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Farm-specific lineages of methicillin-resistant *Staphylococcus aureus* clonal complex 398 in Danish pig farms

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SUMMARY

The objective of this study was to investigate the genetic diversity of methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC) 398 using pulsed-field gel electrophoresis (PFGE). Dust and pigs at five age groups were sampled in six Danish MRSA-positive pig farms. MRSA CC398 was isolated from 284 of the 391 samples tested, including 230 (74%) animal and 54 (68%) environmental samples. PFGE analysis of a subset of 48 isolates, including the six strains previously isolated from farm workers, revealed the existence of farm-specific pulsotypes. With a single exception, human, environmental and porcine isolates originating from the same farm clustered together in the PFGE cluster analysis, indicating that spread of MRSA CC398 in Danish pig farms is mainly due to clonal dissemination of farm-specific lineages that can be discriminated by PFGE. This finding has important implications for planning future epidemiological studies investigating the spread of CC398 in pig farming.

Key words: Antibiotic resistance, MRSA, swine, zoonoses.

INTRODUCTION

The livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) clonal complex (CC) 398 has been reported with increasing frequency in animals (especially in pigs), farm workers and veterinary staff [1–7]. MRSA CC398 is non-typable by standard pulsed-field gel electrophoresis (PFGE) due to methylation of the *Sma*I restriction site [8]. In order to study the epidemiology of this MRSA clone, various PFGE protocols have been developed using alternative endonucleases such as *Cfr*9I [9, 10] or *Apa*I [11]. However, such protocols have not been employed yet to elucidate the population diversity of MRSA CC398 at the farm level.

In The Netherlands, the epidemiology of MRSA CC398 has been extensively studied in pigs. High frequencies of MRSA CC398 have been described in pig-farm workers, and pigs at the slaughterhouse [12, 13]. In Denmark, the prevalence of MRSA CC398-positive pig production holdings ($\geqslant 50$ breeding pigs) appears to be low (3·5%, $n = 198$) compared to the European Union-wide prevalence (25·5%, $n = 3012$) [14]. Screening of participants at the annual meeting of the Danish Pig Production Association in 2008 showed that 3·1% (15/487) of the pig-farm

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workers were positive for MRSA CC398 [15]. One year after this meeting, we visited the farms of six of the 15 positive farm workers and investigated the occurrence of MRSA in different pig age groups and production units within each farm. The objectives of this study were to evaluate the diversity of MRSA CC398 within and between Danish pig farms and to assess the epidemiological relatedness of porcine, environmental and human isolates using a highly discriminatory method such as PFGE.

METHODS

Sampling times and sites

Six of the 15 MRSA-positive farm workers identified in the previous study [15] formally agreed to participate in this study. We visited each farm between October and November 2009. Four farms (nos. 1, 2, 5, 6) were farrow-to-finishing holdings and two farms (nos. 3, 4) were farrow-to-growing holdings (i.e. farms without a finishing unit). All farms exclusively produced pigs born at their facilities and purchased replacement gilts from breeding holdings. Farms 1 and 2 were located on Funen, farm 3 in North Jutland, farms 4 and 5 in South Jutland and farm 6 in West Jutland. A total of 311 animal and 80 environmental samples were collected from three production units: the farrowing unit where sows are kept from 1 week prior to farrowing up until the piglets are weaned; the growing unit where weaned pigs are kept until they reach about 30 kg weight; and the finishing unit where finishers are kept until they are transported to the slaughterhouse at about 100 kg weight. Five age groups of pigs were sampled at each farm: ten pregnant sows <7 days prepartum, ten farrowed sows <7 days postpartum, two piglets <3 days after birth from each farrowed sow, 15 weaned pigs 3 weeks after weaning, and 15 finishers 1 week before slaughter. Fewer than ten pregnant sows were sampled in three farms (farms 3, 5, 6) because no more pregnant sows were present in the farrowing unit at the time of sampling (Table 1). Animals were sampled from the greatest number of pens available within each unit; more than one pig was sampled in a pen if the number of pens was <15 in a unit. Each animal was sampled by introducing a cotton swab in both nares. A vaginal swab was additionally collected from each pregnant and farrowed sow minimizing perineal contamination by avoiding contact with the external vulva with the aid of sterile gloves. The vaginal sample was pooled with its corresponding nasal sample. Nasal samples from the two newborn piglets belonging to the same litter were pooled. In addition to animal samples, five environmental samples were collected from each room under study. Each environmental sample was taken by wiping the dust from four spots on different horizontal surfaces or fences covering a total area of about 25 cm $\times$ 25 cm. Environmental samples were taken using cloth wipes moistened with Ringer's solution (Sodibox, France). All samples were processed within 24 h after collection.

Isolation and genetic characterization of MRSA CC398

Animal swabs and environmental wipes were enriched in 5 and 100 ml Mueller–Hinton broth containing 6·5% NaCl, respectively. After 18 h incubation at 37 °C, 10 μl of the enrichment broth were plated onto Brilliance MRSA agar (Oxoid, UK) and incubated overnight at 37 °C. Presumptive MRSA colonies were subcultured onto blood agar and incubated overnight at 37 °C. MRSA CC398 was confirmed using a multiplex polymerase chain reaction (PCR) as described previously [16]. The association between the carriage status of farrowed sows and that of their piglets was analysed by the GLIMMIX procedure in SAS (SAS Institute Inc., USA).

A subset of 48 isolates from all farms, which included a representative isolate of each age group, one environmental isolate per unit, and the six strains previously isolated from the farm workers, was selected for phenotypic and genotypic characterization. Antimicrobial susceptibility was tested using the commercial broth microdilution minimum inhibitory concentration (MIC) panels Gram Positive All in One format (GPALL1F) (Sensititre, TREK Diagnostics, USA) which includes the clindamycin disk induction test (D test) for detection of inducible clindamycin resistance. The MICs were interpreted according to the Clinical and Laboratory Standards Institute [17], except for tigecycline, for which the breakpoints proposed by the European Committee for Antimicrobial Susceptibility testing were used (http://www.eucast.org/). The polymorphic X region of protein A gene was amplified and processed as described by Harmsen *et al.* [18]. The SCC*mec* was typed by a PCR-based multiplex assay described previously [19]. The same 48 isolates were also typed by PFGE using *Cfr*9I [10]. PFGE cluster analysis [unweighted pair group method with arithmetic mean (UPGMA) based

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Table 1. *Number and percentage of MRSA-positive samples in different production units and age groups at each of the six farms studied*

	Farrowing unit				Growing unit		Finishing unit		
Farm	Dust	Pregnant	Farrowed	Piglets*	Dust	Weaned pigs	Dust	Finishers	Total
1	3/5	7/10	10/10	10/10	1/5	15/15	2/5	14/15	62/75 (83%)
2	3/5	3/10	9/10	7/10	4/5	15/15	5/5	15/15	61/75 (81%)
3	4/5	2/6	8/10	8/10	4/5	15/15	—	—	41/51 (80%)
4	0/5	1/10	4/10	5/10	5/5	15/15	—	—	30/55 (54%)
5	2/5	0/2	6/10	2/10	5/5	14/15	5/5	0/15	34/67 (51%)
6	4/5	1/3	9/10	9/10	3/5	15/15	4/5	11/15	56/68 (82%)
Total	16/30 (53%)	14/41 (34%)	46/60 (77%)	41/60 (68%)	22/30 (73%)	89/90 (99%)	16/20 (80%)	40/60 (67%)	284/391

* Two piglets were sampled from each farrowed sow included in the study.

on the Dice similarity coefficient, with optimization and position tolerance set at 0·1% and 1·0%, respectively] was performed using GelCompar II (Applied Maths, Belgium).

RESULTS

Of the 391 samples tested, 284 (72%) were confirmed to be MRSA-positive, including 230 (74%) animal and 54 (68%) environmental samples. MRSA was isolated from all farms, production units and age groups except farm 5, where all animals located in the finishing unit were negative (Table 1). The carriage status of farrowed sows was highly associated with that of their piglets (odds ratio 16, 95% confidence interval 2·7–91, $P=0·0027$).

The antimicrobial resistance patterns are reported in Figure 1. The susceptibility patterns varied in isolates originating from the same farm. A few differences were observed between farms, e.g. all isolates from farm 4 were resistant to all three fluoroquinolones tested.

Most of the isolates (38/39) from farms 1–5 belonged to *spa* type t034, whereas all isolates from farm 6 ($n=9$) belonged to *spa* type t011. The remaining t011 isolate originated from an environmental sample from the growing unit in farm 1. All isolates harboured SCC*mec* type V. Within each farm, PFGE profiles were indistinguishable or differed by up to 12·3% in porcine, environmental and human isolates with the exception of the human isolate from farm 5, which clustered together with isolates from farm 3. At 87·5% of homology, all isolates were distributed in six farm-specific clusters, except for the human isolate from farm 5 (Fig. 1).

DISCUSSION

MRSA CC398 isolates from the six farms generally displayed farm-specific pulsotypes irrespective of their isolation source, providing evidence of the genetic relatedness of human, porcine and environmental isolates originating from the same farm. The PFGE protocol used in this study was seen to be highly discriminatory as it enabled discrimination between MRSA CC398 isolates displaying the same *spa* type and carrying the same SCC*mec* type. As such it can be employed as a useful tool to track MRSA transmission between farms and to investigate sources of human infection with this zoonotic *S. aureus* lineage. The method also showed indistinguishable PFGE pulsotypes in two strains with different *spa* types isolated from the same farm (farm 1), although the difference between the two *spa* types is either a loss or gain of two repeats, which can be due to one single genetic event.

spa type t034 was the most common type identified in this study. This *spa* type was also reported as the most common MRSA *spa* type in Danish pigs by the European Food Safety report in 2008 [14]. According to the results of this study, *spa*-typing does not provide information explanatory enough to study the epidemiology of MRSA CC398 in Denmark (two *spa* types in six farms) and shows questionable associations between strains (PFGE shows one t011 strain in farm 1 that clusters with the t034 strains in farm 1 and

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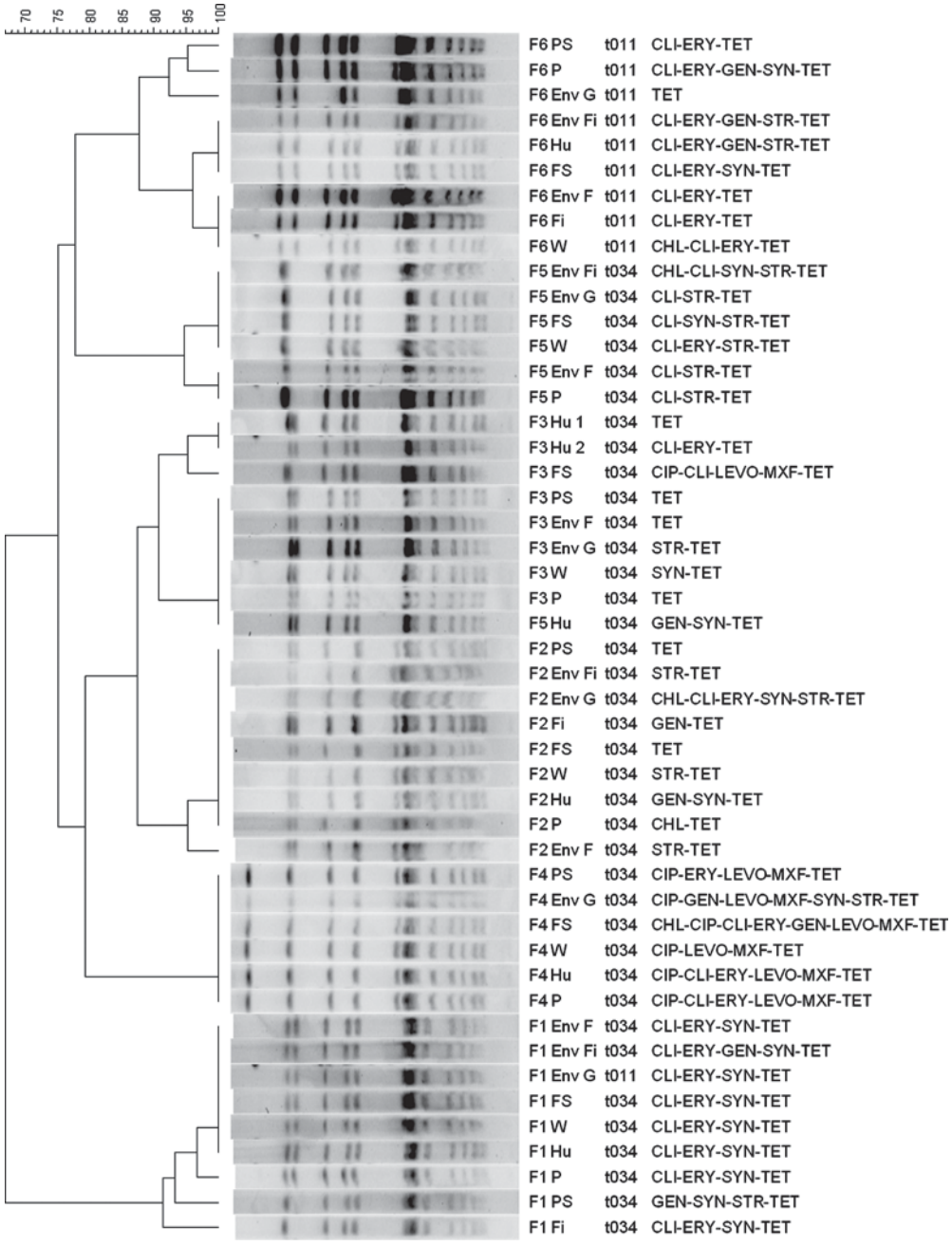

Fig. 1. PFGE profiles in a selection of 48 MRSA CC398 isolates from six Danish pig farms (F1–F6). At each farm, MRSA CC398 was isolated from farm workers (Hu), pregnant sows (PS), farrowed sows (FS), piglets (P), weaned pigs (W), finishers (Fi), and environmental sites at farrowing units (Env F), growing units (Env G) and finishing units (Env Fi). The *spa* types and antimicrobial susceptibility patterns are indicated for all isolates. The susceptibility patterns do not include β-lactam agents tested as all isolates were confirmed to be *mecA*-positive and resistant to all β-lactams. Antimicrobials are shown by their abbreviation: CLI, clindamycin; ERY, erythromycin; LEVO, levofloxacin; MXF, moxifloxacin; STR, streptomycin; SYN, synercid.

not with the other t011 strain in farm 6). Although each farm showed a dominant resistance pattern, the variability in the antimicrobial susceptibility patterns of isolates originating from the same farm was greater than expected according to the genotyping results by PFGE (Fig. 1). For example, the six isolates from farm 4 displayed five distinct susceptibility profiles despite the high similarity (100%) of their PFGE profiles. A possible explanation is that resistance was encoded by mutations or by genes located in small

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plasmids that would not be visible in the PFGE. Only for three isolates were differences in the resistance profile associated with lack of one band compared to other isolates from the corresponding farm (strains F1 PS, F3 FS and F6 Env G in Fig. 1).

The results show that the *Cfr9*I PFGE protocol described by Bosch *et al*. [10] is a highly discriminatory method for studying the epidemiology of MRSA CC398. Except for a single outlier, the band patterns obtained from porcine and environmental isolates were shown to be genetically related to those of human isolates originating from the same farm. As the isolates from farm workers were obtained about 1 year prior to this study, the results suggest that distinct MRSA CC398 sublineages may persist for a long period within the farm or be re-introduced from the gilt supplier. The persistence of strains displaying farm-specific PFGE profiles that appear to be stable over time suggests that each farm may have been contaminated with a distinct MRSA strain that has spread and become established as the dominant strain in the farm. This would then allow strains to be traced back to their origin sources and the mechanisms of spread between farms, e.g. by pig trade or human carriage, could be revealed by PFGE analysis. In addition, introduction of exogenous MRSA strains into the farms may be a rare event or the dominant MRSA strain on a farm is able to displace newly introduced strains. In all circumstances, PFGE should be regarded as a useful tool for future studies aimed at understanding the evolution and epidemiology of MRSA CC398 in pig farming.

In conclusion, the present study reveals the occurrence in Danish pig farms of farm-specific MRSA CC398 lineages that can be discriminated by PFGE. This epidemiological information will be of great value for investigating the mechanisms by which MRSA CC398 evolves and spreads in the pig production system.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Huijsdens XW, *et al.*** Community-acquired MRSA and pig-farming. *Annals of Clinical Microbiology and Antimicrobials* 2006; **5**: 26.
2. **van Loo I, *et al.*** Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. *Emerging Infectious Diseases* 2007; **13**: 1834–1839.
3. **Lewis HC, *et al.*** Pigs as source of methicillin-resistant *Staphylococcus aureus* CC398 infections in humans, Denmark. *Emerging Infectious Diseases* 2008; **14**: 1383–1389.
4. **Khanna T, *et al.*** Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. *Veterinary Microbiology* 2008; **128**: 298–303.
5. **Smith TC, *et al.*** Methicillin-Resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in Midwestern US swine and swine workers. *PLoS ONE* 2009; **4**: e4258.
6. **van den Broek IVF, *et al.*** Methicillin-resistant *Staphylococcus aureus* in people living and working in pig farms. *Epidemiology and Infection* 2009; **137**: 700–708.
7. **van Cleef BA, *et al.*** Prevalence of livestock-associated MRSA in communities with high pig-densities in The Netherlands. *PLoS ONE* 2010; **5**: e9385.
8. **Bens CCPM, Voss A, Klaassen CHW.** Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to uninterpretable results in standard pulsed-field gel electrophoresis analysis. *Journal of Clinical Microbiology* 2006; **44**: 1875–1876.
9. **Argudin MA, *et al.*** High heterogeneity within methicillin-resistant *Staphylococcus aureus* ST398 isolates, defined by *Cfr9I* macrorestriction-pulsed-field gel electrophoresis profiles and *spa* and SCCmec types. *Applied and Environmental Microbiology* 2010; **76**: 652–658.
10. **Bosch T, *et al.*** PFGE diversity within the methicillin-resistant *Staphylococcus aureus* clonal lineage ST398. *BMC Microbiology* 2010; **9**: 10–40.
11. **Kadlec K, *et al.*** Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from diseased swine. *Journal of Antimicrobial Chemotherapy* 2009; **64**: 1156–1164.
12. **Voss A, *et al.*** Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerging Infectious Diseases* 2005; **11**: 1965–1966.
13. **de Neeling AJ, *et al.*** High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. *Veterinary Microbiology* 2007; **122**: 366–372.
14. Analysis of the baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in holdings with breeding pigs, in the EU, 2008, Part A: MRSA prevalence estimates; on request from the European Commission. *EFSA Journal* 2009; **7**: 1376.
15. **Andreasen M, *et al.*** Prevalence of MRSA among Danish participants at a conference for Danish Pig Producers. In: Methicillin-resistant staphylococci in

animals: veterinary and public health implications. Washington: American Society for Microbiology, 2009, pp.27(http://www.asm.org/images/stories/Conferences/mrsa%20program%20book.pdf).

16. **Stegger M, *et al.*** Rapid PCR detection of *Staphylococcus aureus* clonal complex 398 by targeting the restriction-modification system encoding *sau1hsdS1*. *Journal of Clinical Microbiology* 2010; **49**: 732–734.
17. **Clinical and Laboratory Standards Institute (CLSI).** Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. Approved Standard M100-S21. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2011.
18. **Harmsen D, *et al.*** Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *Journal of Clinical Microbiology* 2003; **41**: 5442–5448.
19. **Kondo Y, *et al.*** Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. *Antimicrobial Agents and Chemotherapy* 2006; **51**: 264–274.

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