 (90.0%)	23 (88.4%)
Tetracycline (30µg)	16 (72.7%)	14 (70.0%)	18 (69.2%)
Vancomycin (30µg)	20 (90.9%)	15 (75.5%)	24 (92.3%)

The API Strep System accurately identified 25 of 26 (96.1%) *S. uberis* isolates, 100% of *S. agalactiae* and *S. dysgalactiae* isolates and the 6 reference strains were also correctly identified (Table 2). Accuracy of this system for identification of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* ranges from 96.1% to 100%.

The antibiogram testing patterns of the *Streptococci* isolates are presented in Table 3. Some differences in susceptibility patterns among the isolates and the species were found. All *S. agalactiae*, *S. dysgalactiae* and *S. uberis* isolates were susceptible to nitrofurantoin and oxacillin and about 30% isolates of the three species were resistant to tetracycline. Furthermore, all *S. agalactiae* isolates were highly susceptible to penicillin G, chloramphenicol and methicillin and those of *S. uberis* to gentamycin and methicillin. A high frequency of resistance to cephalexin and kanamycin was observed among the *S. agalactiae* and *S. dysgalactiae* isolates (Table 3). On the other hand, a low frequency of resistance of *S. agalactiae* and *S. uberis* isolates to doxycycline and vancomycin was observed.

DISCUSSION

Biochemical evaluations of bacterial isolates are valuable for initial strain characterization and are still in widespread use. Several phenotypic systems such as enzymatic profiles, serotyping, biotyping and antibiogram have been proposed for typing *Streptococcus* sp. The biochemical characterisation of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* isolates in this study could be effectively performed using cultural characteristics, haemolytic patterns on 5% sheep blood agar, CAMP reactivity, hydrolysis of hippurate and esculin, and fermentation of inulin, mannitol, trehalose, salicin and raffinose. The biochemical reactions were similar to the findings of McDonald and McDonald (1976). In this study, *S. agalactiae* isolates were differentiated from other mastitis streptococci by the CAMP test and hippurate and esculin hydrolysis (100%, 100% and 0% respectively). These results are similar to those of McDonald and McDonald (1976) and Watts (1988). All *S. dysgalactiae* isolates in the present study exhibited phenotypic characteristics similar to those described previously for bovine *S. dysgalactiae* (McDonald and McDonald, 1976; Watts, 1988). McDonald *et al.* (1976) reported that 55.5% of *S. dysgalactiae* strains are capable of hydrolyzing esculin; however it required 240h incubation. Failure of the *S. dysgalactiae* isolates to hydrolyse esculin in the present study is not surprising as the test was read after 24h. Acid production from inulin has been considered as a key diagnostic character for *S. uberis* (McDonald and McDonald, 1976). As shown in Table 1, all *S. uberis* strains in this study fermented inulin. However, Watts (1988) reported only 24.5% *S. uberis* strains utilized inulin while Jayarao *et al.* (1991b) found that 4.8% of *S. uberis* isolates did not utilize inulin. Roguinsky (1969) reported that 16% of the *S. uberis* isolates would not ferment inulin. McDonald and McDonald (1976) described 25.3% of the *S. uberis* isolates were CAMP positive be we observed only 7.6% isolates were CAMP positive.

The API 20 Strep System is perhaps the most widely used systems for identification of *Streptococci* of bovine origin. The results of the present study indicate that a correct identification could be performed within 4h but for more accuracy the recommended incubation period of up to 24h should be maintained. In the present study the biochemical profiles of *S. uberis* were similar to the findings of others (Gravie and Bramely, 1979; McDonald and McDonald, 1976) and the *S. uberis* isolates were differentiated clearly from those of *S. agalactiae* and *S. dysgalactiae*. Some of the *S. uberis* and *S. agalactiae* strains in this study were b-glucoronidase positive, which is in agreement with the studies of Lammler (1999) and Efstratiou *et al* (1994). The alkaline phosphatase reaction was strongly positive for *S. agalactiae* and *S. dysgalactiae* but weakly positive (15.3%) for *S. uberis*. This is in contrast to the results of Collin *et al.* (1984) who reported that all *S. uberis* cultures were clearly PAL negative. According to Lammler (1991), all *S. agalactiae*, *S. dysgalactiae* and *S. uberis* are VP positive. In the present study, all *S. agalactiae*, 96.1% of *S. uberis* and none of the *S. dysgalactiae* strains were VP positive. This finding is in contrast to the report of Lammler (1991) but similar to the results of Collins *et al.* (1984). Percentage of organisms identified by the API 20 Strep System in the present study

is higher than that reported previously (Poutrel and Ryneiwecz, 1984) but similar to the studies of Watts (1989) and Jayarao *et al.* (1991a). Poutrel and Ryneiwecz (1984) showed only 71.4% of 84 mastitis streptococci and Watts (1989) indicated 88.4% of 199 strains and Jayarao *et al.* (1991a) identified 46 of 46 *S. agalactiae*, 48 of 48 of *S. dysgalactiae* and 96.2% of *S. uberis* strains from bovine intramammary infections by the API Strep System. This study characterized accurately 100% of *S. agalactiae* and *S. dysgalactiae* and 96.1% of *S. uberis* isolates. Several factors may influence the identification of organisms by the API 20 Strep System such as, number and diversity of strains representing each species, metabolic state of the organism at the time of test (Watts, 1989) and variation in interpretation of the test results.

The antibiogram patterns revealed that some isolates of *S. agalactiae* and *S. dysgalactiae* showed a high frequency of resistance to cephalexin and kanamycin and susceptible to chloramphenicol and gentamycin. This is in constant to the findings of Wibawan *et al.* (1993) and Sippel *et al.* (1995), who reported a high level of resistance to chloramphenicol and gentamycin. The antibiotic resistance patterns of *S. agalactiae* isolates in the present study are similar to those reported previously (Wibawan *et al.* 1991) and confirmed that resistance to tetracycline is common among *S. agalactiae* isolates. Most of the isolates of *S. agalactiae*, *S. dysgalactiae* and *S.uberis* in the present study were highly susceptible to penicillin G and ampicillin. This is in contrast to the findings of Cantin *et al.* (1992) and Aarestrup *et al.* (1996b), who reported high levels of resistance to ampicillin and penicillin. The differences in occurrence of resistance may reflect the regional differences, frequencies of use of antibiotics but may also, to some extent, be the result of methodological variations between the present and the other studies. The phenotypic characteristics and antibiogram patterns observed in this study would help to identify Streptococci of bovine origin and may be useful for further epidemiological studies.

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RINGKASAN

PENCIRIAN FENOTIP DAN ANTIBIOGRAM STREPTOCOCCI YANG DI PENCIL DARIPADA JANGKITAN INTRAMAMA BOVIN

Pencirian biokimia dan penentuan antibiogram 22 stran *Streptococcus agalactiae*, 20 *Streptococcus dysgalactiae* dan 26 *Streptococcus uberis* daripada jangkitan intramamari dilaksanakan. Skema ujian mudah berdasarkan kepada tujuh ujian biokimia membenarkan pengenalan dan pembezaan pencilan *S. agalactiae*, *S. dysgalactiae* dan *S. uberis* yang menyebabkan mastitis bovin. Kesemua strain *S. agalactiae* adalah b-haemolisis, positif CAMP, menuggunakan hipurat, salicin and rafinosa tetapi gagal menggunakan eskulin, inulin and manitol. Kultura *S. dysgalactiae* adalah a-haemolisis dan hanya menapai trehalosa and rafionose. Kesemua strain *S. uberis* menghidrolisis eskulin and menapai inulin and manitol. API 20 Strep System menciri dengan tepat 100% pencilan *S. agalactiae* dan *S. dysgalactiae* dan 96.1% pencilan *S. uberis.* Penentuan corak kerentanan antimikrobial mendedahkan kebanyakan pencilan rentan kepada kesemua ujian antimikrobial. Lebih daripada 50% pencilan *S. agalactiae* and *S. dysgalactiae* tahan kepada kanamycin dan lebih daripada 50% *S. dysgalactiae* tahan kepada cephalexin. Aras kerentanan tinggi kepada nitrofurantoin and oxacillin adalah setara di antara semua pencilan. Data ini membolehkan pencirian individu *Streptococci* yang berasal daripada bovin dan seterusnya melonjakkan kajian epidemiologi.