Schweizer Archiv für Tierheilkunde
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F. Rüegsegger et al., Band 156, Heft 11, November 2014, 539–542
DOI 10.1024/0036-7281/a000644

Toxic bovine mastitis caused by *Staphylococcus aureus* in twin cows

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Summary

In this report, we describe two cases of bovine toxic mastitis associated with *S. aureus* and we provide DNA microarray based characterization data of the strain causing the disease. Both cows had recently calved and suffered from anorexia, pyrexia, and an elevated heart rate. In both animals, at least one mammary gland was swollen, hardened, sensitive to touch, and produced brownish or bloody secretions. The clinical state of the animals deteriorated quickly and both cows had to be euthanized within 48 hours after presentation. The *S. aureus* strain, which was isolated from the mastitis milk of both cows, was assigned to *spa* type t267, *agr* type I, capsule type 5 and CC97, a clonal complex recently identified as the evolutionary origin of two emerging clones of human epidemic community-associated methicillin-resistant *S. aureus*. The strain did not harbour any genes conferring resistance to antimicrobial agents and we did not detect any genes coding for enterotoxins, toxic shock syndrome toxin, or exfoliative toxins. Taking into consideration that twin cows were affected by this rare disease, we suggest that host factors may play a crucial role in toxic mastitis associated with *S. aureus*.

Keywords: *Staphylococcus aureus*, toxic mastitis, cattle, DNA microarray, CC97

Toxische *Staphylococcus aureus* Mastitiden bei Zwillingsrindern

In dieser Arbeit berichten wir von toxischen Mastitiden bei Zwillingsrindern, die mit *S. aureus* in Verbindung gebracht wurden, und charakterisieren den aus beiden Fällen isolierten *S. aureus* Stamm mithilfe eines DNA Microarrays. Beide Rinder, die kurz vor der Einweisung gekalbt hatten, zeigten Fressunlust, hohes Fieber und eine erhöhte Herzfrequenz. Bei beiden Kühen war mindestens ein Euterviertel geschwollen, verhärtet und schmerzhaft und produzierte bräunliches oder blutiges Sekret. Der klinische Zustand der Tiere verschlechterte sich schnell und die Kühe mussten innerhalb 48 Stunden nach ihrer Vorstellung euthanasiert werden. Der aus Mastitismilch beider Rinder isolierte *S. aureus* Stamm konnte dem *spa* Typ t267, *agr* Typ I, Kapseltyp 5 und dem klonalen Komplex CC97 zugeordnet werden, der kürzlich als evolutionärer Ursprung zweier Klone beim Menschen epidemisch vorkommender sogenannter community-associated MRSA identifiziert wurde. Der *S. aureus* Stamm wies keine Antibiotikaresistenzgene auf. Zudem konnten keine Gene, die für Enterotoxine, das Toxische Schock Syndrom Toxin oder Exfoliativtoxine kodieren, gefunden werden. In Anbetracht dessen, dass Zwillingskühe von dieser seltenen Erkrankung mit dem selben Stamm betroffen waren, könnten Wirtsfaktoren bei der Entstehung der toxischen Mastitis beim Rind eine entscheidende Rolle spielen.

Schlüsselwörter: *Staphylococcus aureus*, toxische Mastitis, Rind, DNA microarray, CC97

Introduction

Bovine mastitis represents a major challenge to the worldwide dairy industry and causes severe economic losses, arising not only from decreased milk production, but also from costs of treatment, premature culling, and death. *Staphylococcus (S.) aureus* can lead to subclinical, acute or chronic mastitis, and has recently been associated with the rare symptom complex of "toxic mastitis" in cattle, which is characterized by a combination of mastitis symptoms and clinical signs of toxemia including lethargy, tachycardia, anorexia, pyrexia or hypothermia, as well as muscle weakness (Andrews et al., 2004; Menzies et al., 2000; Bleul et al., 2006;). However, previous studies investigating bovine toxic mastitis mainly focus on coliforms, as *S. aureus* causes only 3–17% of toxic

mastitis cases (Menzies et al., 2000; Menzies et al., 2003; Bleul et al., 2006). Thus, to date, information on bovine toxic mastitis caused by *S. aureus* is extremely scarce and pathogenesis is unclear. In this report, we describe two cases of *S. aureus* toxic mastitis that occurred in twin cows and we provide characterization data of the strain that caused the disease. Our objective was to (1) provide data on the clinical course of toxic *S. aureus* mastitis and (2) collect information on the genetic traits of an *S. aureus* strain able to cause toxic mastitis in order to identify potential pathophysiological mechanisms.

Case 1 and 2

The two cases of toxic mastitis occurred in 6-year old Simmental twin cows that originated from the same farm and had recently calved (48 h and 24 h). The cows were presented to the Veterinary Ambulatory Clinic (University of Zurich) on December 3rd, 2013, and January 20th, 2014, respectively. Cow1 was presented due to fever and anorexia and had been treated in the previous lactation due to clinical *S. aureus* mastitis following an udder lesion (quarter B) caused by traumatic insult. Cow2 was in sternal recumbency and its skin was cold. Both animals showed anorexia, pyrexia with a rectal temperature of 41.5 °C and 40.0 °C, an elevated heart rate (120 bpm; 112 bpm), and an elevated respiratory rate (40 breaths/min; 44 breaths/min). Cow2 showed reddened oral mucous membranes with a capillary refill time of 2 to 3 seconds. Examination of the digestive system revealed reduced ruminal motility in cow1 and no ruminal motility in cow2. Swinging and percussion auscultation were negative on both sides. The consistency of the feces equaled a Skidmore score of 3 (Skidmore et al., 1996). A gynecological exam showed vaginal discharge of clear mucus. There were no signs of retained fetal membranes. In both cows, examination of the mammary gland revealed painful swelling and hardening in at least one quarter, with grossly changed secretion. In cow1, quarter B was affected with thickened brownish secretions that contained some necrotic tissue. In cow 2, quarter C and D were affected and showed bloody and watery secretion. The clinical exams resulted in no further pathological findings. The preliminary diagnoses were toxic mastitis or hypocalcemia, as the clinical exam did not suggest toxic metritis, right displaced abomasum, ileus, or salmonellosis.

Treatment

A milk sample was collected from the affected quarters for microbiological examination and the mammary gland was hand-stripped and flushed using 0.9 % sodium chloride solution. Both cows were treated using Cloxacoli intramammary injectors containing a combination of cloxacillin and colistin twice daily (Virbac AG, Glattbrugg, Switzerland) and flunixin meglumine (2.2 mg/kg Fluniximin i.v., Dr. E. Graeub AG, Berne, Switzerland). Isotonic sodium chloride solution supplemented with glucose (9 g/L NaCl + 50 g/L glucose) was administered intravenously (40 L/24 h) through an indwelling catheter. In addition, cow 1 received marbofloxacin (2 mg/kg 10 % Marbocyl, Vétoquinol AG, Ittigen, Switzerland) and cow2 received vitamin E + Selen (15 mg sodium selenide, 375 mg α-tocopheryl acetate, s.c., Tocoselenit, Dr. E. Graeub AG, Berne, Switzerland) and 500 mL Calcamyl 40-MP (3.13 g calcium gluconate and borogluconate, 0.55 g magnesium and 1.42 g phosphorus as magnesium hypophosphite per 100 mL, Dr. E. Graeub AG, Berne, Switzerland) intravenously.
The state of both animals deteriorated quickly. On the following day, the cows showed decreased rectal temperatures (39.5 °C; 38.3 °C) and respiratory rates (36 breaths/min; 40 breaths/min). Although therapy was continued, the swelling of the affected mammary glands increased. As cow2 was found in lateral recumbency and exhibited a heart rate of 230 bpm, it was euthanized. In cow1, the parenchyma of gland B was profoundly hardened and the gland produced watery secretion. Its state further deteriorated over night. Cow1 was found in lateral recumbency and showed anorexia and grinding of teeth. The animal exhibited a rectal temperature of 38.2 °C, reddened mucous membranes, a heart rate of 120 bpm and a respiratory rate of 40 breaths/min. The cow was euthanized due to a poor prognosis.

Bacterial isolation and genotyping

The milk samples were plated onto Brolacin agar (Merck, Zug, Switzerland), as well as 5 % sheep blood agar, which yielded white colonies exhibiting double zone hemolysis suggestive of *S. aureus*. The putative *S. aureus* isolates from both cows were finally confirmed using the species-specific markers of the DNA microarray Genotyping Kit (Alere Technologies GmbH, Jena, Germany).
For the microarray based genotyping, cells were lysed and DNA was extracted using the *S. aureus* Genotyping Kit and the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the concentration of nucleic acids. The DNA microarray enabled detection of the absence or presence of 333 genes and their allelic variants including species markers and virulence genes, as well as genes conferring resistance to antimicrobial agents. Microarray results also allowed for assignment of the strain to a clonal complex (Monecke et al., 2008).
We performed *spa* typing as previously described (Wattinger et al., 2012). The polymorphic X region of the *spa* gene was determined using PCR amplification, PCR purification, and subsequent sequencing. The *spa* type was

assigned using the *spa*-server (http://spa.ridom.de/) as described elsewhere (Harmsen and Claus, 2003).

Results

S. aureus was isolated from mastitis milk samples of both cows. The isolates were assigned to *spa* type t267 and CC97. The DNA microarray allows not only for identification of virulence genes, but also for the detection of a vast range of resistance genes, including *blaZ* and *mecA* involved in beta lactam and methicillin resistance, respectively. For SA1608, we did not detect any genes conferring resistance to antimicrobial agents. SA1608 belonged to *agr* type I and capsule type 5. It exhibited various genes encoding hemolysins, leukocidins, adhesion factors, and proteases (Tab. 1). In contrast, no genes of the immune evasion cluster were detected such as *sak* (encoding staphylokinase), *scn* (encoding the staphylococcal complement inhibitor), and *chp* (encoding the chemotaxis inhibitory protein). The strain exhibited neither enterotoxin genes involved in food poisoning, nor genes encoding superantigens associated with the toxic shock syndrome (*tst*) or the staphylococcal scalded skin syndrome (*etA/B/D*).

Discussion

The results of the toxic *S. aureus* mastitis described in this report are highly consistent with the results of a study from Northern Ireland investigating bovine toxic mastitis in general. Menzies et al. showed that toxic mastitis cases occur preferably from November to March (84 %) and within 4 days of calving (29 %) (Menzies et al., 2000).
The isolates were assigned to *spa* type t267. While strains of this *spa* type have been detected among bovine mastitis strains in Switzerland (Johler et al., 2011), no cases of toxic mastitis were associated with these isolates. The isolates were also assigned to CC97, a clonal complex detected amongst 16–21 % of *S. aureus* strains isolated from bovine mastitis milk in Switzerland (Johler et al., 2011; Moser et al., 2013). Bovine *S. aureus* strains of CC97 were recently identified as the evolutionary origin of two emerging clones of human epidemic community-associated methicillin-resistant *S. aureus* (CA-MRSA) (Spoor et al., 2013). Acquisition of mobile genetic elements such as plasmids, phages, and *S. aureus* pathogenicity islands is common among *S. aureus*. It was suggested that after the host jump to humans, the bovine methicillin sensitive CC97 strains acquired various genes encoding virulence factors and antimicrobial resistance genes that facilitate their global spread amongst the human population (Spoor et al., 2013). As the isolates exhibited identical *spa* types and clonal complexes, and highly similar microarray profiles, they were identified as the same *S. aureus* strain (SA1608).
In the DNA microarray analysis, we did not detect any genes in SA1608 that confer resistance to antimicrobial agents, consistent with studies reporting only very low rates of bovine *S. aureus* CC97 strains exhibiting resistance to antibiotic agents (Moser et al., 2013; Spoor et al., 2013). SA1608 exhibited various genes encoding hemolysins, leukocidins, adhesion factors, and proteases that are common among bovine *S. aureus* in Switzerland (Johler et al., 2011; Moser et al., 2013). We also detected several genes coding for virulence factors for which a ruminant host-specific activity was demonstrated, including the lukM/F leukotoxin or the van Willebrand factor binding protein (Guinane et al., 2010; Viana et al., 2010). In contrast, no genes of the immune evasion cluster were detected such as *sak* (encoding staphylokinase), *scn* (encoding the staphylococcal complement inhibitor), and *chp* (encoding the chemotaxis inhibitory protein). This is consistent with the fact that these genes typically are associated with immune evasion in the human and not in the bovine host (van Wamel et al., 2006). Interestingly, the strain exhibited neither enterotoxin genes involved in food poisoning, nor genes encoding superantigens associated with the toxic shock syndrome (*tst*) or the staphylococcal scalded skin syndrome (*etA/B/D*). This could be

Table 1: DNA microarray results for selected virulence genes detected in *S. aureus* strain SA1608.

Group	Gene/Probe	Virulence factor/Function
hemolysins & leukocidins	hla	alpha hemolysin (cytotoxin)
	hlb	beta hemolysin (cytotoxin)
	hlgA, lukS	gamma hemolysin (cytotoxin)
	hld	delta hemolysin (cytotoxin)
	lukM/F	bovine leukocidin (cytotoxin)
	lukD/E	leukocidin (cytotoxin)
Proteases	aur	aureolysin (complement inhibitor)
	splA/B/E	serine protease-like proteins A/B/E (exhibit sequence homology to epidermolytic toxins)
	sspA	glutamylendopeptidase (V8 protease)
	sspP/B	staphopain A/B protease

due to the fact that the strain carries an unknown allelic variant of one of these toxins that is therefore not detected by the DNA microarray.

Conclusion

To date, the pathogenesis of toxic mastitis caused by *S. aureus* is unclear. We were able to show that the cases of toxic mastitis in twin cows described in this report were caused by the same *S. aureus* strain SA1608. Screening of milk samples of all other cows in the herd (n = 30) revealed that the strain was exclusively present in the twin cows suffering from toxic mastitis. While strain SA1608 harbors genes encoding hemolysins, leukocidins and proteases, we did not detect genes encoding well-known superantigenic toxins including toxic shock syndrome toxin, enterotoxins, exfoliative toxins, and panton valentine leukocidin. Taking into consideration that twin cows were affected by this rare disease and the same *S. aureus* strain, we suggest that host factors may play a crucial role in toxic mastitis associated with *S. aureus*.

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Received: 15 January 2014
Accepted: 21 March 2014