Methicillin-Resistant *Staphylococcus aureus* (MRSA) ST398 Associated with Clinical and Subclinical Mastitis in Belgian Cows

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is infrequently reported in mastitis. Yet, as in many other countries, the prevalence of methicillin resistance among *S. aureus* from mastitis is currently unknown in Belgium.

To elucidate this, the presence of *mecA* was investigated in 118 *S. aureus* strains originating from diagnostic mastitis milk samples from 118 different farms experiencing *S. aureus* mastitis. MRSA strains were characterized by disk diffusion susceptibility testing, *spa*-typing, MLST and SCC*mec*-typing. In an additional study, four MRSA-positive farms were selected to assess the in-herd prevalence of MRSA, by sampling all cows in lactation. Isolated MRSA strains were similarly characterized.

The *mecA* gene was detected in eleven (9.3%) of the 118 *S. aureus* isolates, indicating that nearly 10% of the Belgian farms suffering from *S. aureus* mastitis have an MRSA problem. The in-herd prevalence varied between 0% and 7.4%. Characterization of the MRSA strains showed that they were all resistant to tetracycline. Additional resistances to macrolides, lincosamides and aminoglycosides were frequently detected. The strains were ST398, *spa*-types t011 or t567 and had SCC*mec*-types IVa or V, proving they belong to the emerging livestock-associated MRSA (LA-MRSA) strains of CC398.

Our study shows that after detection in Belgian pigs, horses and poultry, LA-MRSA has also attained Belgian cattle. It is the first report on frequent isolation of LA-MRSA from bovine infections. As the in-herd isolation rate resembles that of regular *S. aureus* in farms experiencing *S. aureus* mastitis, the multi-resistance of LA-MRSA strains may cause future treatment problems.

Keywords

methicillin-resistant *Staphylococcus aureus*, MRSA, mastitis, Belgium, ST398, multidrug resistance

Introduction

Staphylococcus aureus is a major pathogen in dairy cattle mastitis (Waage et al., 1998; Tenhagen et al., 2006; Piepers et al., 2007). Resistance of *S. aureus* to antimicrobial agents can complicate treatment of its infections (Lowy, 2003). For treatment of mastitis, methicillin resistance, which is caused by the expression of the *mecA* gene, is of particular interest. Indeed, this mechanism confers resistance to almost all types of β-lactam antibiotics active against *S. aureus*, and these antibiotics are still frequently used in mastitis treatment (Sawant et al., 2005). However, methicillin-resistant *Staphylococcus aureus* (MRSA) has never been important in mastitis. After the very first report of MRSA in mastitis in 1972 (Devriese et al., 1972), MRSA has been described in mastitis only occasionally (Lee, 2003; Kwon et al., 2005; Lee, 2006; Juhász-Kaszanyitzky et al., 2007; Moon et al., 2007; Hendriksen et al., 2008). From such studies, it seems that the prevalence of MRSA in mastitis is generally low. Yet, data on MRSA in mastitis need to be assessed carefully, as there are often ambiguities on presence of *mecA*, level of investigation and origin of the detected MRSA strains.

Recently, a specific MRSA clone, CC398, has been found associated with pigs, veal calves, broiler chickens, companion animals and people in close contact with livestock. MRSA of this type, called Livestock-Associated MRSA (LA-MRSA), typically has closely related *spa*-types (de Neeling et al., 2007; Denis et al., 2009), carries mostly SCC*mec*-types IVa and V (Witte et al., 2007; Van den Eede et al., 2009) and cannot be typed with PFGE using *Sma*I digestion (Bens et al., 2006). In addition, LA-MRSA shows resistance against tetracycline and, to a lesser extent, macrolides, lincosamides, aminoglycosides and fluoroquinolones (Witte et al., 2007). Generally LA-MRSA lacks common virulence factors found in other MRSA (Monecke et al., 2007; Walther et al., 2009). This is remarkable because, although infrequently compared to colonization, LA-MRSA has been isolated from infections, of both animals and humans (e.g. Hermans et al., 2008; Krziwanek et al., 2009). To our knowledge,

so far only one study has reported on the isolation of MRSA ST398 from a case of mastitis (Monecke et al., 2007).

We performed two studies to assess the role of MRSA in Belgian *S. aureus* mastitis. In a first study we investigated how many *S. aureus* isolated from mastitis were resistant to methicillin. Second, we investigated the in-herd prevalence of MRSA in Belgian herds where cows were previously shown to suffer from MRSA mastitis.

Methods

1. Methicillin resistance in S. aureus isolated from mastitis

Strains

From November 2006 through April 2007, the regional veterinary laboratories were asked to send us a representative isolate from all farms on which an *S. aureus*-mastitis problem was detected. Care was taken to include only one strain per visited farm. As such, a collection of 118 non duplicate isolates of *S. aureus*, originating from cases of subclinical or clinical mastitis from different farms was obtained.

DNA extraction

An Eppendorf cup (Eppendorf, Germany) containing a 500 µl Brain Heart Infusion broth (BHI) (BioRad, France) overnight pure culture was centrifuged for 3.0 min at approx. 20,000 x g, at room temperature. After removal of the supernatant, 45 µL of sterile, distilled water and 5 µL of a 1 mg/mL lysostaphin (Sigma-Aldrich, USA) solution at 4°C were thoroughly mixed with the pellet of cells. After incubation for 10 min at 37°C, 45 µL of sterile, distilled water, 5 µL of a 2 mg/mL proteïnase K (Merck, Germany) solution at 4°C and 150 µL of tris-HCl of 0.1 M at pH 8.0 were added. The resulting solution was incubated for 10 min at 60°C, followed by 5 min at 100°C and then centrifuged for 5 min at approx. 20,000 x g, at room temperature. DNA was stored at -20°C until use.

Identification of MRSA

A triplex PCR, targeting a *Staphylococcus*-specific 16S rRNA sequence, the *mecA* gene and the *S. aureus* specific region of the thermonuclease gene (*nuc*), was performed as previously described (Maes et al., 2002). The amplified DNA fragments were separated by electrophoresis on a 2% agarose (Sigma-Aldrich, USA) gel stained with SYBR Safe DNA gel stain (Invitrogen, USA), for 2 h at 80V, using an O'RangeRuler 100bp DNA ladder (Fermentas, Germany).

Characterization of MRSA

Susceptibility testing

Strains proven to be MRSA were tested for susceptibility to non β-lactam antimicrobial agents, by using the disk diffusion method. A panel of 16 antimicrobial agents was used: chloramphenicol, gentamicin, kanamycin, tobramycin, fucidic acid, erythromycin, tylosin, lincomycin, linezolid, quinupristin + dalfopristin, mupirocin, ciprofloxacin, tetracycline, rifampicin, sulfonamides and trimethoprim (NeoSensitabs, Rosco, Denmark). Results were recorded after 24h incubation at 37°C and interpreted according to the directions for use of Rosco with the method described by the CLSI guidelines (document M31-A3).

Spa-typing

Of all MRSA strains, the polymorphic X-region of the *Staphylococcus* protein A (*spa*) gene was amplified according to the Ridom StaphType standard protocol (www.ridom.de/staphtype). Amplicons were purified with a Nucleospin Extract II kit (Macherey-Nagel, Germany) and then sequenced using the same primers. The sequenced DNA was then run on a CEQ 8000 Genetic Analysis System (Beckman Coulter, United Kingdom) according to the manufacturer's instructions. The resulting *spa*-types were assigned by using the Ridom StaphType software package (Ridom GmbH, Germany).

MLST

Multi Locus Sequence Typing was performed on all MRSA strains. In short, seven household genes of *S. aureus* were amplified using primers previously described (Enright et al., 2000).

Amplicons were purified with a Nucleospin Extract II kit (Macherey-Nagel, Germany) and then sequenced using the same primers. The sequenced DNA was then run on a CEQ 8000 Genetic Analysis System (Beckman Coulter, United Kingdom) according to the manufacturer's instructions. Allele numbers and sequence type (ST) were assigned by using the *S. aureus* MLST website (http://saureus.mlst.net).

SCCmec-typing

The SCC*mec* type was determined using three different sets of primers (Oliveira and de Lencastre, 2002; Zhang et al., 2005; Milheiriço et al., 2007). For differentiation among SCC*mec* types I–IV we used all the primers described by Oliveira and de Lencastre (2002). The PCR mix consisted of 25 µL of *Taq* PCR Master Mix (Qiagen Gmbh, Germany), 4 µL of H_2O and 16 µL of the primers, in the reported concentration. To this mix 5 µL DNA was added.

For subtyping SCC*mec* of type IV, we used the primers described by Milheiriço et al. (2007). The PCR-mix consisted of 25 µL of *Taq* PCR Master Mix (Qiagen Gmbh, Germany), 6.4 µL of H_2O, 0.2 µM of primers J IVa forward (F) and reverse (R), 0.2 µM of J IVb F and R, 0.4 µM of ccr B2 F and J IVc F and R, 0.8 µM of ccrB2 R, J IVd F and R, 0.9 µM of J IVg F and R, and 0.9 µM of J IVh F and R. To the mix 5 µL DNA was added.

A third set, meant to detect SCC*mec*-type V and to have a control for SCC*mec*-types IVb, IVc, IVe and IVf, was based on the method described by Zhang et al. (2005). The PCR mix consisted of 25 µL of *Taq* PCR Master Mix (Qiagen Gmbh, Germany), 10.4 µL of H_2O, 0.6 µM of primers Type V F and R, 0.8 µM of Type IVc F and R, and 1.0 µM of Type IVb F and R. To the mix 5 µL DNA was added.

We used the same PCR program for all three sets: an initial denaturation of 4 min at 94°C, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min, followed by a final extension for 4 min at 72°C.

2. MRSA in-herd prevalence

From the results of the first study, four MRSA positive farms were selected for investigation of the in-herd prevalence of MRSA, defined as the number of MRSA-positive cows relative to the total number of lactating cows present in the specific farm. A randomly chosen fifth farm volunteered to serve as control (Table 2). Milk samples were taken from each quarter. All sampling was done by the same person, from February 2008 through April 2008.

Samples were immediately transported to the Veterinary and Agrochemical Research Center (VAR), where each sample was plated on Columbia Colistine Aztreonam Plates (CAP) supplemented with 5% sheep blood (Oxoid, Germany) and on chromID MRSA plates (Biomérieux, France). Suspected *S. aureus* or MRSA colonies were purified. Pure colonies were then subjected to the MRSA triplex PCR, as described above. Strains identified as MRSA were characterized by susceptibility testing, *spa*-typing, MLST and SCC*mec*-typing, as described above.

Results

1 – Methicillin resistance in S. aureus isolated from mastitis

Detection of MRSA

All 118 isolates phenotypically identified as *S. aureus* were confirmed to be *S. aureus* by the triplex PCR. A total of 11 isolates (9.3%) contained *mecA* (Table 1). Two MRSA originated from clinical mastitis, the other nine from subclinical mastitis (Table 1).

Antimicrobial susceptibility testing

Antibiotic resistance patterns of the 11 MRSA strains are shown in Table 1. Nine of them showed additional resistance to at least two different antibiotics. All strains were resistant to tetracycline; nine were resistant to trimethoprim, seven to aminoglycosides and lincomycin, five to macrolides and two to ciprofloxacin (Table 1). No resistance was detected to the other antimicrobial agents tested.

MLST, *spa*- and SCC*mec*-typing

Ten strains were *spa*-type t011. One strain had a different yet related *spa*-type, t567 (Table 1). All MRSA strains were ST398 (Table 1). Five strains had SCC*mec*-type IVa, and five had SCC*mec*-type V. The SCC*mec*-type of one strain could not be determined with the different sets of primers we used (Table 1).

2 – MRSA in-herd prevalence

Identification of MRSA

The percentage of cows carrying MRSA in their milk varied between 0% and 7.4% (Table 2). Quarter level prevalence ranged from 0% to 1.98% (Table 2). Three of the four selected farms were positive. MRSA could not be detected in one farm previously found positive nor in the control farm (Table 2).

One cow from the first farm carried MRSA in three of her quarters. In all other positive cows, MRSA was found in only one quarter, resulting in 14 isolates in total (Table 3). Most isolates were found in the right-hind (6 isolates) and right-front (4 isolates) quarter (Table 3). Of the 11 cows that had MRSA in only one quarter, nine of the MRSA isolates were found in one of the hind-quarters.

Antimicrobial susceptibility testing

All six strains isolated from the first farm had the same susceptibility profile (Table 3). They were all resistant to tetracycline, the tested macrolides and lincomycin, and were susceptible to all other antimicrobial agents tested.

In the second farm, two out of five strains were resistant to trimethoprim, tetracycline and the tested aminoglycosides, and susceptible to all other antimicrobial agents tested. The three other strains had additional resistances to the tested macrolides and lincomycin (Table 3).

The three strains from the third farm were resistant to the tested aminoglycosides, macrolides, lincomycin, tetracycline and trimethoprim (Table 3). They were susceptible to the other antimicrobial agents tested.

MLST, *spa*- and SCC*mec*-typing

All MRSA strains showed *spa*-type t011 (Table 3). The strains originating from the first farm had SCC*mec* type V, while all strains isolated from the cows of the other two farms had SCC*mec* type IVa (Table 3). MLST was performed on one representative MRSA strain per farm. As strains from farm 2 showed two different resistance profiles, one representative strain from each profile was tested. The four strains tested all were ST398 (Table 3).

Discussion

The prevalence of methicillin resistance in *S. aureus* isolated from mastitis in our first study is unexpectedly high. In the abundance of studies investigating the antibiotic resistance of mastitis pathogens, few reports have noted a substantial occurrence of methicillin resistance, meaning MRSA is usually negligible as a mastitis pathogen (Hendriksen et al., 2008). However, we found nearly 10% of our 118 *S. aureus* strains to be MRSA. This means that nearly 10% of the Belgian farms experiencing *S. aureus* mastitis is affected by MRSA. Reports can be found in which a higher prevalence of MRSA among *S. aureus* isolated from mastitis cases is described. In Turkey, Turutoglu et al. (2006) found 18 out of 103 (17.5%) *S. aureus* isolates from mastitis milk samples to be MRSA. However, they did not mention whether all strains were collected from different farms experiencing *S. aureus* mastitis. In addition, their detection method was limited to phenotypic disk diffusion testing. Performing only phenotypic tests has previously been shown to lead to false positive or false negative results (Murakami et al., 1991; De Oliveira et al., 1999). Generally it is now accepted that checking for the presence of *mecA* is the most reliable method for detection of methicillin resistance, and staphylococci carrying *mecA* should be regarded as resistant to almost all types of β-lactam antibiotics (CLSI guidelines, M31-A3). Consequently, to accurately assess our results, only other reports in which *mecA* was proven to be present should be considered. Still, even then, it remains difficult to make viable comparisons, due to differences in sampling methodology or a lack of information on the source of the strains. For example, two South

Korean studies did not mention exactly how many of their samples originated from mastitis (Lee, 2003; Lee, 2006). A Hungarian study sampled only a single farm (Juhász-Kaszanyitzky et al., 2007). In two other studies from South Korea, the data involved quarter-level results (Kwon et al., 2005; Moon et al., 2007).

Despite these difficulties to fully assess our results, it must be acknowledged that the MRSA prevalence we found is quite high. However, some other remarks should be made. First, the burden of MRSA for Belgian milk production cannot be assessed, because we have no data on the total number of farms that were visited during the sampling period. Also, while our study allows us to estimate the importance of methicillin resistance in Belgian *S. aureus* mastitis, we cannot judge the importance of MRSA for mastitis as a whole. A hint to address the latter can be found in a recent study that investigated the importance of *S. aureus* in Belgian mastitis. It was found that *S. aureus* was the most prevalent species in Belgian quarter milk samples from subclinical mastitis, with 25% of culture-positive quarter samples with a geometric mean composite somatic cell count of ≥ 250 000 cells/ml harboring *S. aureus* (Piepers et al., 2007). Regarding this, our result is certainly quite worrying.

Another important fact is presented by our typing data. All our strains had characteristics typical for the emerging livestock-associated MRSA CC398 strains. Consequently, it seems that our findings should rather be regarded as a further expansion of the host range of the CC398 MRSA clone than as an indication of a generally increasing incidence of methicillin resistance in mastitis-associated *S. aureus*. This should however not be less worrying.

In addition to its resistance against all β-lactam antibiotics, which are still the most used antimicrobial agents in the treatment of mastitis, the typical antibiotic resistances of LA-MRSA also include some other antibiotics used to treat or prevent mastitis, such as aminoglycosides and macrolides (Sawant et al., 2005). This could lead to serious treatment problems. Moreover, in our second study we found that the in-herd prevalence of LA-MRSA

ranged between 0% and 7.4%. In the farms where MRSA was found, it varied from 3.9% to 7.4%, with a corresponding quarter level prevalence of 0.97% to 1.98%. This resembles the in-herd quarter level prevalence of *S. aureus* described earlier in a cross-sectional collection of Belgian milk samples (Piepers et al., 2007), suggesting that, considering its spread in farms, LA-MRSA behaves similar to regular mastitis causing *S. aureus*. The possibility that LA-MRSA could become equally important in mastitis as normal *S. aureus* should thus be thoroughly investigated. Unfortunately, we have no data on the individual health status of the cows from which MRSA was isolated in our second study, so we cannot state that the LA-MRSA strains we found were actually involved in mastitis. As it was shown that within-cow transmission between quarters likely occurs in *S. aureus* mastitis (Barkema et al., 1997), the fact that 11 of the 12 cows carried LA-MRSA in only one quarter could mean that the isolates concerned only contaminants. However, *S. aureus* infection of only one quarter also certainly exists (Barkema et al., 1997). Moreover, *S. aureus* was shown to more frequently infect the right and hind quarters (Barkema et al., 1997; Barkema et al., 2006). Of the 11 single-quarter LA-MRSA isolates we found, 10 originated from right quarters and nine from hind quarters. Considering also our first study, which clearly showed the capacity of LA-MRSA to cause mastitis, the actual presence of LA-MRSA in Belgian mastitis should urgently be studied in more depth, in order to profoundly assess its possible burden.

LA-MRSA has been reported only once before in mastitis in cows, one LA-MRSA strain that was found among 128 *S. aureus* isolated from German mastitis cases (Monecke et al., 2007). While this strain was *spa*-type t034, our strains were *spa*-types t011 and t567. It thus seems unlikely that a specific subclone of LA-MRSA is associated with mastitis, but more research is required to confirm this. Until now, it is also unclear whether LA-MRSA has an actual reservoir in dairy cattle. Whereas veal calves have been found carrying LA-MRSA in the

Netherlands (Mooij et al., 2007) and Belgium (unpublished data), the colonization capacity of LA-MRSA in milking cows has not yet been investigated.

The presence of LA-MRSA in infections has been reported substantially less frequent than carriage, and has only been described occasionally in pigs (van Duijkeren et al., 2007), horses (Hermans et al., 2008; Loeffler et al., 2009), humans (e.g. Krziwanek et al., 2009) and a dog (Witte et al., 2007). Our findings thus seem to add new proof of a certain pathogenic potential of LA-MRSA. Remarkably, many common virulence factors, including those considered to be involved in mastitis, such as toxic shock syndrome toxin-1 (tsst-1), haemolysins and enterotoxins (Matsunaga et al., 1993), have been shown to be largely absent in LA-MRSA (Monecke et al., 2007; Walther et al., 2009). However, as we did not check for the presence of virulence factors in our strains, the significance of our data regarding the pathogenic potential of LA-MRSA is hard to assess. Yet, in addition to the other reports on LA-MRSA associated with infections, our findings urge for further research into the virulence capacities of LA-MRSA.

Conclusions

We found an unusual high prevalence of MRSA in Belgian cases of subclinical and clinical *S. aureus* mastitis in cows. All strains belonged to the CC398 clone, which, seen its multi-resistance, may lead to treatment problems. Future research is warranted to assess the actual spread and corresponding burden that LA-MRSA may pose for dairy cattle farming and to elucidate which virulence factors are involved.

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418 Table 1. Resistance profile, Multi-Locus Sequence Type (MLST), *spa*- and Staphylococcal

419 Cassette Chromosome (SCC)*mec*-type per MRSA strain in study 1.

Strain	Type of mastitis	Resistance profile[a]	*spa*	MLST	SCC*mec*[b]
1	Subclinical	AG, TET, TMP	t011	398	IVa
2	Clinical	AG, TET, TMP	t011	398	IVa
3	Subclinical	AG, ML, LM, TET, TMP	t011	398	IVa
4	Subclinical	LM, CIP, TET, TMP	t011	398	V
5	Subclinical	TET	t567	398	NT
6	Subclinical	AG, ML, LM, TET, TMP	t011	398	IVa
7	Subclinical	LM, CIP, TET, TMP	t011	398	V
8	Clinical	AG, ML, LM, TET, TMP	t011	398	IVa
9	Subclinical	KAN, TOB, ML, LM, TET, TMP	t011	398	V
10	Subclinical	AG, ML, LM, TET, TMP	t011	398	V
11	Subclinical	TET	t011	398	V

420 [a] AG: all aminoglycosides tested; KAN: kanamycin; TOB: tobramycin; ML: all macrolides

421 tested; LM: lincomycin; CIP: ciprofloxacin; TET: tetracycline; TMP: trimethoprim

422 [b] NT: not typeable with the primers used

423 Table 2. In-herd prevalence of MRSA • no. 5: control farm

Farm	Herd size	Herd size	Positive cows		Positive quarters	
	(n cows)	(n quarters)	n	%	n	%
1	63	252	4	6.3	6	1.98
2	68	272	5	7.4	5	1.83
3	77	308	3	3.9	3	0.97
4	51	204	0	0	0	0
5	69	276	0	0	0	0

424

425 Table 3. Resistance profile, Multi-Locus Sequence Type (MLST), *spa*- and Staphylococcal

426 Cassette Chromosome (SCC)*mec*-type per MRSA strain in study 2.

Farm	Quarter[a]	Strain	Resistance profile[b]	*Spa*	MLST[c]	SCC*mec*
1	LH	1	ML, LM, TET	t011	ND	V
	RH	2	ML, LM, TET	t011	ND	V
	RF	3	ML, LM, TET	t011	ND	V
	LF	4	ML, LM, TET	t011	398	V
	RH	5	ML, LM, TET	t011	ND	V
	RF	6	ML, LM, TET	t011	ND	V
2	LH	7	AG, TET, TMP	t011	398	IVa
	LF	8	AG, ML, LM, TET, TMP	t011	ND	IVa
	RH	9	AG, TET, TMP	t011	ND	IVa
	RH	10	AG, ML, LM, TET, TMP	t011	398	IVa
	RH	11	AG, ML, LM, TET, TMP	t011	ND	IVa
3	RH	12	AG, ML, LM, TET, TMP	t011	ND	IVa
	RH	13	AG, ML, LM, TET, TMP	t011	ND	IVa
	RF	14	AG, ML, LM, TET, TMP	t011	398	IVa

427 [a] LF: left-front; LH: left-hind; RH: right-hind; RF: right-front

428 [b] AG: all aminoglycosides tested; LM: lincomycin; ML: all macrolides tested; TET:

429 tetracycline; TMP: trimethoprim

430 [c] ND: not determined

431