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Unexpected Sequence Types in livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA): MRSA ST9 and a Single Locus Variant of ST9 in pig farming in China.

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Abstract

In October 2008 nine farrow-to-finish pig farms were visited in Shuangliu County in Sichuan Province, China. One farm was empty for one month but not cleaned after depopulation. Dust samples were collected at each farm and analysed for the presence of methicillin-resistant *Staphylococcus aureus* (MRSA). Dust samples from four farms were also analysed for the presence of methicillin-susceptible *Staphylococcus aureus* (MSSA). On 5/9 farms MRSA was isolated and on 2/4 farms MSSA was isolated. On two farms, including the empty farm, no MRSA or MSSA could be detected. All MRSA isolates (n=43) belonged to *spa* type t899. MSSA isolates belonged to *spa* type t899 (n=12) and *spa* type t034 (n=2). From 4/9 farms the MRSA isolates of *spa* type t899 were assigned to multilocus sequence type (MLST) ST9 whereas on one farm the MRSA *spa* type t899 isolates belonged to a single locus variant of MLST ST9 (ST1376). MSSA isolates with *spa* type t899 belonged to MLST ST9 and the MSSA with *spa* type t034 belonged to MLST ST398.

This is the first report on MRSA in pig farms in China and the first time that MRSA ST9 and a single locus variant of ST9 are detected in pig farms. This study shows that livestock associated MRSA is not restricted to clonal lineage ST398 as found in Europe and Northern America in commercial pigs but that other MRSA lineages are able to spread in livestock as well. The study confirms that livestock may act as a reservoir for MRSA.

Introduction

Methicillin-resistant *Staphyloccocus aureus* (MRSA) is of increasing importance not only as a cause of nosocomial infections but also as a cause of community acquired infections in humans. In 2005, the presence of MRSA in pigs and the transfer to humans was reported for the first time (Voss et al., 2005). Soon thereafter dissemination of a single MRSA clone in pig production in different geographical regions in the world was described (De Neeling et al., 2007; Khanna et al., 2007; Sergio et al., 2007; Smith et al., 2008). The isolates belonging to this clone were not typeable by pulsed-field gel electrophoresis (PFGE) using *Sma*I and therefore initial referred to as non typeable MRSA (NT-MRSA) currently called livestock associated MRSA (LA-MRSA). Virtually all LA-MRSA isolates belong to MLST Clonal Complex 398 (CC398) with Sequence Type 398 (ST398) as the predominant sequence type. Within ST398 different *spa* types, mainly t011, t034, t108, t899 and t1254 have been described (De Neeling et al., 2007; Graveland et al., 2008). The clone is not restricted to pig farming but is also widespread in veal farming and has been found in poultry and horses (Graveland et al., 2008; Persoons et al., 2009; Van Den Eede et al., 2009). The reason for the efficient spread of this specific clone remains unclear. In pig farming a trend was seen with routinely administered antimicrobials being a risk factor for a farm to be MRSA positive (Van Duijkeren et al., 2008). ST398 strains are all resistant to tetracycline and susceptible for trimethoprim/sulfamethoxazole whereas other resistances vary between strains (Weese and Van Duijkeren, 2009). Clinical disease in livestock caused by LA-MRSA is rarely seen. However, livestock acts as an emerging reservoir for MRSA with subsequent transmission to humans (Voss et al., 2005). Therefore, from a public health point of view, the epidemiology and control of the spread of MRSA in livestock is important. In Canada, a common human MRSA clone (CMRSA-2/USA100) was isolated from pigs besides MRSA of ST398 (Khanna et al., 2007). This supports the concern on the potential changes in the epidemiology of

MRSA in livestock by changes in the current clone (e.g. uptake of virulence genes) or a replacement of ST398 by other, more virulent clones (Scientific Opinion EFSA, 2009). Until now only data on the occurrence of MRSA in livestock in Europe and Northern America is available. An important pig industry is present in Asia but data on MRSA prevalence in pigs is lacking. This information is important to understand the epidemiology of MRSA and is needed to assess the risk for transmission to humans.

The aim of this study was (i) to determine if MRSA is present on commercial pig farms in China, and (ii) to characterize the MRSA isolates.

Materials and Methods

Collection of samples

Nine commercial pig farms all located in Shuangliu County in Sichuan Province were visited in October 2008. All farms were farrow-to-finish pig farms with >1000 animals and they all started < 5 years ago except for the empty farm (Farm I) that was considerably older. Farm I was empty for one month but not cleaned after depopulation. Dust samples were collected using dry viscose/polypropylene clothes (Zeeman, the Netherlands). The number of dust samples that were analysed varied per farm (Table 1). Dust was collected from pen partition walls, ventilator, tubes and other horizontal flat surfaces. Samples were transported to the lab in plastic bags and processed within 10 days after sampling.

MRSA and methicillin-susceptible *S. aureus* (MSSA) isolation and identification

Pre-enrichment broth

Samples were incubated at 37˚C overnight in containers with 50-100 ml Mueller Hinton Broth (MHB) containing 6.5% NaCl and subsequently used for the procedure for MRSA and MSSA isolation.

106

107 *MRSA isolation and identification (all farms)*

108 One ml of this pre-enrichment broth was transferred into 9 ml phenyl mannitol broth (PHMB)
109 (bioMérieux, Marcy l'Etoile, France) with 5 µg/ml ceftizoxime and 75 µg/ml aztreonam. After
110 overnight incubation at 37˚C, 10 µl of the PHMB broth was plated onto Heart Infusion agar
111 with 5% sheep blood (sheep blood agar) (Biotrading, Mijdrecht, The Netherlands) and
112 Brilliance MRSA agar (Oxoid, Badhoevedorp, the Netherlands).
113 Suspected colonies were identified as *S. aureus* using standard techniques: colony
114 morphology, Gram staining, catalase production, coagulase production, and by a latex
115 agglutination test (Pasteurex Staph Plus, Bio-Rad Laboratories, Hercules, USA). MRSA
116 suspected colonies were confirmed by PCR specific for a *S. aureus* DNA fragment
117 (Martineau et al., 1998), the *mecA* gene (de Neeling et al., 1998), and the Panton-Valentine
118 leukocidin toxin (PVL) genes (Lina et al., 1999).

119

120 *MSSA isolation and identification (farms D, G, H, I)*

121 Ten µl of MHB pre-enrichment broth was plated onto SA-select agar (Bio-Rad, the
122 Netherlands) and sheep blood agar. Suspected colonies were identified as *S. aureus* by using
123 standard techniques as mentioned before. From samples that were also MRSA-positive, up to
124 18 *S. aureus* colonies were checked for the absence of the *mecA* gene by PCR, because on the
125 SA-select agar both MRSA and MSSA can grow.

126

127 **Genotyping**

128 *Spa*-sequence typing was performed as described before, utilizing sequencing the variation in
129 the tandem repeat region of the protein A encoding *spa* gene (Harmsen et al., 2003). Data
130 were analyzed by using the Ridom Staphtype software version 1.4 (http://www.ridom.de).

From farms A, B, C, D, E, F, and G one isolate was further characterized with PFGE using *Sma*I as restriction enzyme according to the Harmony protocol (Murchan et al., 2003) and by multilocus sequence typing (MLST) (Enright et al., 2000), (www.saureus.mlst.net). As MLST assigned a ST9 variant to isolates of farm A, all 7 isolates of this farm were analysed by MLST.

Susceptibility testing

From each MRSA or MSSA positive farm two isolates were randomly selected for susceptibility testing. Susceptibility was tested quantitatively by broth microdilution with cation-adjusted Mueller Hinton broth according to ISO standard 20776-1:2006. For broth microdilution, microtitre trays were used with custom made panels of dehydrated dilution ranges of antibiotics (Sensititre®, Trek Diagnostic Systems, Basingstoke, UK). ATCC strains *Enterococcus faecalis* 29212 and *S. aureus* ATCC 29213 were included for quality control. The Minimum Inhibitory Concentrations (MICs) were defined as the lowest concentrations without visible growth. EUCAST clinical breakpoints (www.eucast.org) and CLSI breakpoints (M100-S17) were used for classification of Resistant, Intermediate and Susceptible.

Results

Dust samples of 5/9 (55.6 %) farms were MRSA-positive (Table 1). On 2/4 farms MSSA could be detected (Table 1). On both MSSA-positive farms (D and G) no MRSA could be detected. On 2 farms (H and I), including the empty farm, no MRSA or MSSA could be detected.

All MRSA and MSSA isolates were negative for Panton-Valentine leukocidin toxin genes.

All MRSA isolates (n= 43) belonged to *spa* type t899. MSSA isolates detected at farm D belonged to *spa* types t899 (n=1) and t034 (n=2). MSSA isolates on farm G were all assigned to *spa* type t899 (n=11).

From each farm B, C, E, and F one MRSA isolate was assigned to MLST ST9 based on its allelic profile, 3-3-1-1-1-1-10. All MRSA isolates of farm A were assigned to a novel single locus variant of MLST ST9 with the allelic profile, 3-73-1-1-1-1-10 (ST1376). From each farm D and G one MSSA isolate of *spa* type t899 was also assigned to MLST ST9. One isolate with *spa* type t034 (farm D) was assigned to MLST ST398.

ST9 isolates, both MRSA and MSSA, were typeable with *Sma*I macrorestriction PFGE whereas the ST398 MSSA isolate was not typeable with *Sma*I PFGE (Figure 1). All ST9 isolates revealed a different PFGE pattern (Figure 1; farm F not shown).

Susceptibility testing revealed that 9/10 tested MRSA isolates were resistant to amikacin, ciprofloxacin, clindamycin, erythromycin, gentamicin, neomycin, and tetracycline. These strains were susceptible to fusidic acid, linezolid, mupirocin, rifampicin, and trimethoprim/sulfamethoxazole. One MRSA strain (farm A) was resistant only to amikacin, gentamicin, and tetracycline. The MSSA strains tested (n=4) generally showed the same susceptibility pattern as the majority of the MRSA isolates. The exception was for neomycin to which two strains were resistant and two were susceptible.

Discussion

This is the first report of MRSA on Chinese pig farms. The most remarkable finding was the presence of the *mecA* gene in *S. aureus* of MLST ST9 and a single locus variant of ST9 in dust samples from commercial pig farms. Although this was a pilot study and the number of sampled farms was relatively small, this newly described pig-related MRSA type seem to be

common among pig farms in this region. MRSA MLST ST9 was found on four farms and its single-locus variant ST1376 on one farm. This finding is remarkable as until now the most prevalent MRSA clone in livestock in Europe, Canada and the US belongs to CC398 (Weese and Van Duijkeren, 2009). One study on MRSA in experimental pigs in Singapore showed the presence of MRSA ST398 (Sergio et al., 2007). To date, this is the only report of livestock associated MRSA in Asia. Studies investigating the presence of MRSA on commercial pig farms have not been published before. In our study we did not sample pigs themselves but dust samples from the environment. Environmental samples are an indicator for colonisation in pigs (Scientific Opinion EFSA, 2009). MSSA of ST9 has been described in humans, in pigs, and in humans working with the ST9 pigs showing that ST9 is able to colonize human and can be transmitted between pigs and humans (Armand-Lefevre et al., 2005; Kehrenberg et al., 2009). Although *S. aureus* of CC9 can colonize humans, it was found only sporadically in nasal samples of nonhospitalized elderly individuals in the United Kingdom (Grundmann et al., 2002) and among Irish students (Collery et al., 2008). In pigs and pig farmers MSSA of ST9 seems to be common. In one study in France, 7/14 porcine *S. aureus* isolates and as many as 18/44 pig farmer *S. aureus* isolates belonged to ST9, but ST9 was not found amongst *S. aureus* isolates from bank- or insurance workers indicating that pigs act as reservoir for transmission to humans (Armand-Lefevre et al., 2005). In our study the farmers were not included in sampling and transfer from pigs to humans could not be confirmed. However, it is likely that these farmers were at risk of being positive for MRSA. The virulence of ST9 MRSA is still unclear. Three studies on the prevalence of MRSA in Chinese hospitals do not describe ST9 indicating that ST9 is not the dominant ST in Chinese hospitals (Yu et al., 2008; Xu et al., 2009; Zhang et al., 2009). Liu et al., (2009), however, have described an MRSA isolate with *spa* type t899 and MLST ST9 from a patient with severe clinical illness. This indicates that MRSA ST9 is associated with disease in humans. Unfortunately no information

was available if this patient had been in contact with animals. Generally, MRSA of ST5 and ST239 are the most prevalent sequence types in Chinese hospitals and MRSA of ST9 are still rare (Liu et al., 2009). To date no information is available in the literature on MRSA colonization rates of pig farmers in China. In addition, we do not know if MRSA ST9 emerged in Chinese pigs recently. In that case it could be expected to find more human carriers and infections in the future. Whether MRSA ST9 also causes disease in pigs needs further investigation.

The limitation of this study is that only nine farms were sampled in one specific geographical area and the history of trade on these farms was not available. As most of the farms were relatively new (< 5 years) there may be a common source of pigs when the farms were populated. It has been shown that trade of colonized pigs plays an important role in the spread of MRSA within the pig population (Van Duijkeren et al., 2008).

This study shows that for understanding the epidemiology of MRSA we should not rely solely on *spa* typing. MRSA of *spa* type t899 have been found in Dutch pigs, but these isolates all belonged to MLST ST398 (De Neeling et al., 2007; Van Duijkeren et al., 2008). This study shows MRSA of *spa* type t899 belonging to a different Clonal Complex (CC9). This confirms that in addition to *spa* typing MLST typing provides important information to get insight in the epidemiology of MRSA. PFGE of the ST398 isolate confirmed the specific characteristic of this clonal cluster to be non-typeable with *Sma*I macrorestriction. Isolates of ST9, both MRSA and MSSA, could be typed and revealed a different PFGE pattern for each farm. The PFGE patterns of farm B and farm E isolates showed a high degree of homology. The diversity in PFGE patterns of the MRSA isolates may suggest either that the acquisition of the *mecA*-gene has been a multiple event or the molecular variation of MRSA ST9 over time.

In this study the concurrent on-farm occurrence of MSSA and MRSA did not occur and may suggest a competition between MSSA and MRSA. However, this may be partly biased by the

method used. On MRSA positive farms, the *S. aureus* selective plates (SA-select) showed heavy growth of MRSA and it can not be excluded that MSSA could have been present but have been missed as only up to a maximum of 18 colonies have been tested for the *mecA* gene. This aspect of competition definitively needs more attention in further studies.

The presence of MSSA belonging to ST9 and ST398 strengthen former studies that these STs are able to colonize pigs. The origin of MRSA ST398 and ST9 remains unclear but the efficient colonisation of pigs of MSSA ST398 and ST9, the probable co-colonisation with *mecA* donor species like coagulase-negative staphylococci and the abundant use of antimicrobials in pig production may favour the development of MRSA. In the present study no MRSA ST398 could be detected. Although our study is limited in size, it shows the presence and suggests a spread of the *mecA* positive ST9 in pigs in China. Further studies with samples from other areas are needed to investigate the prevalence of this clone and potentially other MRSA STs on pig farms in China. The finding that on farm A a single locus variant of ST9 was identified, supports the idea that pig farms may act as a reservoir for evolution of existing clones and emergence of new MRSA lineages. China has about 50% of the world pig population and the presence of MRSA in this pig population may act as an enormous reservoir for MRSA. In view of the national and international travel and trade, this is a potential risk for humans and pigs.

In conclusion: this study shows that MRSA is also present on commercial pig farms in China. On this limited number of farms the clone is different from the one that is widely spread in Europe, the US and Canada. The fact that within this small study a variant of ST9 was identified confirms that MRSA in livestock is not a static happening but a continuous evolution in a big animal reservoir. As *S. aureus* of ST9 is very common in pigs, the occurrence of MRSA of this sequence type is worrying. The implications of this finding for

the public health need to be determined but it is highly likely that this clone can be transmitted to humans and is therefore a threat for public health.

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Conflict of interest statement

None of the authors has any financial and personal relationships with other people or organisations that could inappropriately influence or bias the work.

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359
360

Table 1

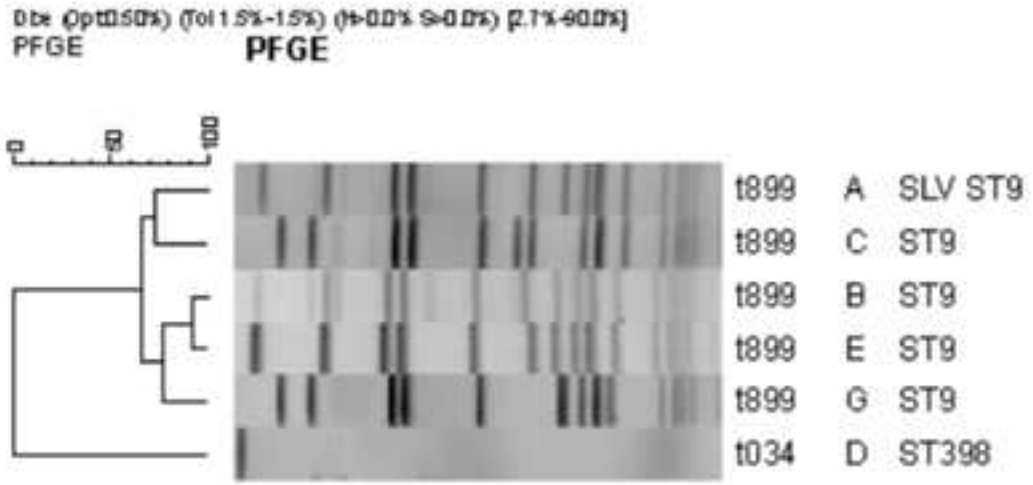
Characteristics of isolates from dust samples from 9 pig farms in China, October 2008

Farm	Number of samples tested	Number of MRSA [1]	Number of MSSA [1]	Spa type (number)	MLST
A	2	7	n.d	t899 (7)	ST1376
B	2	12	n.d	t899 (12)	ST9
C	2	8	n.d	t899 (8)	ST9
D	8	0	3	t034 (2)	ST398
				t899 (1)	ST9
E	4	9	n.d	t899 (9)	ST9
F	4	7	n.d.	t899 (7)	ST9
G	8	0	11	t899 (11)	ST9
H	8	0	0		
I	8	0	0		

[1] n.d: not determined, Number of positive MRSA colonies that were characterized with *spa* typing, multi locus sequence typing and pulsed field gel electrophoresis.

Figure 1: PFGE patterns of ST9 and single locus variant of ST9 MRSA and MSSA isolates, and one ST398 MSSA isolate. The farm of origin is indicated.

Figure 1


PFGE
PFGE
t899 A SLV ST9
t899 C ST9
t899 B ST9
t899 E ST9
t899 G ST9
t034 D ST398