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Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance

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ABSTRACT

This study was conducted to determine the occurrence of zinc and copper resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) from swine and veal calves in a global strain collection.

The test population consisted of 476 porcine MRSA isolates from ten European countries, 18 porcine MRSA isolates from Canada and seven MRSA from China, 92 MRSA and 60 methicillin-susceptible *S*. *aureus* (MSSA) isolates from veal calves in the Netherlands and 88 porcine MSSA isolates from four European countries. Most porcine MRSA (n=454) and all bovine MRSA belonged to clonal complex (CC) 398 whereas 37 of the pig MRSA from Europe and the seven Chinese isolates belonged to other CCs and 3 isolates were not classified into a CC.

All isolates were tested for susceptibility to zinc chloride and copper sulphate using agar dilution and tested by PCR for the *czrC* gene encoding zinc resistance.

Phenotypic zinc resistance (MIC>2mM) was observed in 74% (n=324) and 42% (n=39) of European MRSA CC398 from pigs and veal calves respectively, and in 44% of the Canadian isolates (n=8), but not among the Chinese isolates. Almost all (99%) zinc-resistant MRSA carried *czrC*. Of the 37 European non-CC398 MRSA, 62% were resistant to zinc, but only 46% of them carried *czrC*,. The MICs of the MSSA isolates to zinc chloride ranged from 1 to 4 mM and none carried *czrC*. The MICs of copper sulphate were neither associated with methicillin resistance nor with the detection of *czrC*.

This study showed that zinc resistance and the *czrC* gene is widespread among CC398 MRSA isolates. This suggests that the use of zinc in feed might have contributed to the emergence of MRSA.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has recently emerged as a zoonotic pathogen in the animal production around the world (Smith et al., 2008; Khanna et al., 2008; Wulf and Voss, 2008; Lewis et al., 2008).

The most frequently livestock associated MRSA (LA-MRSA) is clonal complex 398 (CC398) although other clonal complexes (CC1, CC5, CC8, CC9, CC30 and CC97) have also been described (Wagenaar et al., 2009; Sunde et al., 2009; Pomba et al., 2009; Cui et al., 2009; Battisti et al., 2009). The prevalence of the different MRSA clonal complexes might be related to differences in the population of methicillin-susceptible *Staphylococcus aureus* (MSSA) (Hasman et al., 2010). The factors contributing to the success of LA-MRSA are poorly understood and still under investigation (Vanderhaeghen et al., 2010).

It has been suggested that tetracycline resistance could have driven selection of MRSA CC398 (de Neeling et al., 2007). In fact, MRSA CC398 have been found resistant to tetracycline (Kadlec et al., 2009; Aarestrup et al., 2010; Fessler et al., 2010) However, tetracycline resistance is equally common to MRSA and MSSA of this clonal complex suggesting other selective mechanisms. (Aarestrup et al., 2010).

Metal-containing compounds are widely used as feed supplements for prevention of gastro-intestinal diseases in food animals. We have previously found the phenotypic resistance to zinc and methicillin resistance were strongly associated in *S. aureus* CC398 from pigs in Denmark (Aarestrup et al., 2010). A gene, *czrC,* was cloned and found to confer resistance to zinc and cadmium in *S. aureus* and it was found genetically located within the type V SCC*mec* elements highly prevalent among CC398 MRSA (Cavaco et al., 2010).

The purpose of this study was to assess whether the prevalence of zinc resistance among MRSA and MSSA isolates in a worldwide collection and to investigate the role of the recently described *czrC* gene in metal resistance in MRSA of animal origin.

MATERIALS AND METHODS

Strains

A total of 476 MRSA isolates originating from dust samples in pig farms were obtained from ten European countries (Belgium, Denmark, France, Germany, Hungary, Italy, Poland, Portugal, Spain, and The Netherlands) from the collection of isolates obtained within the European Baseline Study performed under decision of the European Commission (EC Decision 2008/55/EC). The same sampling and isolation methods were used in the different countries across Europe allowing comparisons between the results. Furthermore, 88 MSSA isolates from diagnostic samples of pig origin were obtained from four countries in Europe (Denmark, Germany, Ireland and Poland) for comparison. In addition to the European isolates, 18 MRSA CC398 and seven MRSA CC9 isolates of swine origin obtained from Canada (Khanna et al. 2008), and China (Cui et al., 2009), respectively, were included for comparison.

The bovine isolates were obtained from recently performed screenings in veal calf farms in The Netherlands (Graveland et al., 2010) and included 92 MRSA and 60 MSSA isolates obtained from nasal swabs of the veal calves.

All MRSA strains were previously genotyped by *spa* typing and assigned to a clonal complex using their *spa* typing data in Ridom *spa* server (http://www.ridom.de/spaserver/) or by multi-locus sequence typing (MLST) performed

in the countries of origin and SCCmec typing was performed in 73 selected MRSA isolates.

Susceptibility testing

Minimum inhibitory concentrations (MIC) of metals were determined by agar dilution assays using Mueller Hinton agar plates (Becton Dickinson) supplemented with either zinc chloride (Sigma-Aldrich, MW=136.30) in a range of concentrations (0.25-16 mM) at twofold dilutions including an additional concentration of 12mM or copper sulphate (Merck, MW=249.70) in a range of concentrations (1-28 mM) at twofold dilutions including additional concentrations of 12mM, 20mM, 24mM and 28mM. The pH of the medium was adjusted to 5.5 or 7.2, respectively, as previously described (Aarestrup and Hasman, 2004). The strains *S. aureus* SO385 (range: 4-8 mM), *S. aureus* C10682 (range: 4-8 mM) *Enterococcus faecium* A17sv1 HHAS210 (range: 4-16 mM for zinc chloride and 16-28mM for copper sulfate) and *S. aureus* ATCC 29213 (range: 1-2 mM for zinc chloride and 8-20mM for copper sulfate) have been tested extensively in previous studies (Aarestrup and Hasman, 2004) and were used on every test plate as internal control of the susceptibility testing procedures. Although there are currently no approved interpretive criteria available for the classification of staphylococci as susceptible or resistant to zinc chloride or copper sulphate, the following tentative threshold values for resistance were used: zinc chloride (MIC>2 mM) and copper sulphate (MIC>12 mM), respectively (Aarestrup and Hasman, 2004).

PCR screening

The isolates were tested for the presence of *czrC* by PCR amplification with primers *czrC* fw 5'- TAGCCACGATCATAGTCATG -3' and *czrC* rev 5'- ATCCTTGTTTTCCTTAGTGACTT -3' using a previously described protocol (Cavaco et al., 2010). The PCR results were compared with the MICs of zinc chloride and copper sulphate.

Statistical analysis

The association between the zinc susceptibility/resistance phenotype, PCR screening and methicillin resistance was investigated by statistical analysis of the data in contingency tables using Fisher´s exact test. Agreement between susceptibility testing results and PCR detection of the *czrC* gene was investigated using the Kappa test (Thrusfield, 2010).

RESULTS

Metal susceptibility testing

A total of 73% (N=348/476) of the MRSA from swine in Europe were resistant to zinc chloride at MICs of >2mM. A prevalence of 74% (n= 324) of zinc resistance was observed among MRSA isolates of CC398 of pig origin and 42% (n=39) among those originating from veal calves in Europe. All porcine MSSA were found to be susceptible to zinc chloride and only two of the bovine MSSA showed an MIC of 4mM (Table 1 and Table 2, respectively).

Twenty percent (N=94/476) of the MRSA isolates from pigs and 21% (N=19/92) of the MRSA isolates from veal calves were resistant to copper sulphate at MICs>12mM. Among the MSSA isolates 66% (N=58/88) from pig origin were resistant to copper

sulphate whereas only 3% (N=2/60) of the MSSA from veal calves were found resistant to copper sulphate at MICs> 12mM (Tables 4 and 5).

PCR screening

The PCR results correlated significantly with the results obtained by MIC determination since 95% of the zinc-resistant pig isolates and 97% of the zinc-resistant veal calf isolates were found to carry *czrC* (agreement 96.1%, Kappa coefficient 0.91) whereas only 0.6% (n= 3) of the isolates were found positive for *czrC* but found phenotypically susceptible (MIC= 2mM). Among the 37 European non-CC 398 MRSA pig isolates 62% (n= 24) of the isolates were resistant to zinc chloride, but only 46% (n=11) of these isolates were positive for *czrC*, suggesting the presence of other resistance determinants. The *czrC* gene was associated to SCC*mec* cassette in all 55 isolates were SCC*mec* typing was performed, whereas among 34 *czrC* negative isolates tested most carried either type V or type IV SCC*mec* elements (n=18 and n=14) and only one each carried type II and type III SCC*mec*. Among the non–European isolates, phenotypic zinc resistance associated with the gene *czrC* and SCCmec type V was observed in 44% (n=8) of the 18 Canadian isolates, but neither phenotypic zinc resistance nor the *czrC* gene were detected among the seven ST9 Chinese MRSA isolates (Tables 1, 2 and 3).

By country, the prevalences of *czrC* varied between 35% and 92% among MRSA CC398 from pigs and from 0% to 50% in non-CC398 MRSA isolates (Table 3). A prevalence of *czrC* of 41% was observed among MRSA CC398 isolates from veal calves (Table 2). None of the MSSA isolates tested carried the *czrC* gene and the

corresponding MICs varied between 1 to 2mM, except for two isolates which showed a MIC of 4mM.

Susceptibility testing towards copper sulphate of both MRSA and MSSA resulted in MIC values between 4 and 24mM and the resistance observed was not correlated with the presence of *czrC* ($p>0.05$) (Table 4).

DISCUSSION

The factors related to the emergence of the MRSA subpopulations from the previously adapted MSSA clones remains to be explained. In our previous study it was observed that most MRSA CC398 strains isolated from pigs in Denmark had a high prevalence of zinc resistance and harboured SCC*mec* type V (74%), whereas the corresponding MSSA were susceptible (Aarestrup et al., 2010). Thus, it was hypothesized that zinc resistance could be a factor involved in the selection of MRSA of this clonal complex (Aarestrup et al., 2010). Furthermore, the *czrC* gene found in the SCC*mec* cassette type V was cloned and found to confer resistance to zinc chloride (Cavaco et al., 2010). The results of the present study are in agreement with the previous results and indicate that zinc resistance is highly prevalent among MRSA from pigs and veal calves in Europe and mainly associated with the presence of the *czrC* gene (Cavaco et al., 2010). Although there is an excellent agreement (96.8%) between zinc resistance and the detection of the gene *czrC*, our results also indicate that other zinc resistance determinants may be present in few CC398 isolates and in a larger proportion of non-CC398 (mainly CC1) isolates. Furthermore, the results indicate that zinc resistance is not the only factor contributing to the spread of MRSA in livestock as a considerable proportion of the MRSA isolates was found to be zinc-susceptible. Nevertheless, the

193 *czrC* gene is genetically located within the type V SCC*mec* elements (Cavaco et al.,
194 2010) and thus resistance to metals such as zinc and cadmium may play a role in co-
195 selection of methicillin resistance in *S. aureus*.
196 The results of susceptibility testing of copper sulphate were neither significantly
197 associated with the *czrC* gene nor methicillin resistance in this study which agrees with
198 the expected phenotype for *czrC* expression in *S. aureus* which was found to confer
199 only zinc and cadmium resistance (Cavaco et al., 2010).
200 The MRSA CC398 strains from pigs had, a higher prevalence of zinc resistance (74%)
201 compared to the strains from veal calves (42%). Although the veal calf isolates were
202 obtained from only one country, the calves fattened in the Netherlands were originated
203 from different European countries and therefore the origin of the MRSA in the veal
204 calves could not be determined. Therefore and due to differences in the production
205 systems, the zinc resistance prevalence observed in the veal calve isolates can only be
206 compared to the prevalence observed in the pig isolates from the Netherlands or from
207 other European countries with caution.
208 Metal containing compounds such as copper sulphate and zinc oxide are widely used
209 as feed supplements in food producing animals for prevention of diarrhoeal diseases.
210 However, the total amounts and concentrations used in feed might differ among
211 countries, due to restrictions imposed by national legislation. As a consequence,
212 different selective pressures might be exerted within the different countries. Our results
213 indicate that the potential selective effect of zinc might have impact on the flora of
214 production animals by providing a competitive advantage of resistant strains. This
215 could also be of relevance to humans, as zinc compounds are used in ointments and
216 skin preparations that are used widely from a very early age, which could potentially

influence the skin flora, although the potential selective effects are unexplored. The use of zinc in animal production and for humans might be an important factor to consider in the future when investigating the selection dynamics of MRSA.

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Table 1. Distribution of results of *czrC* screening and MIC of zinc chloride among 476 MRSA and 88 MSSA isolated from pigs in Europe.

Isolates	CC	N isolates	No. of isolates with a MIC of zinc chloride of (mM)					
			1	**2**	**4**	**8**	**12**	**16**
czrC negative MSSA	ns	88	22	66				
czrC negative MRSA	CC398	115	49	61	2	2	1	
	CC1	19	2	5	1		1	10
	CC5	1		1				
	CC9	1		1				
	CC97	4		3			1	
	nd	3	1	2				
czrC positive MRSA	CC398	321		2	15	189	81	34
	CC1	2				1		1
	CC97	10		1	1	6	2	

Legend: Nd- not determined; Ns- not specified

Table 2.Distribution of results of *czrC* screening and MIC of zinc chloride among 92 MRSA and 60 MSSA isolated from veal calves in the Netherlands.

Isolates	CC	N isolates	Nr of isolates with a MIC towards Zn chloride of (mM)					
			1	**2**	**4**	**8**	**12**	**16**
czrC negative MSSA	ns	60	14	44	2			
czrC negative MRSA	CC398	54	18	35	1			
czrC positive MRSA	CC398	38				32	6	

Legend: Ns- not specified

Table 3-Prevalence of *czrC genes* in MRSA isolates from pig farms by country of origin.

Country	N MRSA isolates	N MRSA isolates of CC398	N *czrC* positive CC398 isolates	N *czrC* positive non-CC398 isolates	Positive to *czrC* (%)
Belgium	100	97	59	n.a.	59.0%
Denmark	7	7	5	n.a.	71.4%
France	8	7	5	0	62.5%
Germany	16	13	12	1	81.3%
Hungary	5	5	3	n.a.	60.0%
Italy	77	46	16	10	33.8%
Netherlands	59	59	37	n.a.	62.8%
Poland	9	9	7	n.a.	77.8%
Portugal	21	21	19	n.a.	90.5%
Spain	174	172	158	1	91.4%
Canada	18	18	8	n.a.	44.5%
China	7	0	n.a.	0	0%

n.a. - not applicable

Table 4- Distribution of results of *czrC* screening and MIC of copper sulphate among 476 MRSA and 88 MSSA isolated from pigs in Europe.

Isolates	CC	N isolates	No. of isolates with a MIC of copper sulphate of (mM)							
			4	**8**	**12**	**16**	**20**	**24**	**28**	**>28**
czrC negative MSSA	ns	88		9	21	14	5	14	12	13
czrC negative MRSA	CC398	115		81	20	1	3	10		
	CC1	19		14			4	1		
	CC5	1		1						
	CC9	1			1					
	CC97	4		2		1	1			
	nd	3		2	1					
czrC positive MRSA	CC398	321	1	249	9	15	23	22	2	
	CC1	2		1		1				
	CC97	10				8	2			

Legend: Nd- not determined; Ns- not specified

Table 5-.Distribution of results of *czrC* screening and MIC of copper sulphate among 92 MRSA and 60 MSSA isolated from veal calves in the Netherlands.

Isolates	**CC**	**N isolates**	Nr of isolates with a MIC towards copper sulphate of (mM)			
			8	**12**	**16**	**20**
czrC negative MSSA	ns	60	54	4	2	
czrC negative MRSA	CC398	54	14	39	1	
czrC positive MRSA	CC398	38	19	1	12	6

Legend: Ns- not specified