Acta vet. scand. 1999, *40,* 315-321.

Identification of Coagulase-Positive Staphylococci Isolated from Bovine Milk

By *A. Capurro*[1], *C. Concha*[2], *L. Nilsson*[2] and *K. Östensson*[1]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, and [2]Department of Mastitis, National Veterinary Institute, Uppsala, Sweden.

Capurro A, Concha C, Nilsson L, Östensson K: Identification of coagulase-positive staphylococci isolated from bovine milk. Acta vet. scand. 1999, 40, 315-321. – A total of 414 coagulase-positive staphylococcal strains obtained at the mastitis laboratory, National Veterinary Institute, Uppsala, Sweden, were studied. One hundred and seventy seven strains were used for a frequency study. Ninety-seven per cent were identified as *Staphylococcus aureus,* 2% as *Staphylococcus intermedius* and 1% as *Staphylococcus hyicus.* Two hundred and thirty seven strains with atypical hemolysis reactions on bovine blood agar were randomly selected, with the aim to increase the number of *S. intermedius* and *S. hyicus* strains available for testing. Eight different characteristics, including physiological, enzymatical and biochemical properties, were used to identify the coagulase-positive *Staphylococcus species.* The results of this study suggest that the following tests should be included for correct identification of the 3 different species of coagulase-positive staphylococci: P agar supplemented with acriflavin, ß-galactosidase and hemolytic reaction on chocolate agar. These 3 tests are simple and quick to perform and enable accurate for easy differentiation of the 3 coagulase-positive *Staphylococcus* species.

cow; mastitis; differentiation.

Introduction

In the last edition of the Bergey's Manual of determinative bacteriology (1986) three species of coagulase-positive staphylococci (CPS) are listed; *S. aureus (Buchanan & Gibons* 1974), *S. intermedius* (*Hajék* 1976) and *S. hyicus* subsp. *hyicus* (*Devriese et al.* 1978). CPS are often isolated from bovine milk samples, and in routine laboratory diagnostics, generally, all strains of CPS have been considered as *S. aureus.* However, it is today generally considered that CPS in milk include not only *S. aureus,* but also *S. intermedius* and *S. hyicus* (*Harmon et al.* 1993, *National Mastitis Council,* 1987). Historically, species in the genera *Staphylococcus* have been divided into coagulase-positive and coagulase-negative staphylococci, differentiated by the use of one single test, the coagulase test (*Evans* 1965). However, since it has been shown that different strains of *S. aureus* can have different coagulase reaction (*Watts et al.* 1984; *Fox et al.* 1996) and that *S. hyicus* subsp. *hyicus* is coagulase variable (*Devriese et al.* 1978), additional tests have to be used for an accurate differentiation.

Studies, using complementary tests to the coagulase test, have been carried out during recent years to determine a good combination of tests for differentiation of the 3 CPS at specie level (*Botha & Brand* 1987, *Roberson et al.* 1992). Several studies in different regions and countries (*Rampone et al.* 1993, *Calzolari et al.* 1995, *Roberson et al.* 1996) have reported dif-

ferent frequencies for *S. aureus, S. intermedius,* and *S. hyicus,* among CPS from bovine milk, identified by the use of a combination of tests. In all studies there were a high predominance of *S. aureus.*

The main objectives of the present report were to study the relative occurrence of *S. aureus, S. intermedius,* and *S. hyicus* among CPS, isolated from milk samples collected from bovine mastitis cases in Sweden and to establish a relevant and accurate typing scheme for identification of the 3 CPS species.

Materials and methods

Bacterial strains

The CPS strains were obtained from bovine milk samples from mastitis cases, sent to a mastitis routine laboratory (National Veterinary Institute, Uppsala, Sweden). The total number of CPS strains included in this study was 414, among which 327 were from subclinical and 87 from clinical mastitis. The samples were classified in material A and B according to the hemolysis reaction on bovine blood agar of each strain. The terminology by *Elek & Levy* (1950) was used. Material A, used for frequency study, consisted of the 177 strains irrespective of type of hemolysis reaction presented. In this material all CPS isolated from milk samples during a specific period of time were selected, with the restriction of just one strain per herd. Material B consisted of 237 strains, without the bovine *S. aureus* strains typical double hemolysis zones on bovine blood agar. The strains were CPS randomly selected from different herds, with the aim to increase the number of strains other than *S. aureus,* available for testing.

Identification of isolates

Gram-positive strains of non-motile cocci which produced catalase (*National Mastitis Council* 1987) and coagulase (*National Mastitis Council* 1987, *Quinn et al.* 1994) were classified as CPS. Isolates which were identified as CPS were additionally tested.

Storage of isolates

Each of the isolates was harvested in a tube (Nalgene cryogenics vials, Nalge Company, Rochester, NY, USA) containing 1 ml of Trypticase soy broth (TSB) +15% glycerol, (Becton-Dickinson, San Jose, CA, USA) for prolonged storage at -31°C. After storage each isolate was subcultured twice, before it was tested.

Differentiation of staphylococcal species

Differentiation of staphylococcal species was carried out using the following tests in the given order.

In the test of ability to grow on P agar (*Phillips & Nash* 1985), the medium was supplemented with 7 μg of acriflavin per ml according to *Harmon et al.* (1991). The inoculation was performed according to *Roberson et al.* (1992). Growth indicated *S. aureus.* In the acetoin test the medium was prepared according to *Roberson et al.* (1992). *S. aureus* is in general positive in this test. Anaerobic fermentation of mannitol was performed as described by the *Subcommittee on Taxonomy of Staphylococci and Micrococci* (1965). *S. aureus* is considered to be positive. Culture on purple agar (Difco, Detroit, IL, USA) with 1% maltose was prepared and interpreted according to *Quinn et al.* (1994). *S. aureus* and *S. intermedius* show positive reactions. The β-galactosidase test (*Robertson et al.* 1992) was performed using a commercially available substrate tablet (Rosco, Taastrup, Denmark) (*Mac Faddin* 1980), according to instructions by the company. *S. intermedius* is considered to be positive.

Hemolytic reaction on chocolate agar was performed as described by *Lämmler* (1991). *S. hyicus* has a positive reaction. Synergistic hemolysis (CAMP-like) test was performed as described by *Hébert & Hancock* (1985). *S. hyi-*

Table 1. Number and frequency of coagulase-positive staphylococcal species in material A (all coagulase-positive strains) and B (strains without the bovine coagulase-positive *S. aureus* typical double hemolysis zone), divided into clinical and subclinical cases of mastitis.

	Material A				Material B			
Species	Clinical	Subclinical	Total	%	Clinical	Subclinical	Total	%
S. aureus	36	137	173	97	48	182	230	97
S. intermedius	1	2	3	2	1	4	5	2
S. hyicus	0	1	1	1	1	1	2	1
Total	37	140	177	100	50	187	237	100

cus has a positive reaction. Aerobic fermentation of mannitol was tested in mannitol salt agar (Acumedia, Ljusne, Sweden) and interpreted according to *Botha & Brand* (1987). *S. aureus* and *S. intermedius* show positive reactions.

Control strains

Control strains used in all tests were obtained from a collection at the Department of Mastitis, National Veterinary Institute, Uppsala, Sweden. The control strains were: *Staphylococcus aureus* Newman A275, *Staphylococcus aureus* Oxford 209, *Staphylococcus intermedius* L13, *Staphylococcus hyicus* 8610193, and *Staphylococcus hyicus* 8612948.

Accuprobe® method

Ten, 8 and 3 strains of *S. aureus, S. intermedius* and *S. hyicus,* respectively, were tested with Accuprobe® *Staphylococcus aureus* culture identification test (Gen-probe, San Diego, CA, USA), specific for *S. aureus* of human origin. The test was performed according to *Freney et al.* (1993). This is a nucleic acid hybridization test which is based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes. A chemiluminescent labelled, single-stranded DNA probe, complementary to the ribosomal RNA of the target organism, combines with the RNA to form a stable DNA:RNA hybrid. The labelled DNA:RNA hybrids are measured in a Gen-Probe luminometer.

Results

Table 1 shows the number and frequencies of the different species obtained among the CPS. In samples from clinical and subclinical mastitis cases and in both materials high predominance of *S. aureus* was seen. There were no differences in the frequencies of the 3 species between material A and B.

A simplified key (Fig. 1) was compiled for the identification of the different CPS species.

Table 2 shows the proportion of the tested strains positive in the 8 different tests. Four hundred and three strains that were positive on P agar with acriflavin, and positive to the aerobic and anaerobic mannitol fermentation test, among which 98% and 80% were positive to acetoin test and purple agar test, respectively, were determined as *S. aureus.* All these *S. aureus* strains were negative in the other tests used.

The 8 strains with positive reaction in the aerobic mannitol fermentation test and ß-galactosidase test, were considered as *S. intermedius.* Among these, 2 and 6 strains were also positive to the acetoin test and purple agar test, respectively. All *S. intermedius* strains were negative to the test on P agar with acriflavin, anaerobic

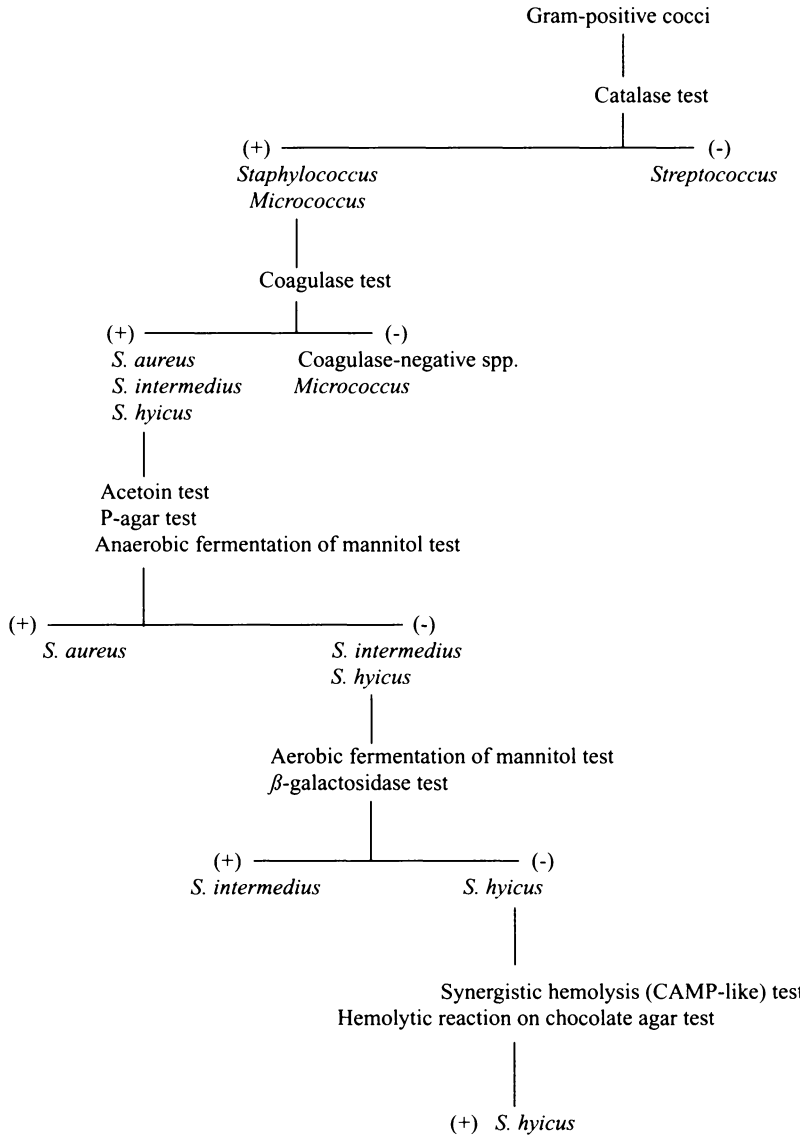

Figure 1. A simplified key for differentiating coagulase-positive staphylococci.

mannitol fermentation test, and the CAMP-like test and showed no hemolytic reaction on chocolate agar.

Only 3 strains were positive in the CAMP-like test and gave a hemolytic reaction on chocolate agar and were determined as *S. hyicus*. These strains were negative in all the other tests.

The results of Accuprobe® test are shown in Ta-

Table 2. Results of analyses of coagulase-positive staphylococci by 8 different tests for characterization of *S. aureus, S. intermedius* and *S. hyicus*. The figures are given as percentages of positive strains.

Species	Test							
	P agar	Anaerobic fermentation of mannitol	Aerobic fermentation of mannitol	ß-galactosidase	Acetoin	Purple agar	CAMP-like	Hemolysis on chocolate agar
S.aureus	100	100	100	0	98	80*	0	0
S.intermedius	0	0	100	100	25	75**	0	0
S.hyicus	0	0	0	0	0	0	100	100

*A +++ positive test. **A ++ positive test.

Table 3. Percentages of positive reactions among 21 coagulase-positive staphylococcal strains tested by the Accuprobe® test.

Species	Number of strains stested	Accuprobe® positive strains (%)
Staphylococcus aureus	10	100
Staphylococcus intermedius	8	0
Staphylococcus hyicus	3	0

ble 3. All *S. aureus* strains were positive and the other CPS were negative.

Discussion

The 3 species were, more or less, equally distributed among clinical and sub-clinical cases (Table 1). The frequencies of the different CPS species were similar in material A and B but slightly different to what has been reported in other studies. *El-Sukhon,* (1995) found higher frequency of *S. hyicus* (3.8%). This may be explained by differences in the characterization of various species. It is also known that in the population of *S. hyicus* subsp. *hyicus* some strains have an irregular, often negative, but sometimes delayed positive coagulase reaction (*Devriese et al.* 1978, *Sperber* 1979).

According to differences between the materials and tests used, the results from studies by *Botha & Brand* (1987), *Rampone et al.* (1993), and *Calzolari et al.* (1995) are not really comparable. However, the frequencies of the different species reported in these studies, as well as in the present, are rather similar.

Roberson et al. (1996) found, compared with our findings, higher frequency of *S. hyicus* (17.7%) and lower frequency of *S. intermedius* (0.2%). The differences might be due to the fact that *Roberson et al.* (1996) had 41% of primiparous cows in their trial. According to *Nickerson et al.* (1995), primiparous cows have higher prevalence of *S. hyicus*. Furthermore, *Roberson et al.* (1996) used a different chromogenic compounds in the ß-galactosidase test.

In the present study different tests for identification and differentiation of the 3 CPS species were evaluated, in order to find accurate but also simple, inexpensive and quick tests. The tests should have a high accuracy in identifying each species and identification should be based on positive, rather than negative test results. The tests in the present study (Table 2) were evaluated according to the these criteria.

According to the results of the present work a combination of the following tests is suggested to accurately differentiate the 3 CPS species from each other: P agar supplemented with acriflavin test, ß-galactosidase test and hemolytic reaction on chocolate agar. These tests are meant to supplement rather than replace the coagulase test. They are rather quick, easily per-

formed and interpreted, and relatively inexpensive.

To identify a strain on the basis of a small number of biochemical characteristics may be considered a problem because it increases the risk of misidentification. Moreover, regarding *S. aureus* and *S. intermedius,* there are strains that have weak utilisation, or fail to utilise certain substrates in these 3 tests. Therefore we suggest that anaerobic fermentation of mannitol also should be used. To distinguish between *S. intermedius* and coagulase-positive *S. hyicus* with atypical or weak reactions, we recommend aerobic fermentation of mannitol to be used.

The Accuprobe® test (Table 3), specific for human *S. aureus,* worked well for the 10 *S. aureus* bovine strains tested. This seems to be a simple and secure method but needs further evaluation for CPS species isolated from bovine milk.

Acknowledgements

The laboratory work was carried out at the mastitis laboratory, Department of Mastitis, National Veterinary Institute, Uppsala, Sweden. Dr. Capurro was awarded a scholarship by the Swedish Foundation for International Co-operation in Research and Higher Education (STINT).

References

Botha FS, Brand PAJ: A simplified key for identification of coagulase-positive staphylococci isolated from bovine milk. S. Afr. J. Dairy Sci. 1987, *1,* 39-44.

Buchanan RE, Gibbons NE: Bergey's manual of determinative bacteriology. 8th ed. The Williams & Wilkens Company. Baltimore. 1974.

Calzolari A, Bettera S, Frigeiro C, Rampone H, Giraudo J: Taxonomic classification and antibiotic resistance of staphylococci isolated from bovine milk. Proceedings of the 3rd international mastitis seminar, Tel Aviv, Israel, 1995, session 2, 78-79.

Devriese LA, Hajek V, Oeding P, Meyer SA, Schleifer KH: Staphylococcus hyicus (*Sompolinsky* 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. *nov.* Int. J. Syst. Bacteriol. 1978, *28,* 482-490.

Elek SD, Levy E: Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. J. Pathol. Bacteriol. 1950, *62,* 541-554.

El-Sukhon SN: Characterization of staphylococci isolated from mastitic cows in Jordania. Bull Anim. Health Prod. Afr. 1995, *43,* 231-235.

Evans JB: Current views and problems relating to the taxonomy of the Micrococcaceae. Int. Bull. bact. Nomencl. Taxon. 1965, *15,* 111-112.

Fox LK, Besser TE, Jackson SM: Evaluation of a coagulase-negative variant *Staphylococcus aureus* as a cause of intramammary infections in a herd of dairy cattle. J. Am. Vet. Med. Assoc. 1996, *209,* 1143-1146.

Freney J, Meugnier H, Bes M, Fleurette J: Identification of *Staphylococcus aureus* using a DNA probe: Accuprobe®. Ann. Biol Clin. 1993, *51,* 637-639.

Hájek V: Staphylococcus intermedius, a new species isolated from animals. Int. J. Syst. Bacteriol. 1976, *26,* 401-408.

Harmon RJ, Eberhart RJ, Jasper DE: Microbiological procedures for the diagnosis of bovine udder infection. Arlington VA, National Mastitis Council Inc, 1993.

Harmon RJ, Langlois BE, Akers K: A simple medium for the verification of identity of *Staphylococcus aureus* of bovine origin. J. Dairy Sci 1991, *74,* (Suppl 1), 202.

Hébert GA, Hancock AH: Synergistic hemolysis exhibited by species of staphylococci. J. Clin. Microbiol. 1985, *22,* 409-415.

Lämmler C: Characterization of *Staphylococcus hyicus* with the ATB 32 Staph System and with Conventional Tests. J. Clin. Microbiol. 1991, *29,* 1221-1224.

Mac Faddin JF: In: Biochemical tests for identification of medical bacteria. 2nd edn. Williams & Wilkins, Baltimore, Maryland, USA, 1980, pp 120-128.

National Mastitis Council: Staphylococci. In: Laboratory and field handbook on bovine mastitis. Arlington, VA, 1987, p. 80.

Nickerson SC, Owens WE, Boddie RL: Mastitis in Dairy Heifers: Initial studies on prevalence and control. J. Dairy Sci. 1995, *78,* 1607-1618.

Phillips E, Nash P: Culture media. In: Lennette, Ballows, Hausler Jr, Shadomy (eds.): Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, DC 1985, 1051-1092.

Quinn PJ, Carter ME, Markey B, Carter GR: Staphylococcus species. In: Clinical Veterinary Micro-

biology, Quinn, Carter, Markey and Carter (eds.) Wolfe publishing, London, 1994, p. 118.

Rampone H, Bogni C, Giraudo J, Calzolari A: Identification of staphylococci from milk in Argentina. Zbl. Bakt. 1993, *279,* 537-543.

Roberson JR, Fox LK, Hancock DD, Besser TE: Evaluation of methods for differentiation of Coagulase-Positive staphylococci. J. Clin. Microbiol. 1992, *30,* 3217-3219.

Roberson JR, Fox LK, Hancock DD, Gay JM, Besser TE: Prevalence of coagulase-positive staphylococci, other than *Staphylococcus aureus,* in bovine mastitis. Am. J. Vet. Res. 1996, *57,* 54-58.

Sperber WH: The identification of staphylococci in clinical and food microbiology laboratories. Crit. Rev. Clin. Lab. Sci., 1979, *7,* 121-184.

Anonymous: Subcommittee on Taxonomy of Staphylococci and Micrococci: Recommendations. Int. Bull. Bacteriol. Nomencl. Taxon. 1965, *15,* 109-110.

Watts JF, Pankey W, Nickerson SC: Evaluation of Staph- Evaluation Ident and STAPHase Systems for Identification of Staphylococci from Bovine Intramammary Infections. J. Clin. Microbiol. 1984, *20,* 448-452.

Sammanfattning

Identifiering av koagulaspositiva stafylokocker isolerade från bovin mjölk.

En undersökning har genomförts där sammanlagt 414 koagulaspositiva stafylokockstammar studerats. Stammarna var isolerade från mjölk från fall av mastit i samband med rutinverksamheten vid Avdelningen för mastit, Statens Veterinärmedicinska Anstalt, Uppsala, Sverige. Av dessa 414 stammar ingick 177 stammar i en frekvensstudie, varvid 97% av stammarna identifierades som *Staphylococcus aureus,* 2% som *Staphylococcus intermedius* och 1% som *Staphylococcus hyicus.* Ytterligare 237 stammar med atypisk hemolysreaktion på nötblodagar valdes ut i syfte att öka antalet *Staphylococcus intermedius*- och *Staphylococcus hyicus*-stammar tillgängliga för testning. Åtta olika karaktärisktika, inkluderande fysiologiska, enzymatiska och biokemiska egenskaper, användes för att identifiera de koagulaspositiva stafylokockarterna. Baserat på resultatet av denna studie föreslås att följande tester kan användas för en säker identifiering av de 3 olika arterna av koagulaspositiva stafylokocker: P agar med acriflavin, β-galactosidas och hemolytisk reaktion på chokladagar. Dessa 3 tester är okomplicerade och snabba att genomföra vilket möjliggör en enkel, snabb och korrekt differentiering av de 3 koagulaspositiva stafylokockarterna.

(Received May 25, 1998; accepted August 2, 1999).

Reprints may be obtained from: C. Concha, Department of Mastitis, National Veterinary Institute, S-751 89 Uppsala, Sweden. E-mail: carlos.concha@sva.se, tel: +46 18 67 42 60, fax: +46 18 30 94 02.