Schweizer Archiv für Tierheilkunde
A. Moser et al., Band 155, Heft 6, Juni 2013, 333–338
DOI 10.1024/0036-7281/a000468

Species distribution and resistance profiles of coagulase-negative staphylococci isolated from bovine mastitis in Switzerland

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Summary

Coagulase-negative staphylococci (CNS) are the predominant cause of bovine intra-mammary infections. They can lead to chronic infections and were reported to significantly increase milk somatic cell counts. The goal of our study was to determine the species distribution and antimicrobial susceptibility profiles of CNS in bovine mastitis milk samples in Switzerland. Between March 2011 and February 2012, a total of 120 CNS were isolated from mastitis milk samples from 117 different animals at 77 farms. The isolates were identified by matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) and subsequently tested for sensitivity to various antibiotic agents by disk diffusion. Antimicrobial agents were selected mainly based on their relevance to the treatment of bovine mastitis in Switzerland. MALDI-TOF MS assigned the 120 isolates to 12 different staphylococcal species – *S. chromogenes* (33 %), *S. xylosus* (28 %), *S. sciuri* (13 %), *S. haemolyticus* (9 %), *S. epidermidis* (4 %), *S. simulans* (4 %), *S. warneri* (3 %), *S. equorum* (2 %), *S. hyicus* (2 %), *S. cohnii* (1 %), *S. succinus* (1 %), and *S. fleuretti* (1 %). Resistance rates in CNS were high, with 39% of isolates exhibiting resistance to ampicillin and penicillin, 6% of isolates being resistant to amoxicillin with clavulanic acid, ceftiofur, cephalothin, and cefoxitin, and 5 % being resistant to erythromycin. In rare cases resistance to gentamicin (2 %), kanamycin (2 %), and kanamycin-cefalexin (1 %) was detected.

Keywords: coagulase-negative staphylococci, bovine-mastitis, resistance profiles, MALDI-TOF

Speziesverteilung und Resistenzprofile Koagulase-negativer Staphylokokken isoliert von Rindern mit Mastitis in der Schweiz

Koagulase-negative Staphylokokken (CNS) stellen die häufigste Ursache intramammärer Infektionen beim Rind dar. Sie können chronische Infektionen verursachen und den somatischen Zellgehalt der Milch deutlich erhöhen. Ziel dieser Studie war es, die Speziesverteilung und Resistenzprofile von CNS in bovinen Mastitismilchproben zu bestimmen. Zwischen März 2011 und Februar 2012 wurden 120 CNS aus Mastitismilchproben von 117 verschiedenen Kühen auf 77 landwirtschaftlichen Betrieben isoliert. Die Isolate wurden mittels MALDI-TOF MS (matrix-assisted laser desorption ionization – time of flight mass spectrometry) Technologie identifiziert und anschließend mittels Agardiffusion auf ihre Empfindlichkeit gegenüber diverser Antibiotika getestet. Die getesteten Antibiotika wurden vor allem anhand ihrer Relevanz in Bezug auf die Behandlung boviner Mastitiden in der Schweiz ausgewählt. MALDI-TOF MS ordnete die 120 Isolate 12 verschiedenen Staphylokokken Spezies zu – *S. chromogenes* (33 %), *S. xylosus* (28 %), *S. sciuri* (13 %), *S. haemolyticus* (9 %), *S. epidermidis* (4 %), *S. simulans* (4 %), *S. warneri* (3 %), *S. equorum* (2 %), *S. hyicus* (2 %), *S. cohnii* (1 %), *S. succinus* (1 %) und *S. fleuretti* (1 %). Insgesamt betrachtet lag der Anteil resistenter CNS hoch, wobei 39 % der Isolate als resistent gegen Ampicillin und Penicillin, 6 % als resistent gegen Amoxicillin mit Clavulansäure, Ceftiofur, Cephalothin und Cefoxitin und 5 % als resistent gegen Erythromycin eingestuft wurden. In seltenen Fällen wurden auch Resistenzen gegen Gentamicin (2 %), Kanamycin (2 %) und Kanamycin-Cefalexin (1 %) gefunden.

Schlüsselwörter: Identifizierung, CNS, Resistenzprofile, MADLI-TOF

Introduction

Mastitis remains a major challenge to the worldwide dairy industry. While coagulase-negative staphylococci (CNS) are often referred to as "minor pathogens" limited to subclinical or mild cases of mastitis, their clinical relevance is controversially discussed (Supré et al., 2011; De Vliegher et al., 2012). CNS are the most common bacteria associated with bovine intramammary infections (IMI) (Makovec and Ruegg, 2003; Tenhagen et al., 2006; Schukken et al., 2009). They can cause chronic infections (Gillespie et al. 2009), exhibit virulence factors such as cytotoxins (Allaker et al., 1991; Zhang and Maddox, 2000; Taponen and Pyörälä, 2009), and were shown to represent a risk factor for acquiring *Staphylococcus (S). aureus* intramammary infections (Reyher et al., 2012).
Regarding the effect of CNS IMI on milk production, different studies yield conflicting results. While some reported decreased milk production (Green et al., 2006), others observed a slight but significant increase in milk production in cows with CNS IMI compared to culture-negative cows (Gillespie et al., 2009; Schukken et al., 2009).
CNS were shown to increase milk somatic cell counts (SCC) in individual cows and can contribute significantly to bulk milk SCC on herd level, espcecially in herds with a bulk milk SCC below 200'000 cells/mL (Schukken et al., 2009). The effect of CNS on SCC differs between species (Supré et al., 2011), stressing the need to gain more information on the distribution of different CNS. However, the prevalence of CNS isolated from bovine milk varies greatly in different geographical locations (Gillespie et al., 2009; Piessens et al., 2011; Waller et al., 2011; Pate et al., 2012) and to date, there is no data on the prevalence and species distribution of CNS in Switzerland. As CNS tend to be more resistant to antimicrobial agents than *S. aureus* and develop multiresistance easily (Taponen and Pyörälä, 2009), close surveillance of antimicrobial resistance is necessary to ensure the reasonable use of antibiotic agents and to control the development and spread of resistant strains. Therefore, the goal of our study was to determine the species distribution and antimicrobial susceptibility of CNS in bovine milk samples in Switzerland.

Material and Methods

Bacterial Isolates

A total of 120 coagulase-negative staphylococci (CNS) from clinical and subclinical cases of bovine mastitis were isolated from 117 different animals (77 different farms distributed in the cantons Zürich, Zug, Graubünden, Aargau, Thurgau, St. Gallen). The isolates were collected between March 2011 and February 2012. Milk samples were collected from the affected quarter of each cow in an aseptical manner during farm calls by the attending veterinarian. Milk samples were streaked onto sheep blood agar (Oxoid, Pratteln, Switzerland), as well as onto bromothymol blue lactose cystine agar (VWR International AG, Dietikon, Switzerland) and incubated at 37 °C over night. All gram-positive, catalase-positive cocci forming black colonies without an opaque zone on rabbit plasma fibrinogen agar (Oxoid, Cambridge, United Kingdom) were classified as presumptive CNS. The isolates were stored at –80 °C.

Species Identification

CNS species identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Briefly, one colony of a fresh overnight culture of all 120 isolates grown on sheep blood agar (Oxoid, Pratteln, Switzerland) was spotted in duplicate on a steel target plate, overlaid with 1 µl 25 % formic acid, air-dried and again overlaid with 1 µl matrix consisting of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Buchs, Switzerland) in 33 % acetonitrile (Sigma-Aldrich), 33 % ethanol and supplemented with 3 % trifluoroacetic acid, and air-dried within minutes at room temperature. Spectra were acquired using an Axima™ confidence (Shimadzu-Biotech Corp., Kyoto, Japan) MALDI-TOF mass spectrometer in linear positive mode at a laser frequency of 50 Hz and within a mass range from 2000–20000 Da. Acceleration voltage equalled 20 kV, and the extraction delay time was 200 ns. A minimum of 10 laser shots was used to generate each ion spectrum. For each bacterial sample, a total of 50 ion spectra per spot were averaged and processed using the Launchpad™ v.2.8 Software (Shimadzu-Biotech Corp., Kyoto, Japan). Peaks lists were imported in ASCII format into SARAMIS™ (Spectral Archive And Microbial Identification System, AnagnosTec, Potsdam-Golm, Germany) for automated species identification.
To confirm species identification, 23 randomly chosen strains were identified by sequencing an internal fragment of the *sod*A gene following a protocol of Poyart et al. (2001) with slight modifications. PCRs were performed in a T3000 thermocycler (Biometra, Goettingen, Germany) in a final volume of 50 µL containing a minimum of 150 ng of DNA, 2 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 2 U of FastStart High Fidelity polymerase (Roche, Basel, Switzerland), and 1x reaction buffer containing 18 mM $MgCl_2$. PCR mixtures underwent an initial denaturation for 3 min at 95 °C and were subsequently subjected to 30 cycles of amplification: 30 s denaturation at 95 °C, 60 s annealing at 39 °C, and 45 s elongation at 72 °C. Amplicons were purified, sequenced and the results were blasted against the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Susceptibility Testing

The isolates were subjected to antimicrobial susceptibility testing using the standard disk diffusion method to

classify isolates as susceptible, intermediate, or resistant depending on respective zone diameters following CLSI standard protocols (Clinical and Laboratory Standards Institute, 2008). All antimicrobial agents were chosen with regard to their relevance in mastitis therapy. Antibiotic agents tested included ampicillin (30 µg), amoxicillin (20 µg) with clavulanic acid (10 µg), cephalothin (30 µg), ceftiofur (30 µg), erythromycin (15 µg), cefoxitin (30 µg), gentamicin (10 µg), kanamycin (30 µg), kanamycin-cefalexin (30 µg/15 µg), penicillin (10 U.I.), and penicillin-novobiocin (10 U.I/30 µg). Mueller-Hinton agar, as well as disks containing ceftiofur and penicillin-novobiocin were provided by Oxoid, while disks containing cefalexin-kanamycin (Ubrolexin®) were provided by Boehringer Ingelheim (Basel, Switzerland). All other disks containing antibiotic agents were obtained from Becton Dickinson (Basel, Switzerland). The direct colony suspension method (recommended by CLSI for CNS) was used to prepare bacterial suspension of 0.5 Mac Farland turbidity. Subsequently, bacterial suspensions were swabbed uniformly across a Müller-Hinton agar plate and filter paper disks impregnated with the respective antimicrobial agents were placed on the surface of the agar using a dispenser. Plates were incubated at 35 °C for 18 h and the zone of inhibition (in mm) was assessed and measured using a calliper. Accuracy of the test system was monitored by including the reference strain *S. aureus* ATCC 25923. The presence of the *mecA* gene was confirmed by PCR for all cefoxitin resistant strains using the primers and cycling conditions published by Mehrotra et al. (2000).

Results and Discussion

Using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), the 120 strains belonged to twelve different staphylococcal subspecies – *S. chromogenes* (33 %), *S. xylosus* (28 %), *S. sciuri* (13 %), *S. haemolyticus* (9 %), *S. epidermidis* (4 %), *S. simulans* (4 %), *S. warneri* (3 %), *S. equorum* (2 %), *S. hyicus* (2 %), *S. cohnii* (1 %), *S. succinus* (1 %), and *S. fleuretti* (1 %) (Fig. 1). MALDI-TOF MS identification was confirmed by sequencing *sod*A in a subset of 23 isolates, proving MALDI-TOF MS to be a fast and reliable tool for species identification of CNS that is suitable for screening large numbers of samples.

The antimicrobial susceptibility data generated by disk diffusion is listed in Table 1. Overall, resistance rates in CNS were high, with 39 % of isolates exhibiting resistance to ampicillin and penicillin, 6 % of isolates being resistant to amoxicillin with clavulanic acid, ceftiofur, cephalothin, and cefoxitin, and 5 % being resistant to erythromycin. In rare cases resistance to gentamicin (2 %), kanamycin (2 %), and kanamycin-cefalexin (1 %) was detected. The only antimicrobial agent that all CNS isolates were susceptible to was penicillin-novobiocin, which represents the single antimicrobial agent tested in this study that has not been licenced for bovine mastitis therapy in Switzerland yet. *S. sciuri* and *S. haemolyticus* exhibited especially high rates of multiresistant strains, with one strain each being susceptible to only one of the licensed antimicrobial agents tested (*S. sciuri*: erythromycin, *S. haemolyti-*

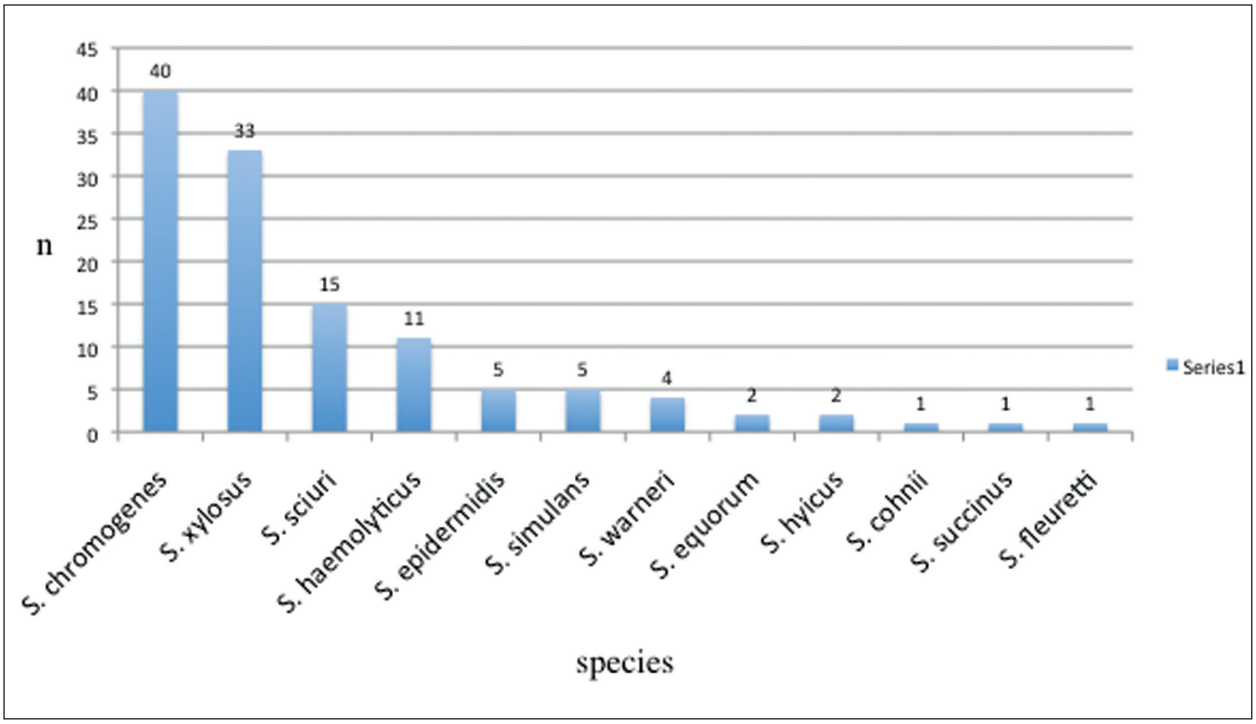

Figure 1: Species distribution of the 120 CNS strains.

Table 1: Overview on the antimicrobial susceptibility of the 120 CNS strains tested.

Species	No. of strains	No. of resistant strains[1]										
		AM	P	AMC	EFT	CF	FOX	E	GM	K	K/CFX	P/NB
S. chromogenes	n = 40	9	9	0	0	0	0	1	0	0	0	0
S. xylosus	n = 33	20	20	0	0	0	0	(2)[2]	0	0	0	0
S. sciuri	n = 15	6	6	5	5	5	5	1 (1)[2]	1	1	1	0
S. haemolyticus	n = 11	5	5	1	1	1	1	1	1	1	0	0
S. epidermidis	n = 5	3	3	0	0	0	0	0	0	0	0	0
S. simulans	n = 5	0	0	0	0	0	0	0	0	0	0	0
S. warneri	n = 4	2	2	0	0	0	0	0	0	0	0	0
S. equorum	n = 2	0	0	0	0	0	0	2	0	0	0	0
S. hyicus	n = 2	0	0	0	0	0	0	0	0	0	0	0
S. cohnii	n = 1	0	0	0	0	0	0	1	0	0	0	0
S. succinus	n = 1	1	1	1	1	1	1	(1)[2]	0	0	0	0
S. fleuretti	n = 1	1	1	0	0	0	0	0	0	0	0	0
Total	120	47	47	7	7	7	7	6	2	2	1	0

[1] Abbreviations: AM = ampicillin, P = penicillin, AMC = amoxicillin with clavulanic acid, EFT = ceftiofur, CF = cephalothin, FOX = cefoxitin, E = erythromycin, GM = gentamicin, K = kanamycin, K/CFX = kanamycin-cefalexin, P/NB = penicillin-novobiocin

[2] Numbers in brackets equal the percentage of strains that exhibited intermediate resistance to the antibiotic agents tested

cus: kanamycin-cefalexin). All *S. simulans* and *S. hyicus* isolates were susceptible to all antimicrobial agents tested. Prevalence and antimicrobial resistance rates that can be found in the literature need to be treated with caution, as most authors relied on biochemical/phenotypic characteristics that were recently shown not to be suited for CNS species identification (Taponen and Pyörälä, 2009; De Vliegher et al., 2012). *S. chromogenes*, the species most frequently isolated from bovine milk samples in our study, was reported to represent the most common CNS species in bovine IMI (Taponen and Pyörälä, 2009). In contrast, we found only low numbers of *S. simulans*, a CNS species very frequently identified by previous studies (Jarp, 1991; Waage et al., 1999). In our study, we also detected a high rate of *S. xylosus* among the total number of CNS (28 %). *S. chromogenes*, *S. simulans*, and *S. xylosus* were reported to induce a particularly strong increase in milk SCC, comparable to *S. aureus* (Supré et al., 2011).

The antimicrobial resistance rates that we determined among CNS isolated from bovine milk samples in Switzerland resemble those of other European studies. Penicillin resistance for CNS obtained from mastitis equalled 36 % in Norway, 25 % in Denmark, 41–61 % in the Netherlands, and 32 % in Finland (Taponen and Pyörälä, 2009). We detected the *mecA* gene in 6 % of all CNS isolates, comprising five of the 15 *S. sciuri*, one of the 11 *S. haemolyticus*, and the only isolated *S. succinus* strain. This is in accordance with data from Finland and the Netherlands, reporting higher prevalence of *mecA* in CNS compared to *S. aureus* (Taponen and Pyörälä, 2009). Moreover, the species distribution of strains harbouring the *mecA* gene fits with the data of a recently published study with a focus on methicillin resistant CNS (Huber et al., 2011).

The antimicrobial resistance rates determined for different species varied greatly in our study. Thus, the common research practice of investigating CNS as a group, while omitting discrimination on a species level, seems highly questionable. MALDI-TOF MS was shown to enable fast and reliable species identification of CNS, suitable for its application in the routine mastitis diagnostic.

Acknowledgements

The authors would like to thank Dr. Sabrina Corti for her kind support in collecting the CNS isolates.

Répartition des espèces et profils de résistance des staphylocoques coagulase négatifs isolé sur des vaches souffrant de mammites en Suisse

Les staphylocoques coagulase négatifs (CNS) sont la cause la plus fréquente d'infections intra mammaires

Distribuzione delle specie e profili di resistenza degli stafilococchi coagulasi-negativi isolati da bovini affetti da mastite in Svizzera

Gli stafilococchi coagulasi-negativi (CNS) rappresentano la causa più comune delle infezioni intramam-

chez les bovins. Ils peuvent causer des infections chroniques et augmenter nettement le nombre de cellules somatiques du lait. Le but de la présente étude était de déterminer la répartition des espèces et les profils de réséstance des CNS dans des échantillons de lait mammiteux bovins. De mars 2011 à février 2012, 120 CNS ont été isolé à partir d'échantillons de lait issus de 117 vaches provenant de 77 exploitations. Les isolats ont été identifiés par la technologie MALDI-TOF MS (matrix-assisted laser desorption ionization – time of flight mass spectrometry) puis testés par diffusion sur gel d'agar quant à leur sensibilité vis à vis de divers antibiotiques. Les antibiotiques ont été choisis principalement par rapport à leur importance quant aux traitements des mammites bovines en Suisse. Le test MALDI-TOF MS a classifié les 120 isola en 12 espèces différentes de staphylocoaques: *S. chromogenes* (33 %), *S. xylosus* (28 %), *S. sciuri* (13 %), *S. haemolyticus* (9 %), *S. epidermidis* (4 %), *S. simulans* (4 %), *S. warneri* (3 %), *S. equorum* (2 %), *S. hyicus* (2 %), *S. cohnii* (1 %), *S. succinus* (1 %) et *S. fleuretti* (1 %). D'une façon générale, la proportion de CNS résistants était élevée, 39 % des échantillons étant résistants à l'ampicilline et à la pénicilline, 6 % à l'amoxicilline avec acide clavulanique, au ceftiofur, à la céphalothine et à la céfoxitine et 5 % à l'érythromycine. Dans de rares cas on a trouvé des resistances à la gentamicine (2 %), à la kanamycine (2 %) et à la kanamycine-céfalexine (1 %).

marie nei bovini. Essi possono causare infezioni croniche e aumentare significativamente il contenuto di cellule somatiche del latte. Lo scopo di questo studio è di determinare la distribuzione delle specie e i profili di resistenza dei CNS in campioni di latte con mastite bovina. Tra marzo 2011 e febbraio 2012 sono stati isolati 120 CNS da campioni di latte con mastite di 117 diversi bovini provenienti da 77 aziende agricole. Gli isolati sono stati identificati mediante la tecnologia MALDI-TOF MS (matrix-assisted laser desorption ionization – time of flight mass spectrometry) e infine è stata testata la loro sensibilità, per diffusione su agar, ai vari antibiotici. Gli antibiotici testati sono stati selezionati principalmente sulla base della loro rilevanza per il trattamento della mastite bovina in Svizzera. MALDI-TOF MS ha associato i 120 isolati a 12 specie diverse di stafilococchi – *S. cromogeni* (33 %), *S. xylosus* (28 %), *S. sciuri* (13 %), *S. haemolyticus* (9 %), *S. epidermidis* (4 %), *S. simulans* (4 %) *S. warneri* (3 %), *S. equorum* (2 %), *S. hyicus* (2 %), *S. cohnii* (1 %), *S. succinus* (1 %) e *S. fleuretti* (1 %). Nel complesso, la percentuale di CNS resistenti era alta, anche se il 39 % degli isolati sono stati classificati come resistenti alla ampicillina e alla penicillina, il 6 % erano resistenti all'amoxicillina con acido clavulanico, ceftiofur, cefalotina e cefoxitina e il 5 % erano resistenti alla eritromicina. In rari casi sono stati trovati anche resistenze alla gentamicina (2 %), canamicina (2 %), e canamicina-cefalexina (1 %).

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Received: 19 September 2012
Accepted: 7 November 2012