OCCURRENCE AND CHARACTERIZATION OF LIVESTOCK-ASSOCIATED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract. Methicillin resistant *Staphylococcus aureus* causes a wide range of severe and economically-important diseases in humans and animals. Different types of MRSA are associated with different hosts but the transmission occurs between them. The aim of this study was to investigate possible spread of MRSA among livestock in Lithuania and to determine their types and antimicrobial resistance. Cattle (n=120), horses (n=120) pigs (n=160) and poultry (pooled samples, n=120) were tested for MRSA prevalence. From a total of 520 samples tested, 4 isolates of methicillin resistant *Staphylococcus aureus* (0.8 %) were identified. All isolates were obtained from the finisher pigs delivered from the same farm complex. Multiplex PCR demonstrated presence of *mec*A, *nuc* and 16S genes in all tested cultures. All MRSA isolates were identified as ST398. Sequencing of spa genes and *SCCmec* typing revealed that all strains belonged to the spa type t011 and *SCCmec* V. PFGE revealed two different clones among the isolates. Susceptibility testing revealed resistance to tetracycline in all MRSA isolates attributed to *tet*K and *tet*M genes. All tested isolates were resistant to erythromycin owing to the presence of *erm*B gene as well as resistances to azithromycin, clindamycin and quinupristin/dalfopristin. One isolate was resistant to trimethoprim/sulfamethoxazole and carried the resistance gene *dfrK* while the other isolate was resistant to the combination of amoxicillin and clavulanic acid. All of the isolates were susceptible to fluoroquinolones, cefotaxime, chloramphenicol, fosfomycin, fusidic acid, gentamicin, linezolid, vancomycin, mupirocin and teicoplanin.

Keywords: MRSA; ST398; livestock; antimicrobial resistance.

Introduction

Staphylococcus (*S.*) *aureus* causes a wide range of severe and economically-important diseases in humans and animals (Safdar and Bradley, 2008). In livestock *S. aureus* is an important cause of mastitis, soft tissue and skin infections and, to a lesser extent, infections of the locomotory system (Catry et al., 2010). *S. aureus* is intrinsically susceptible to beta-lactam agents that inhibit cell wall formation due to binding with peptidoglycan (Haesebrouck et al., 2009).

Different types of MRSA may be distinguished based on epidemiological grouping although it is acknowledged that this is a simplistic approach since there are cases where strains of MRSA had spread between the groups (Morgan, 2008) and thus it might be difficult to determine what epidemiologic pattern a certain MRSA strain is associated with. Livestock Associated MRSA (LA-MRSA) refers mainly to the clonal spread of a certain MRSA strain (CC398) that colonise different mammal species including horses and may cause infections in humans.

During the period 1970–2000, MRSA has been sporadically isolated from animals, in particular cows, small companion animals, and horses. With the exception of some equine isolates, the nature of these isolated cases suggested a human origin and no epidemics have been reported (Leonard and Markey, 2008). In the last years, the situation has changed, with an increased number of reports on LA-MRSA in livestock, especially swine and veal calves.

In 2005, a high prevalence of LA-MRSA was found in Dutch pigs in slaughterhouses (de Neeling et al., 2007). This study was undertaken following high colonization rates of farmers and relatives without known risk factors for MRSA (Voss et al., 2005). Other reports have confirmed these findings in different countries like Denmark (Guardabassi et al., 2007), Germany (Meemken et al., 2008), Canada (Khanna et al., 2008), Belgium (Denis et al., 2009) and Portugal (Pomba et al., 2009), and the predominant *spa* types found were t108, t034, and t011, all close relatives within CC398. The SCC*mec* element predominantly found was IV.

To date, clinical infections with LA-MRSA in food producing animals have been described (Van Duijkeren et al., 2008; Pomba et al., 2010). The first report described a post-weaning dermatitis with 20% mortality in swine from the Netherlands and in which *spa* type t011 was found (Van Duijkeren et al., 2008). So far, the LA-MRSA strains in the living animal have not been reported to possess PVL (Van Duijkeren et al., 2007; Denis et al., 2009). On the contrary, in CC398 from predominantly healthy persons, a prevalence of 9.4% for the PVL toxin was reported (van Loo et al., 2007) although an Asian subclone was suggested for these isolates (Yu et al., 2008). Other MRSA clones harbouring PVL have nevertheless been found among living animals (Morgan, 2008).

Different typing techniques are used for MRSA differentiation. In addition to SCC*mec* typing, two important molecular typing techniques for differentiation

of MRSA are pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). In contrast to MLST, PFGE is less expensive but has the disadvantage of limited inter-laboratory exchangeability of the results. *Spa* typing is the third important genotyping method which can differentiate strains that are indistinguishable by PFGE or MLST (Catry et al., 2010). ST398 belongs to CC398, and examples of *spa* types found herein are t108, t011, t034 (van Loo et al., 2007).

In spite of different methodologies described for the differentiation of MRSA, the data about MRSA type prevalence in Baltic countries among livestock are missing.

The aim of this study was to investigate possible spread of MRSA among livestock in Lithuania, to determine their types and antimicrobial resistance.

Materials and Methods

The investigations were carried out at the Lithuanian University of Health Sciences and Laboratory of Antimicrobial and Biocide Resistance of the Technical University of Lisbon.

Collection of samples

Samples collection was performed in 2011–2012 on animal farms located in different regions of Lithuania. Nasal swabs from healthy calves (n=120) and horses (n=120) on six and eight farms respectively were collected using sterile cotton swabs and transport media (TRANSWAB, Polysciences Inc.). One hundred and twenty nasal samples of sows were taken from 6 closed-cycle pig farms. Additionally, nasal swabs from finisher pigs (n=40) representing two different pig farms were obtained from two slaughterhouses just before slaughtering. One hundred and twenty pooled samples (1 pool contained 5 individual samples of chickens) from chicken nasal cavity were taken in two slaughterhouses, representing 2 large and two medium-sized chicken farms (including broilers and layers). The samples were delivered to the laboratory at the same day.

Bacteriological testing and DNR extracting

The samples were inoculated into Mueller Hinton Broth (Oxoid) supplemented with 6.5% NaCl and incubated at +35ºC for 24 hours followed by inoculation onto chromogenic media: Brilliance™ MRSA 2 Agar (Oxoid) and chromID™ MRSA medium (bioMérieux). Suspected MRSA colonies were identified up to species level using RapID STAPH PLUS (Thermo Scientific) identification system and software ERIC® (Remel). The DNR material for molecular testing was obtained after DNR lysis prepared following the Danish National Food Institute (DTU) protocol (DTU CRL-AR, 2009) for detection of the *mec*A gene.

Detection of mecA genes and classification of SCCmec type

Multiplex PCR was used for the detection of *mec*A-1, *mec*A-2, *nuc*-1, *nuc*-2, *16S*-1 and *16S*-2 genes according to the method described by Poulsen at al. (2003) with slight modifications. Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment was used according to the method described by Kondo et al. (2007).

Sequencing of spa genes

PCR protocol according to Shopsin et al. (1999) was used for the evaluation of protein A gene polymorphic region (spa typing) of isolated strains of MRSA. The product was purified using JETQUICK PCR Product Purification Spin Kit (GenoMed) and sent for the sequencing to the Portugal sequencing centre. Data were analysed using Ridom spa server database (http://spa.ridom.de/).

Identification of S.aureus ST398 and genotyping of the isolates

PCR described by van Wamel et al. (2010) was used for the identification of *S. aureus* sequence type 398. Clonality was assessed by Pulse Field Gel Electrophoresis (PFGE) with Cfr9I restriction as performed by Pomba et al. (2010).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the broth microdilution method (MicroScan® PM21; Dade Behring, Deerfield, IL) for ampicillin, amoxicillin/clavulanate, azithromycin, cefotaxime, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fosfomycin, fusidic acid, gentamicin, levofloxacin, linezolid, moxifloxacin, mupirocin, netilmicin, oxacillin, penicillin, quinupristin/dalfopristin, tetracycline, teicoplanin, trimethoprim/sulfamethoxazole and vancomycin. The Promt™ Inoculation System and RENOK® Rehydrating Inoculator (Siemens) was used for the plates inoculation. The results of the susceptibility testing were interpreted according to the standard of Clinical and Laboratory Standards Institute (CLSI, 2006). The quality control strain *S. aureus* ATCC 29213 was included in each assay for validation purposes.

The genes encoding antimicrobial resistance *(tet*K, *tet*M, *erm*A, ermB and *erm*C, *vga*A, *vga*B, *vga*C and *dfr*K) were determined using PCR. Isolates were also tested for the *lukF/lukS* genes encoding Panton-Valentine leukocidin (PVL).

Results

From a total of 520 samples tested, 4 isolates of methicillin resistant *S. aureus* (0.8 %) were identified among Lithuanian livestock. All isolates were obtained from the finisher pigs delivered from the same farm complex located at the central part of the country. All isolates demonstrated typical growth on chromogenic MRSA media and had typical biochemical properties for *S. aureus*. Multiplex PCR demonstrated presence of *mec*A, *nuc* and 16S genes in all tested cultures. All MRSA isolates were identified as ST398 (Table 1).

Sequencing of spa genes and *SCCmec* typing revealed that all strains belonged to the spa type t011 and *SCCmec* V (Table 1). None of the MRSA isolates carried the PVL genes. Analysis obtained by PFGE revealed that three isolates had similar profiles, while the other belonged to a different cluster (Table 1).

Antimicrobial resistance patterns between the isolates slightly varied (Table 2). Susceptibility testing revealed resistance (MIC>8mg/L) to tetracycline in all MRSA

isolates attributed to *tet*K and *tet*M genes. All tested isolates were resistant (MIC>4mg/L) to erythromycin owing to the presence of *erm*B gene as well as resistances to azithromycin, clindamycin and quinupristin/dalfopristin. Isolate 02 was resistant to the combination of trimethoprim/sulfamethoxazole (MIC>2/38) and carried the resistance gene *dfr*K while the isolate 03 was the only strain that demonstrated resistance (MIC>4/2) to the combination of amoxicillin and clavulanic acid. All of the isolates were susceptible to fluoroquinolones, cefotaxime, chloramphenicol, fosfomycin, fusidic acid, gentamicin, linezolid, vancomycin, mupirocin, and teicoplanin.

Table 1. **Characteristics of MRSA isolates**

Isolate	Sample source, Origin	SCC*mec*	ST	spa	PVL	PFGE pattern[1]	Resistance patterns[2]	Resistance genes
01	Pig nasal swab	V	398	t011	neg	A1	AMP, AZI, CD, E, OX, P, TET, Q/D	tetM, tetK, ermB
02	Pig nasal swab	V	398	t011	neg	A2	AMP, AZI, CD, E, OX, P, TET, Q/D, SXT	tetM, tetK, ermB, dfrK
03	Pig nasal swab	V	398	t011	neg	B	AUG, AMP, AZI, CD, E, OX, P, TET, Q/D	tetM, tetK, ermB
04	Pig nasal swab	V	398	t011	neg	A1	AMP, AZI, CD, E, OX, P, TET, Q/D	tetM, tetK, ermB

[1]The definition of a PFGE cluster was based on a similarity cutt-off value of 80% using the unweighted pair group method (UPGMA); [2]AMP – Ampicillin; AZI – Azithromycin; CD – Clindamycin; E – Erythromycin; OX – Oxacillin; P – Penicillin; TET – Tetracycline; Q/D – Quinupristin/Dalfopristin; AUG – Amoxicillin/Clavulanate; SXT – Trimethoprim/Sulfamethoxazole

Table 2. **Minimal inhibitory concentrations of antimicrobials to MRSA isolates**

Isolate	AU	AM	AZ	CF	C	CI	CD	E	FO	FU	GE	LF
01	4/2	>8	>4	≤8	16	≤0.5	>2	>4	≤32	≤2	4	≤0.5
02	4/2	>8	>4	≤8	≤4	≤0.5	>2	>4	≤32	≤2	≤2	≤0.5
03	>4/2	>8	>4	≤8	16	≤0.5	>2	>4	≤32	≤2	8	≤0.5
04	≤2/1	>8	>4	≤8	8	≤0.5	>2	>4	≤32	≤2	≤2	≤0.5

	LZ	MX	MU	NE	NI	OX	P	TE	Q/D	TI	S/T	VA
01	2	≤0.1	≤4	8	≤32	>4	>8	>8	>2	≤1	≤2	≤1
02	2	≤0.1	≤4	≤ 4	≤32	>4	>8	>8	>2	≤1	>2	≤1
03	2	≤0.1	≤4	8	≤32	>4	>8	>8	>2	≤1	≤2	≤1
04	2	≤0.1	≤4	≤ 4	≤32	>4	>8	>8	>2	≤1	≤2	≤1

*AU – Amoxicillin/Clavulanic Acid; AM – Ampicillin; AZ – Azithromycin; CF – Cefotaxime; C – Chloramphenicol; CI – Ciprofloxacin; CD – Clindamycin; E – Erythromycin; FO – Fosfomycin; FU - Fusidic Acid; GE – Gentamicin; LF – Levofloxacin; LZ – Linezolid; MX – Moxifloxacin; MU – Mupirocin; NE – Netilmicin; NI – Nitrofurantoin; OX – Oxacillin; P – Penicillin; TE – Tetracycline; Q/D - Quinupristin/Dalfopristin; TI – Teicoplanin; S\T – Trimethoprim/Sulfamethoxazole; VA – Vancomycin.

Discussion

Recent studies carried out in the EU demonstrated the prevalence of MRSA in different Member States among holdings with breeding pigs. However, no methicillin resistant staphylococci were found in the Baltic countries (EFSA, 2009). The current study demonstrates the first occasion of MRSA ST398 in the Baltic countries among livestock. ST398 of MRSA in pigs and pig farms has been found in Austria, France, Italy, Netherlands, Portugal and other EU countries with the highest rate in Spain, Belgium and Germany (EFSA, 2009). The prevalence among pigs varied according to the group of animals (piglets, sows and finishers) and farming type. For instance, a lower prevalence of MRSA was found among sows compared to piglets and finishers in a Belgian survey. In addition, this survey revealed a marked difference in the number of MRSA positive animals between open (94%) and closed farms (56%) (Denis et al., 2009). This is in line with a Dutch survey which indicated transmission of LA-MRSA within the production chain, e.g. from multiplier to finisher farms (Van Duijkeren et al., 2008). Piglets in these multiplier farms can be colonized by different routes or vectors, and longitudinal studies are needed to indicate if the environment, e.g. feed or dust or the sows are the primary source of colonization (de Neeling et al., 2007).

In 2002, the isolation of CC398 clone from pigs was reported in France for the first time, but the isolate was

susceptible to methicillin (MSSA) (Armand-Lefevre et al., 2005). MRSA CC398 has been found in other animal species including man. Previous reports from France, Denmark, The Netherlands, Germany Canada and Portugal, indicate that CC398 is widespread among animals and transmissible from animals to humans (Armand-Lefevre et al., 2005; Lewis et al., 2008; vanBelkum et al., 2008; Pomba et al., 2010). Persons in direct contact with MRSA-positive animals have shown to have an increased risk of carrying the same MRSA strains as the animals (Weese et al., 2008). For instance, epidemiological studies in the Netherlands have indicated that human contact with veal calves or pigs was significantly associated with carriage of CC398 (Voss et al., 2005; van Rijen et al., 2008). The occupational hazard for LA-MRSA colonization through (the intensity of) pig contact has been confirmed in Belgian farmers (Denis et al., 2009), regional German investigations (Meemken et al., 2008) and is well described in reflection paper of SAGAM (Catry et al., 2010). Human infections with LA-MRSA have been described since 2004 (Voss et al., 2005), with an increasing frequency in the Netherlands (van Rijen et al., 2008) and Denmark (Lewis et al., 2008). Examples of severe infections are an aggressive soft tissue infection of a pig inflicted bite wound (Declercq et al., 2008) and endocarditis (Ekkelenkamp et al., 2006).

Besides CC398, other types of MRSA had been found among pigs in other countries. In a recent Canadian study an endemic HA-MRSA (US100) strain was found in pigs in addition to CC398 (Weese et al., 2008). Such data demonstrates possible spread of different MRSA types among pigs and man.

Other animal species, such as horses, companion animals and poultry are also known as a reservoir of MRSA and can be potentially dangerous for men. For example, the transmission of a PVL positive MRSA strain between humans and a dog has been reported (van Duijkeren et al., 2005). Several studies report that MRSA isolates from horses and people working with horses are indistinguishable and differ from MRSA isolates from humans in the general population (Seguin et al., 1999; Weese et al., 2005; Cuny et al., 2008). To date, MRSA has been isolated from horses in Europe, Asia and North America (Shimizu et al., 1997; Baptiste et al., 2005; Weese et al., 2005; Witte et al., 2007; van den Eede et al., 2009). Facts of the prevalence of MRSA in poultry are also described. In recent study carried out in Belgium a rather low within broilers flock prevalence of MRSA varied between 0% and 28% (Pletinckx et al., 2011). *S. aureus* inscluding MRSA prevalence in livestock varied according to different tissues or organs. For instance, in broilers MRSA was most frequently isolated from the cloaca and nose shell and to a lesser extent from the skin beneath the wing and the pharynx thus, an appropriate sampling place should be selected for screening (Pletinckx et al., 2011).

MRSA susceptibility to antimicrobials excluding beta-lactams varies depending on type, source of isolation, geographical distribution or other factors. A causal relationship between the use of antimicrobial drugs and MRSA has been demonstrated in human medicine for different antimicrobial compounds in a recent meta-analysis (Tacconelli et al., 2008). It is probable that similar conditions apply also to animals, in particular since LA-MRSA are often co-resistant to several antimicrobial agents (Catry et al., 2010).

ST398 strains isolated from pigs in different countries usually had the similar resistance patterns with expressed resistances to tetracycline and erythromycin. *tet*K, *tet*M and *erm*C genes were the predominant coding resistance to tetracyclines and macrolides respectively (Guardabassi et al., 2007; Pomba et al., 2009). Isolates obtained during this study were also resistant to tetracyclines and macrolides. However, resistance to erythromycin was mediated by *erm*B. Resistance to erythromycin encoded by *erm*B was demonstrated in some other studies, for example, in MRSA isolated from pigs in Germany (Kadlec et al., 2009). All Lithuanian isolates also were resistant to the combination of quinupristin and dalfopristin, the compounds that are exclusively used in human medicine. This fact may be explained by possible transmission through humans or by phenomenon of co-resistance. Slight differences in antibioticograms and different patterns of antimicrobial resistance between the isolates demonstrate MRSA lability in changing of antimicrobial resistances even within the same farm.

Conclusions

1. The prevalence of MRSA during 2011–2012 among livestock in Lithuania was very low: this agent was found only in one pig farm.

2. Isolated MRSA was characterized as ST398, spa type t011 and *SCCmec* V.

3. Susceptibility testing of isolated strains demonstrated resistance to tetracyclines, macrolides, quinupristin/dalfopristin and susceptibility to glycopeptides, fluoroquinolones, amphenicols, fosfomycin, fusidic acid, gentamicin, linezolid and mupirocin.

Acknowledgements

This study was partially supported by the Interdisciplinary Centre of Research in Animal Health, from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal, and by a grant (No. MIP-061/2012) from the Research Council of Lithuania.

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Received 4 March 2013
Accepted 16 April 2014