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ORIGINAL ARTICLE

Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis

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Significance and Impact of the Study: This study confirms the presence of ST398 MRSA in milk from bovine mastitis in Belgium. Moreover, the isolated MRSA strains were described for genotypic and phenotypic characteristics potentially implicated in virulence. This study highlights that Belgian bovine could be a reservoir of MRSA for human.

Keywords
Belgium, genotyping, mastitis, MRSA, phenotyping.

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Abstract

The aim of this study was to evaluate the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) among a (*S. aureus*) collection ($n = 430$) isolated from milk of cows suffering from mastitis in Belgium and to compare their genotypic as well as phenotypic characteristics. Pulsed field gel electrophoresis (PFGE) and PCR-based typing techniques (MLST, *spa*, SCC*mec*, and *agr* typing) have been applied and supplemented by capsule serotyping, biofilm production quantification and antimicrobial susceptibility testing. Nineteen MRSA were isolated. Seven distinct *Apa*I PFGE patterns were observed. All isolates, except one, were identified as ST398 strains. Three *spa* types (t011, t567 and t108) and two SCC*mec* types (IV and V) were identified. All isolates belonged to *agr* type I and capsule type 5 and were Panton-Valentine leukocidin (PVL) negative. All isolates produced biofilm in TSB_{glc}, whereas the majority did not in milk serum. Twelve resistance patterns were observed, with almost two-thirds of the isolates being resistant to at least six antibiotics, including penicillin and tetracycline. Our study confirms that the emerging ST398 LA-MRSA clone has attained Belgian cattle. With regard to genotypic and phenotypic typing, the 19 MRSA isolated in this study form a homogenous group and do not differ much from one another, neither from what has been previously described.

Introduction

Staphylococcus aureus is recognized worldwide as a pathogen causing many serious diseases in humans and animals. In the last five decades, methicillin-resistant *S. aureus* (MRSA) have spread as human hospital-acquired pathogens (HA-MRSA) throughout the world. More recently, community-acquired (CA-MRSA) and livestock-associated (LA-MRSA) MRSA have emerged. MRSA isolated from livestock (such as pigs, cattle or poultry) have been increasingly reported, and the zoonotic risk of transmission to human has already been demonstrated. Indeed, MRSA ST398, the prototype clone of LA-MRSA, has already been found in human, often in close contact with animals (such as veterinarians or farmers) (Van Cleef *et al.* 2011). Moreover, methicillin resistance, which is encoded by the *mecA* gene, could complicate the treatment of bovine mastitis caused by *S. aureus* (Lowy 2003). Indeed, this mechanism also confers resistance to almost all types of beta-lactam

antibiotics still commonly used in mastitis treatment. Although little is known about MRSA isolated from cattle, several studies suggest that the prevalence of MRSA in mastitis is generally low (Juhasz-Kaszanyitzky *et al.* 2007; Moon *et al.* 2007; Hendriksen *et al.* 2008).

As MRSA-contaminated bovine milk could be a source of human MRSA infection, the screening of milk sampled from bovine mastitis cases and the genotyping of the isolates are essential to estimate the MRSA emergence and the related zoonotic hazard (Turkyilmaz *et al.* 2010). Many different typing methods have been developed for *S. aureus* epidemiological studies: pulsed field gel electrophoresis (PFGE); multilocus sequence typing (MLST) of seven housekeeping genes; polymorphism of protein A gene (*spa* typing) or accessory gene regulator gene (*agr* typing); and staphylococcal cassette chromosome *mec* (SCC*mec*) typing.

Besides these molecular techniques, the virulent character of *S. aureus* strains can be evaluated by the presence or absence of specific features. The Panton-Valentine leukocidin (PVL) is a cytotoxin involved in leucocyte destruction and tissue necrosis and could help the bacteria to persist and spread (Lina *et al.* 1999). The expression of capsular polysaccharide (CP) and the ability to produce biofilm are also two characteristics that could enhance the bacterial persistence in the host by avoiding the immune system. In addition, resistance to a panel of antibiotics is a well-known way to persist inside the host.

The aims of this study were then to (i) evaluate the presence of MRSA in a *S. aureus* collection isolated from milk of cows suffering from mastitis in Belgium; (ii) epidemiologically compare the isolated strains using PFGE, MLST, *spa*, SCC*mec* and *agr* typing and (iii) study their genotypic (presence of PVL- and capsular-encoding genes) and phenotypic characteristics (capsule serotyping, biofilm production quantification and antimicrobial susceptibility testing) potentially involved in the pathogenicity.

Results and discussion

Of the 430 *Staphylococcus aureus* isolates collected from bovine mastitis cases, 19 isolates (4·4%) were identified as methicillin-resistant *S. aureus*, and the presence of the *mecA* gene was confirmed by PCR for all 19 isolates.

As all except one isolates were observed to be nontypeable by *Sma*I PFGE, they were compared after *Apa*I digestion (Fig. 1). The 19 isolates showed seven distinct pulsotypes, which were grouped in three clusters namely A (n = 16), B (n = 2) and C (n = 1), using a cut-off at 80% similarity. According to MLST, all isolates of clusters A and B were identified as ST398 strains (Table 1). The cluster C strain was identified as ST8 strain. The ST398 strains were further associated with three different yet related *spa* types: t011 (n = 3), t567 (n = 4) and t108 (n = 11). The only ST8 strain was identified as *spa*-type t008. The detection of the SCC*mec* types identified two types of cassettes: a type V SCC*mec* (n = 15) and a type IV SCC*mec* (n = 4). Furthermore, all 19 isolates were shown to belong to *agr* type I. Interestingly, some indistinguishable PFGE pulsotypes present different *spa* types or resistance patterns. These could be easily explained by single genetic events (loss or gain of repeats for the *spa* typing and genes mutations for the antibiotic resistance) not visible using PFGE method.

With regard to molecular typing, 16 of the 19 MRSA isolated in this study formed a homogeneous group and did not differ much from one another, even with 11 appearing to be identical. According to MLST, 18 isolates belonged to the previously described CC398 clonal complex. This type of LA-MRSA has been incidentally responsible for hospital outbreaks in humans (Wulf *et al.* 2008; Kluytmans and Struelens 2009). Similarly to other studies, our ST398 strains predominantly belonged to t011 or related t108 and t567 *spa* types, as well as to IV or V SCC*mec* types (Witte *et al.* 2007; Fessler *et al.* 2010; Huber *et al.* 2010; Vanderhaeghen *et al.* 2010). The only

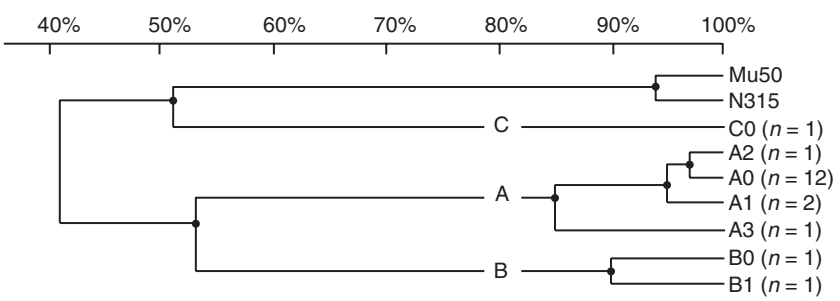

Figure 1 Pulsed field gel electrophoresis (PFGE) cluster analysis. The *ApaI* fragment patterns of the methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were compared with Mu50 and N315 reference strains. The numbers in brackets indicate the numbers of isolates that exhibited the respective fragment pattern. Using a cut-off at 80% similarity, the seven distinct pulsotypes were grouped in three major clusters (A–C).

non-ST398 isolate of our MRSA was identified as CC8-ST8-IV strain. This clone has already been described as one of the most predominant clones of HA-MRSA in the South of Belgium (Denis *et al.* 2006). This observation therefore raises the question of possible human-to-animal transmission of MRSA. Also in accordance with other studies, all of our MRSA isolates belonged to *agr* type I (Kadlec *et al.* 2009; Fessler *et al.* 2010). This *agr* type was recently associated with penicillin resistance (Melchior *et al.* 2009). Similarly, we could here state the same association between methicillin resistance and *agr* type I.

As reported in other studies, none of the isolates harboured the PVL-encoding genes (*lukF-PV* and *lukS-PV*), molecular marker of CA-MRSA strains, whereas all isolates were shown to carry the *cap5H* gene, gene involved in the type 5 CP (CP5) biosynthesis (Monecke *et al.* 2007; Kadlec *et al.* 2009; Fessler *et al.* 2010; Turkyilmaz *et al.* 2010). Using ELISA analysis, the majority of the isolates ($n = 16$) expressed this CP5, whereas the three remaining isolates were defined as nontypeable (NT). Concerning biofilm production, we observed that all isolates produced biofilm, at least weakly, in TSB_{glc}, whereas the majority did not in milk serum (Table 1).

We confirmed here that ST398 MRSA isolates from mastitis milk are exclusively capsule type 5. In addition, the majority of our isolates expressed this CP5 capsule phenotypically. Although its role in mastitis is still controversy, capsule may enhance host invasion due to its antiphagocytic properties. The high prevalence of *agr* type I strains in bovine mastitis has already been described, and the correlation between *agr* type I and CP5 expression was also observed (Buzzola *et al.* 2007). In addition, typically of *agr* type I strains, the majority of our isolates did not produce biofilm in bovine milk serum (Ikonomidis *et al.* 2009; Melchior *et al.* 2009).

As already documented, in addition to their resistance to almost all types of beta-lactam antibiotics, MRSA strains show resistance to a wide range of other antimicrobial agents, also used to treat or prevent mastitis (Sawant *et al.* 2005). Susceptibility tests revealed here a wide variety of 12 different resistance patterns among the 19 isolates (Table 1). All isolates were resistant to at least three antibiotics, and two-thirds of the strains (58%) were resistant to at least six of the sixteen antibiotics. In accordance with other studies, in addition to oxacillin, all of our MRSA were resistant to penicillin and tetracycline, but also to spectinomycin (Kadlec *et al.* 2009; Fessler *et al.* 2010; Huber *et al.* 2010; Vanderhaeghen *et al.* 2010). Contrastingly, they showed less resistance to erythromycin (7/19) and clindamycin (7/19) than previously described (Huber *et al.* 2010; Turkyilmaz *et al.* 2010; Vanderhaeghen *et al.* 2010). This high resistance percentage to some antibiotics can be explained by their frequent use in veterinary practice these recent years, and could lead to serious treatment problems in the future.

In conclusion, emergence of MRSA in cattle could hence complicate the treatment of bovine mastitis caused by *S. aureus*, with also a potential risk of animal-to-human

Table 1 Comparison of the characteristics of the 19 MRSA isolates

PFGE type	MLST	*spa* type	SCC*mec* type	*agr* type	Capsule serotype	Biofilm in TSB_{glc}	Biofilm in milk serum	Resistance pattern
C0 ($n = 1$)	8	t008	IV	I	NT	+	–	PEN, SPC, TET, AMP, ML, GEN, KAN, CHL, SXT, CFP
A2 ($n = 1$)	398	t567	V	I	CP5	+	+	PEN, SPC, AMP, CEX, ML
A0 ($n = 12$)	398	t108 ($n = 11$) t567 ($n = 1$)	V	I	CP5	+	–	PEN, SPC, TET ($n = 5$) PEN, SPC, TET, AMP ($n = 2$) PEN, SPC, TET, CEX ($n = 1$) PEN, SPC, TET, AMP, CEX ($n = 1$) PEN, SPC, TET, AMP, CEX, ML ($n = 1$) PEN, SPC, TET, CEX, ML, GEN, CHL ($n = 1$) PEN, SPC, TET, ML, GEN, KAN, CHL, SXT ($n = 1$)
A1 ($n = 2$)	398	t567	V	I	CP5 ($n = 1$) NT ($n = 1$)	+	+ ($n = 1$) – ($n = 1$)	PEN, SPC, TET, AMP, CEX, ML
A3 ($n = 1$)	398	t011	IV	I	CP5	+	–	PEN, SPC, TET, AMP, CEX, GEN, KAN
B0 ($n = 1$)	398	t011	IV	I	NT	+	–	PEN, SPC, TET, GEN, KAN, CHL
B1 ($n = 1$)	398	t011	IV	I	CP5	+	–	PEN, SPC, TET, AMP, CEX, GEN, KAN, CFP, XNL, CIP

NT, nontypeable; CP5, capsular polysaccharide type 5; AMP, ampicillin; CEX, cephalexin; CFP, cefoperazone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; ML, macrolide/lincosamide; MRSA, methicillin-resistant *Staphylococcus aureus*; MLST, multilocus sequence typing; PEN, penicillin; SPC, spectinomycin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; XNL, ceftiofur.

transmission. The screening of milk sampled from bovine mastitis cases and the genotypic analysis of the isolated strains are therefore essential to estimate MRSA spread and elucidate transmission mechanisms.

Since the first publication in 1972 (Devriese *et al.* 1972), most studies report a low prevalence of MRSA in mastitis (Juhasz-Kaszanyitzky *et al.* 2007; Moon *et al.* 2007; Hendriksen *et al.* 2008). Contrastingly, an unexpectedly high prevalence of mastitis-associated MRSA was recently reported in Belgium (Vanderhaeghen *et al.* 2010). As extensively discussed by these authors, comparison of prevalence data between studies is difficult, and this mainly due to differences in sampling methodology. Moreover, LA-MRSA prevalence may show large regional differences that reflect proximity with animals (van Cleef *et al.* 2011; Wassenberg *et al.* 2011; Vandendriessche *et al.* 2012). Even though reassuring, recent surveillance studies of MRSA carriage upon hospital admission in Belgium found a relatively low prevalence of LA-MRSA among hospitalized patients, suggesting that LA-MRSA contributes to only a minor fraction of all MRSA in humans (van Cleef *et al.* 2011; Wassenberg *et al.* 2011; Vandendriessche *et al.* 2012).

To further monitor epidemiology of MRSA in Belgian cattle, as well as to measure the impact of the recent campaigns to promote hand hygiene or rational use of antibiotics by farmers and veterinarians, more studies should be carried out based on systematic and well-defined sampling methodology.

Materials and methods

Bacterial isolates and MRSA identification

A total of 430 bovine *Staphylococcus aureus* isolates, isolated from different bovine mastitis cases between 2005 and 2008 in different areas of Wallonia (southern part of Belgium), were tested for methicillin resistance. These isolates were either obtained from the *Association Régionale de Santé et d'Identification Animales* (ARSIA) or isolated from milk samples directly received at the Bacteriology laboratory. All isolates were streaked onto chromogenic chromID™ MRSA agar (bioMérieux) and incubated for 24 h at 37°C. Blue green colonies, considered as positive MRSA colonies, were stored at −80°C in LB broth with 50% (v/v) glycerol until further characterization.

PCR-based genotyping

DNA extraction was carried out using the ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen) according to the manufacturer's instructions for staphylococci and stored at −20°C.

PCR detection for the penicillin-binding protein 2a gene (*mecA*), the PVL-encoding genes (*lukS-PV* and *lukF-PV*) and two genes involved in capsule biosynthesis (*cap5H* and *cap8H*) was performed as previously described (Ote *et al.* 2011).

Molecular typing based on the sequence of seven housekeeping genes (MLST) and typing of the polymorphic region of protein A (*spa* typing) were performed according to protocols described elsewhere (Shopsin *et al.* 1999; Enright *et al.* 2000). Each gene was amplified and sequenced using the same primers. The allele number and sequence type (ST) were assigned using the MLST website (http://saureus.mlst.net), whereas the numeric *spa* repeats and *spa*-type codes were determined using the Ridom SpaServer website (http://www.spaserver.ridom.de).

SCC*mec* and *agr* typing were carried out using multiplex PCR strategies described elsewhere (Shopsin *et al.* 2003; Milheirico *et al.* 2007). Amplification patterns were compared with the patterns of prototype strains used as positive controls.

Pulsed field gel electrophoresis

All of the isolates were analysed by pulsed field gel electrophoresis (PFGE) as already described (Kadlec *et al.* 2009). Chromosomal DNA was digested with 20U of *Sma*I or 10U of *Apa*I according to the manufacturer's instructions. Restricted fragments were separated through a 1% pulsed field certified agarose gel using the following electrophoresis conditions: 6·0V per cm for 20 h, with pulse times ranging from 2 to 5-s, an angle of 120° and a linear ramp factor. The fragment patterns were compared with patterns of two human MRSA strains Mu50 and N315. The dendrogram was prepared by the Unweighted Pair Group Method using arithmetic average Algorithm (UPGMA), with Dice coefficient and an optimization and position tolerance of 1% using Biogene Software (Vilber Lourmat).

Capsular serotyping

Capsular serotyping was performed through the detection of capsular polysaccharides types 5 and 8 (CP5 and CP8). Overnight culture bacteria were collected off agar plates and adjusted to 10^{10} CFU ml^{-1} by optical density (OD) measure. The capsular serotype of 10^9 CFU of each strain was determined by enzyme-linked immunosorbent assay (ELISA) in 96-well microplates, using specific monoclonal and polyclonal antibodies (kindly provided by GSK Biologicals, Belgium) and horseradish peroxidase-based quantification. The OD of each well was measured at 490 nm using a microplate reader. The results were collected from at least two independent experiments in

which the capsule production of each tested culture was evaluated in triplicates. OD values were compared with those obtained with *S. aureus* CP reference strains, namely the CP5-positive strain Lowenstein (ATCC 49521) and CP8-positive strain Wright (ATCC 49525), and isolates that tested negative for CP5 and CP8 ELISA were defined as nontypeable (NT).

Biofilm production

Biofilm formation was evaluated by spectrophotometry in microplates using safranin staining. Overnight TSB cultures were diluted 1:100 either in TSB containing 0·25% glucose (TSB_{glc}) or in milk serum (Melchior *et al.* 2009) and transferred and incubated overnight into wells of sterile 96-well flat-bottom tissue culture (TC) plates. TSB with glucose or milk serum served as negative control. The supernatant was then discarded, and the plates were stained, after washing, with safranin 0·1% (w/v). To reveal, a mixture of 50% ethanol–50% acetic acid was added to each well and plates. The OD of each well was then measured at 490 nm using a microplate reader. The results were collected from at least two independent experiments in which the biofilm formation of each culture tested was evaluated in triplicates. The quantitative classification of biofilm production based on OD values was carried out according to literature recommendations (Stepanovic *et al.* 2007).

Antimicrobial susceptibility testing

Susceptibility tests were performed by the disc diffusion method of Bauer *et al.* (Bauer *et al.* 1966) on Mueller-Hinton agar according to the SF-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) 2010 recommendations. In addition to oxacillin, susceptibility to 15 antimicrobial agents was determined. These comprised penicillins (penicillin and ampicillin), cephalosporins (cephalexin, cefoperazone and ceftiofur), tetracycline, a macrolide (erythromycin), a lincosamide (clindamycin), a folate pathway inhibitor (sulfamethoxazole/trimethoprim 19 : 1), aminoglycosides (gentamicin, streptomycin and kanamycin), an aminocyclitol (spectinomycin), chloramphenicol and a fluoroquinolone (ciprofloxacin).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PFGE gel. The *ApaI* fragment patterns of the MRSA isolates were compared to Mu50 and N315 reference strains.