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Prevalence and Genetic Diversity of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* on Belgian Pork

MARIJKE VERHEGGHE,[1,2]* FLORENCE CROMBÉ,[2,3] KAAT LUYCKX,[1,4] FREDDY HAESEBROUCK,[2] PATRICK BUTAYE,[2,3] LIEVE HERMAN,[1] MARC HEYNDRICKX,[1,2] AND GEERTRUI RASSCHAERT[1]

[1]*Food Safety Research Group, Technology and Food Science Unit, Institute for Agricultural and Fisheries Research, Brusselsesteenweg 370, 9090 Melle, Belgium;* [2]*Department of Pathology, Bacteriology, and Avian Diseases and* [4]*Department of Obstetrics, Reproduction, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; and* [3]*Department of Bacteriology and Immunology, Veterinary and Agrochemical Research Centre, Groeselenberg 99, 1180 Brussels, Belgium*

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ABSTRACT

Since the first description of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), a high prevalence was observed in pigs. At present, questions remain about the transmission of LA-MRSA to the general human population through pork. The objectives of the present study were to determine the prevalence of LA-MRSA in Belgian pork and to determine the role of the pork production chain and butcheries in transmission of LA-MRSA to the human population. Pig meat samples (chops, bacon, minced pork, ribs, forelimbs, and ears; $n = 137$) originating from four butcheries (A through D) were spread plated on ChromID MRSA plates both before and after overnight enrichment culture. Suspect colonies were confirmed using a MRSA-specific triplex PCR assay and a CC398-specific PCR assay. The isolates ($n = 147$) were further characterized by SCC*mec* typing, multiple-locus variable number tandem repeat analysis, and antimicrobial susceptibility testing, a selection of isolates were subjected to pulsed-field gel electrophoresis and *spa* typing. Direct plating revealed a MRSA prevalence of 8%. After enrichment, MRSA was isolated from 98 (72%) of 137 samples of which the majority were from rib, ear, and forelimb. The majority (97%) of obtained isolates belonged to CC398, the main LA-MRSA type. A high level of genetic diversity was noted among the isolates from one butchery. Thirty antimicrobial susceptibility profiles were found; 13 and 9% of the isolates had Cip-Tet-Tri and Gen-Kan-Tet-Tob-Tri profiles, respectively. These results indicate the importance of enrichment for MRSA detection of pork. The observed genetic diversity of the isolates indicated that the pork production chain can be considered a source of multiple MRSA types that could be transmitted to the human population through cross-contaminated meat.

In 2005, Voss and colleagues *(31)* reported on a new methicillin-resistant *Staphylococcus aureus* (MRSA) type. This livestock-associated MRSA (LA-MRSA) and the main European clone MRSA ST398 (the dominant type in clonal complex [CC] 398) have been isolated from various livestock animals. High LA-MRSA prevalence was observed in pigs, which led to the hypothesis that pigs are a MRSA reservoir. Transmission from pigs to people working with them was reported, and LA-MRSA infections in people with and without animal contact have been occasionally described *(27, 32)*. As a result, concerns have been expressed about LA-MRSA entering the food chain and subsequently infecting the general human population.

Meat is an important vehicle for the transmission of both beneficial and pathogenic microorganisms to the general human population. Because this transmission has been described for zoonotic pathogens such as *Salmonella* and *Campylobacter* on pork, the question arose whether pork is also an important vehicle for LA-MRSA *(7)*. To date, only a limited number of studies (German, Dutch, Danish, and U.S.-Canadian) have investigated the presence of LA-MRSA on pork and pork products. Low levels of LA-MRSA (in CFU per gram) have been reported, which led to the suggestion that the possibility of transmission of LA-MRSA might be rather low *(1, 2, 5, 6, 10, 17, 28, 33)*. In previous studies, pigs and carcasses were sampled, and the isolates obtained were typed using molecular methods *(29, 30)*. Comparison of these isolates suggested the presence of one MRSA genotype in the pork production chain. The aims of the present study were (i) to determine the LA-MRSA (CC398) prevalence in various Belgian pork meat types using culture with and without enrichment and (ii) to study the genetic diversity of the isolates to determine the role of the pork production chain and butcheries in the transmission of MRSA to the human population. Samples of various pork types were collected from several butcheries on several occasions. The MRSA isolates were characterized using molecular methods and compared.

MATERIALS AND METHODS

Sample collection and sample processing. From February to April 2012, two local butcheries (A and B) and two supermarket butcheries (C and D) in the region of Ghent (Belgium) were

* Author for correspondence. Tel: +32 9 272 30 89; Fax: +32 9 272 30 00; E-mail: marijke.verhegghe@ilvo.vlaanderen.be.

arbitrarily chosen for study. The inclusion criteria were open for business on Monday and availability of ears and forelimbs. Local butcheries sell only fresh meat, which is handled by the butcher upon purchase and are wrapped in small packages. Supermarket butcheries are part of a retail chain, and the meat is packed before sale in the store. Every week (total of six successive weeks), six pork samples (pork chop, bacon, minced pork meat, rib, forelimb, and ear) were collected from each butchery ($n = 137$). Pork chops, bacon, and ribs are common items for consumption. The forelimb was a good site for isolation of MRSA at the slaughterhouse *(30)*. The ears were chosen because singeing of the lower carcass at the slaughterhouse is not always sufficient to prevent the survival of MRSA in the ears *(30)*. No minced pork was available at butchery D, and on one occasion no ear sample was available at butchery B. After purchase, the meat samples were transported directly to the laboratory and processed immediately upon arrival (within 1 h after purchase). Twenty-five grams of each chop, bacon, and minced pork sample were diluted 10-fold with salt-enriched (6.5% sodium chloride, 1.06404, Merck, Darmstadt, Germany) Mueller-Hinton (MH) broth (CM0405, Oxoid, Basingstoke, UK). Each dilution was mixed mechanically in a stomacher for 1 min, and subsequently a 10-fold dilution series was made until dilution 10^{-5} was reached. Because rib, forelimb, and ear could not be homogenized mechanically, these samples were measured to determine the area (in square centimeters) per sample, weighed, and diluted twofold. After homogenizing manually for 1 min, the rib, forelimb, or ear was removed, and a 10-fold dilution series was made until dilution 10^{-5} was reached. For all dilutions, 100 μl was spread plated onto Chrom-ID MRSA (bioMérieux, Marcy l'Etoile, France) plates. After incubation (18 to 20 h at 37°C), the number of characteristic green colonies was counted. The CFU per gram was calculated for the chop, bacon, and minced pork, and the CFU per square centimeter was calculated for the rib, forelimb, and ear. After subsequent incubation of the dilutions, 100 μl of the enrichment broth was plated onto Chrom-ID MRSA. After incubation, growth on the plates was examined. The highest dilution for which growth was observed was used to define the CFU per milliliter. This value was noted as smaller than the dilution factor CFU per milliliter of enrichment broth (e.g., growth up to 10^{-2} dilution was <100 CFU/ml enrichment broth). Depending on the number of characteristic green colonies on the plates, one to five colonies were removed and purified on Chrom-ID MRSA, and pure colonies were stored at –20°C in brain heart infusion (BHI) broth (CM0225, Oxoid) supplemented with glycerol (15% wt/vol, Fisher Scientific, Leicestershire, UK) until further analysis.

MRSA identification and MRSA CC398 confirmation. DNA was isolated from each colony and stored at –20°C until further use *(25)*. Isolates were identified as MRSA as described by Maes et al. *(13)*, targeting a 16S rRNA gene specific for the genus *Staphylococcus,* a *nuc* gene specific for *Staphylococcus aureus,* and the *mecA* gene specific for methicillin resistance. The presence of three bands confirmed the presence of MRSA. To confirm the presence of LA-MRSA and specifically the main European clone, a CC398-specific PCR targeting the restriction-modification system encoded by *sau1-hsdS1* was conducted with the confirmed MRSA isolates *(24)*.

Molecular typing. SCC*mec* typing and multiple-locus variable number tandem repeat analysis (MLVA) was performed on all isolates confirmed as MRSA ($n = 147$). The majority of CC398 MRSA isolates carry SCC*mec* types IVa and V *(29)*, so SCC*mec* typing focused on these types and was based on the combination of three protocols *(14, 18, 34)*. MLVA was performed and results were analyzed according to Verhegghe et al. *(29)*. After capillary electrophoresis of the PCR products, the MLVA data were transformed into numeric codes (a string of five integers) using the MLVA plugin of Bionumerics (version 7.5, Applied Maths, St.-Martens-Latem, Belgium). Each MLVA numeric code was converted to an MLVA type with a unique number (e.g., MLVA numeric code 36-56-36-7-3 was converted to MLVA type 1). In addition, a minimum spanning tree based on the numeric code was generated for all butcheries together with the Bionumerics program. Clustering of the dominant type with single-locus variants (MLVA types with one difference in one repeat region compared with the dominant type) was performed as described previously *(29)*. The results were compared with those for isolates from pig farms and from the slaughterhouse *(29, 30)*. A selection of CC398 isolates was chosen for pulsed-field gel electrophoresis (PFGE) and *spa* typing to represent the dominant MLVA cluster(s) and other nondominant MLVA types per butchery. Thirty-seven isolates (35 CC398 and 2 non-CC398) were selected: 10 isolates from butchery A (2 from forelimb, 3 from minced pork, and 5 from rib), 7 isolates from butchery B (1 from minced pork, 1 from rib, and 5 from chop), 14 isolates from butchery C (1 from chop, 5 from forelimb, and 7 from ear), and 6 isolates from butchery D (1 from rib and 5 from bacon). PFGE with *Bst*ZI restriction enzyme (Promega, Madison, WI) was performed, and the restriction profiles obtained were analyzed with Bionumerics 7.5 using the unweighted pair group method using averages with the Dice coefficient (tolerance, 1%; tolerance change, 1%; optimization, 1%) *(22)*. Pulsotypes were determined based on a delineation level of 97% and were given a Roman numeral. The *spa* typing was done according to the Ridom StaphType standard procedure (Ridom Bioinformatics, Munich, Germany, www.ridom.de/staphtype). The results were compared with those for isolates from pig farms and the slaughterhouse.

Antimicrobial susceptibility testing. The disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) procedure *(4)* was used to determine the antimicrobial susceptibility of all isolates. Neo-sensitabs (Rosco Diagnostica, Taastrup, Denmark) for 16 antimicrobial agents and the interpretation tables of the manufacturer (based on the CLSI method) were used. The agents were chloramphenicol (Clr; 60 μg), ciprofloxacin (Cip; 10 μg), erythromycin (Ery; 78 μg), fucidin (Fuc; 100 μg), gentamicin (Gen; 40 μg), kanamycin (Kan; 100 μg), lincomycin (Lin; 19 μg), linezolid (Line; 30 μg), mupirocin (Mup; 10 μg), quinupristin-dalfopristin (Syn; 15 μg), rifampin (Rif; 30 μg), sulfonamides (Sul; 240 μg), tetracycline (Tet; 80 μg), tobramycin (Tob; 40 μg), trimethoprim (Tri; 5.2 μg), and tylosin (Tyl; 150 μg). *S. aureus* ATCC 25923 and a MRSA ST398 strain (MB4360) were used as reference strains. Because tetracycline resistance was expected for all CC398 isolates, the presence of the tetracycline resistance genes *tetM* and *tetK* was assessed for the 20 CC398 tetracycline-sensitive isolates by a PCR assay as described by Ng et al. *(15)*.

RESULTS

After direct plating of the pork homogenates, MRSA was detected in 11 (8%) of the 137 meat samples: 1 bacon (butchery A), 1 rib (butchery B), 2 chop (butcheries A and D), 3 forelimb (butcheries A, B, and C), and 4 ear (butcheries A, B, and C) samples. The level of MRSA bacteria was 200 to 80,000 CFU/g or 6 to 14,776 CFU/cm^2 depending on the sample type (Table 1).

TABLE 1. *Results of MRSA isolation by pork meat type on six sampling occasions after direct plating (DP) or enrichment (E)*[a]

		Chop		Bacon		Minced meat		Rib		Forelimb		Ear	
Butchery	Sample event	DP (CFU/g)	E (CFU/ml)	DP (CFU/g)	E (CFU/ml)	DP (CFU/g)	E (CFU/ml)	DP (CFU/cm^2)	E (CFU/ml)	DP (CFU/cm^2)	E (CFU/ml)	DP (CFU/cm^2)	E (CFU/ml)
A	1	–	–	–	<100	–	<1,000,000	–	<1,000	3,557	<1,000,000	–	<1,000,000
	2	–	–	–	–	–	–	–	<10	–	<100	–	<100
	3	8,100	–	200	–	–	–	–	<10	–	<10	541	<100,000
	4	–	–	–	–	–	–	–	<10	–	<10	–	<10
	5	–	<10	–	<1,000,000	–	<10	–	<10	–	–	–	<10
	6	–	–	–	–	–	–	–	<10	–	<10	–	<100
B	1	–	<10	–	–	–	–	–	<100	–	<10	14,776	<1,000,000
	2	–	<10	–	<10	–	<10	–	<10	–	<100	–	<10
	3	–	<10	–	<1,000	–	<10	930	<10	6,704	<1,000	184	<1,000
	4	–	–	–	<10	–	<10	–	<100	–	<100	–	<100
	5	–	<10	–	–	–	<10	–	<10	–	<10	–	<100
	6	–	<10	–	–	–	<10	–	<10	–	<10	NA	NA
C	1	–	<10,000	–	–	–	<10	–	–	–	<10	–	<10
	2	–	–	–	<10	–	<10	–	–	–	<10	–	<10
	3	–	–	–	<10	–	–	–	<10	–	<10	–	<1,000
	4	–	<10	–	–	–	<10	–	<10	6	<10	6	<1,000,000
	5	–	–	–	–	–	<10	–	–	–	<10	–	<10
	6	–	–	–	–	–	–	–	–	–	<10	–	<10
D	1	–	<10	–	<10	NA	NA	–	<10	–	<10	–	<100
	2	–	<10	–	<10	NA	NA	–	<10	–	<10	–	<10
	3	–	–	–	<10	NA	NA	–	<10,000	–	<100	–	<100
	4	80,000	–	–	-	NA	NA	–	<100	–	–	–	<10
	5	–	–	–	<10	NA	NA	–	<10	–	<10	–	<10
	6	–	<10	–	<10	NA	NA	–	<10	–	–	–	<10
No. of positive/ total samples		2/24	11/24	1/24	12/24	0/18	11/18	1/24	20/24	3/24	21/24	4/23	23/23
% of positive samples		8	46	4	50	0	61	4	83	12	88	16	100

[a] –, MRSA negative; NA, not available. For the enrichment cultures, the highest dilution where growth was observed was noted as less than the dilution factor of the enrichment broth.

After enrichment, MRSA was isolated from 98 (72%) of the 137 samples: 100% of ear, 88% of forelimb, 83% of rib, 61% of minced meat, 50% of bacon, and 46% of chop samples. MRSA-contaminated ears, forelimbs, and ribs were found in all four butcheries and on all six sampling occasions. For 8 of the 11 samples from which MRSA was isolated after direct plating, MRSA also was detected after enrichment (Table 1). Estimation of the number of MRSA bacteria led to the following results: less than 10 CFU/ml of enrichment broth was found in 70 samples, less than 100 CFU/ml was found in 14 samples, less than 1,000 CFU/ml was found in 5 samples, and less than 1,000,000 CFU/ml was found in 6 samples. Less than 10,000 CFU/ml of enrichment broth was found in one chop sample, and less than 100,000 CFU/ml of enrichment broth was found in one rib and one ear sample. There was no clear link between the high MRSA levels and meat type or butchery (Table 1).

In total, 147 MRSA isolates (48 from butchery A, 40 from butchery B, 30 from butchery C, and 29 from butchery D) were obtained, and 97% (143 isolates) belonged to CC398. An overview of the available typing results for each individual isolate is given in Supplementary File 1 for the CC398 isolates and Supplementary File 2 for the four non-CC398 isolates (see https://drive.google.com/folderview?id=0B8HpP8npsL_mfkI1cUVDUUxxdWlpNGJZZnVYT1BvSWo1Wjlkc2IwcEV4UG1WT3VfcWVjT0U&usp=sharing for both supplementary files). Four SCC*mec* cassette types were detected among the 143 MRSA CC398 isolates: SCC*mec* type V (110 isolates), IVa (29 isolates), IV (1 isolate), and a nontypeable cassette type 3 (3 isolates, *mec*A complex NT/*ccr* complex C). The four non-CC398 isolates carried SCC*mec* type IV (two isolates), IVa (one isolate), and V (one isolate). In total, 43 MLVA types were isolated; 21, 15, 15, and 13 types were observed in butcheries A through D, respectively. Twenty percent of all MRSA CC398 isolates belonged to a dominant MLVA type (type 1, MLVA code 33-56-36-7-3), and three MLVA clusters were defined (Fig. 1; Supplementary File 1). Two clusters were found in isolates from butcheries A and B, and three were found in isolates from butcheries C and D (Fig. 1A; Supplementary File 1). A wide variety of MLVA types was seen within each butchery and within each meat type, and no MLVA cluster was unique to any butchery (Fig. 1A and 1B). These findings were consistent for all six sampling events (data not shown).

Table 2 gives an overview of the typing results for 35 selected CC398 isolates. These isolates were divided into 12 pulsotypes, and pulsotype VIII predominated in half of the isolates (Table 2). Four *spa* types were found: t011 (88% of isolates), t034 (6%), t2370 (3%), and t3423 (3%). Within the four non-CC398, two isolates belonged to *spa* type t127, one belonged to *spa* type t011, and one was nontypeable (Supplementary File 2). Combined typing results revealed that one overall MRSA genotype (MLVA cluster I, SCC*mec* V, *spa* type t011, and pulsotype VIII), found in 11 (32%) of the 35 tested isolates, was found on meat from all butcheries, whereas various other MRSA types occurred more sporadically. In three butcheries, a related genotype (MLVA cluster II, SCC*mec* V, *spa* type t011, and pulsotype VIII) was also isolated (6 [16%] of 35 isolates). In most cases, isolates originating from the same meat type collected at various sampling events in one butchery did not belong to the same MRSA genotype, and isolates retrieved from different meat types collected at the same sampling event in one butchery had different MRSA genotypes. Genotype MLVA cluster II, SCC*mec* V, *spa* type t011, and pulsotype VIII was also found in the pig and carcass isolates in two previous studies *(29, 30)*. The dominant genotypes isolated on three of four pig farms all belonged to pulsotype VIII and closely related MLVA types (e.g., 33-57-37-7-3 and 33-57-36-7-3) (data not shown).

Antimicrobial agent susceptibility testing revealed 30 antimicrobial susceptibility profiles (Table 3). Antibiotypes Cip-Tet-Tri and Gen-Kan-Tet-Tob-Tri were found in 19 MRSA isolates each (13%), and antibiotype Tet-Tri was found in 13 isolates (9%). The remaining profiles were found in seven or fewer isolates (Table 3; Supplementary File 1). Twenty CC398 isolates (15%) were not resistant to any of the tested antimicrobial agents, although these 20 isolates all carried the *tetM* and *tetK* genes. Within one butchery, most isolates belonging to the same MRSA genotype different antimicrobial resistance profiles.

DISCUSSION

The increased prevalence of LA-MRSA in pigs worldwide has given rise to concerns about the role of pork in the transmission of this potential pathogen to the human population. Handling of contaminated meat by butchers or consumers may be considered a risk factor for contracting MRSA. Meat is an important vector in the transmission of zoonotic bacteria, but little is known about meat and the transmission of LA-MRSA. To our knowledge, this report is the first on the presence of MRSA and specifically MRSA CC398 in Belgian pork.

At present, only three studies have been conducted to determine the MRSA levels in pork by direct plating *(5, 6, 33)*. Compared with those study results, where MRSA levels of 0.06 to <100 CFU/g were detected in pork, the levels in the present study were higher (200 to 80,000 CFU/g). The percentage of pork samples from which MRSA was isolated in the present study (8%) was comparable to that in other studies (approximately 10%). Pork can thus be considered a potential vehicle of MRSA exposure for the general human population. Ingestion of LA-MRSA is less likely to occur because all pork types are heated before consumption (except for minced meat, which is sometimes consumed raw), and MRSA is killed by heating. Nevertheless, after manipulating meat, good hand hygiene and cleaning of kitchen tools should be practiced to prevent transmission of pathogens to other food products (which might be consumed raw) and throughout the household *(16)*. Because only a few MRSA ST398 strains have been found to harbor enterotoxin genes, foodborne illness associated with these strains is unlikely *(23)*.

In many studies, the MRSA prevalence in pork was determined after enrichment of the samples, which resulted in a MRSA prevalence of 0% (Switzerland) to 11% (the Netherlands) in European pork and lower than 10% in U.S.-Canadian pork *(5, 11, 33)*. After enrichment, the overall MRSA prevalence in the present study (72%) was

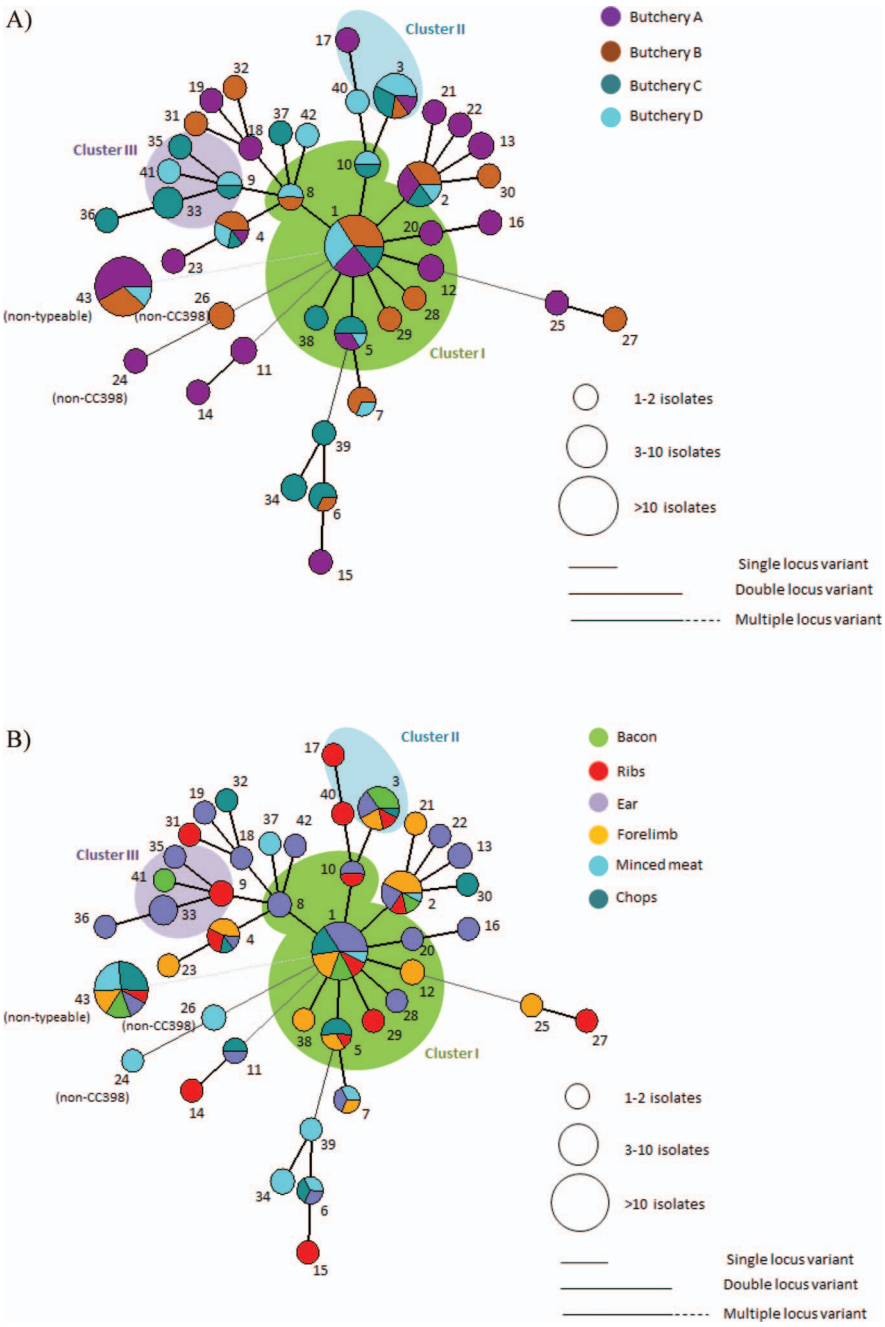

FIGURE 1. *Minimum spanning tree of the MLVA types according to butchery(A) and according to meat type (B). The MLVA types are indicated by numbers. The three MLVA clusters are indicated by colored spheres.*

considerably higher. For each pork type separately, the MRSA prevalence after enrichment was also higher than that in other European and U.S.-Canadian studies. For example, a MRSA prevalence of 46% was observed for chops compared with 5% (United States), 6% (The Netherlands), 6% (United States), and 14% (Canada), respectively, in other studies *(2, 12, 17, 33)*. MRSA was isolated from 61% of the minced pork samples, whereas in Canada and the United States it was found in 6.3 and 9%, respectively, of the minced meat samples *(17, 33)*. MRSA was detected on 83% of the rib samples compared with 8 to 9% in U.S. samples *(12, 17)*. One of the reasons for the differences in MRSA prevalence between the present study and others could be the MRSA isolation protocols, such as the choice of enrichment medium and chromogenic medium. Various enrichment protocols have been used in other studies. In the present study, a one-step enrichment culture in 6.5% salt-enriched MH broth was used, whereas Beneke et al. *(2)* used a two-step enrichment in salt-enriched broth followed by an antibiotic-enriched broth. Not all enrichment broths and supplements are equally efficient for MRSA isolation, which could lead to different estimates of MRSA (CC398) levels on pork. Pang et al. *(19)* found that the use of MH broth yielded higher levels of MRSA than did other broths (e.g., BHI and tryptic soy). Enrichment supplements such as aztreonam (100 mg/liter), polymyxin B (10 mg/liter), salt (4%), and NaN_3 (100 mg/liter) can have a positive effect on MRSA growth. Various chromogenic media also have been used for MRSA detection. Not all chromogenic media perform well for the detection of MRSA (CC398) from meat samples, as has been seen for pig nasal samples *(9, 20)*. The differences in MRSA isolation protocols among the various

TABLE 2. *Overview of the molecular typing results of the 35 selected MRSA CC398 isolates*

Butchery	Meat type[a]	Sample event	MLVA numeric code	MLVA type (cluster)[b]	SCC*mec* type[b]	*spa* type[b]	Pulsotype[b]	Antimicrobial agent resistance profile[c]
A	Mm, R, Fo	1	33-56-36-7-3	1 (I)	IVa	t011	V	Gen, Kan, Tet, Tob, Tri
	R	6	32-56-36-7-3	**1 (I)**	**V**	**t011**	**VIII**	Cip, Tet, Tri
	R	2	32-57-37-7-3	*3 (II)*	*V*	*t011*	*VIII*	Cip, Ery, Lin, Tet, Tri, Tyl
	R	3	33-57-37-7-3	3 (II)	V	t011	IX	Tet, Tri
	R	4	32-56-37-37-4	15	V	t034	V	Clr, Cip, Lin, Syn, Tet, Tri
	Fo	1	33-54-38-38-5	25	V	t2370	VIII	Ery, Lin, Tet, Tri, Tyl
	Mm	3	Nontypeable	43	V	t011	II	
B	C	1	33-56-36-7-3	**1 (I)**	**V**	**t011**	**VIII**	Tet, Tri
	C	3	33-56-36-7-3	**1 (I)**	**V**	**t011**	**VIII**	Cip, Lin, Tet, Tri
	C	5	33-56-36-7-3	**1 (I)**	**V**	**t011**	**VIII**	Cip, Tet, Tri
	C	2	34-53-37-7-3	32	IVa	t011	III	Gen, Kan, Tet, Tob, Tri
	C	6	32-53-36-7-3	30	V	t011	VIII	Lin, Tet
	R	3	34-57-36-7-3	4	V	t011	V	Ery, Gen, Kan, Lin, Tet, Tob, Tri, Tyl
C	C	3	33-56-37-7-3	1 (I)	V	t011	XI	Cip, Lin, Tet, Tri
	Fo	1	32-56-36-7-3	**2 (I)**	**V**	**t011**	**VIII**	Tet, Tri
	Fo	3	33-56-37-7-3	**1 (I)**	**V**	**t011**	**VIII**	Cip, Lin, Tet, Tri
	Fo	4	32-56-36-7-3	**2 (I)**	**V**	**t011**	**VIII**	Cip, Tet
	Fo	5	39-56-36-7-3	38 (I)	V	t3423	VIII	Lin, Syn, Tet
	E	3	33-56-36-7-3	**1 (I)**	**V**	**t011**	**VIII**	Cip, Lin, Syn, Tet, Tri
	E	4	33-56-36-7-3	1 (I)	IVa	t011	V	Gen, Kan, Tet, Tob, Tri
	E	5	32-56-36-7-3	**2 (I)**	**V**	**t011**	**VIII**	Cip, Ery, Lin, Tet, Tri, Tyl
	E	1	33-57-37-7-3	*3 (II)*	*V*	*t011*	*VIII*	Ery, Lin, Tet
	E	2	33-57-37-7-3	*3 (II)*	*V*	*t011*	*VIII*	Ery, Lin, Syn, Tet, Tri, Tyl
	Fo	2	33-57-37-7-3	*3 (II)*	*V*	*t011*	*VIII*	Lin, Tet, Tri
	E	6	33-57-36-7-3	**10 (I)**	**V**	**t011**	**VIII**	Cip, Tet, Tri
	E	3	34-61-34-7-3	33 (III)	V	t011	VII	Cip, Tet, Tri
	Fo	6	34-57-36-7-3	4	IVa	t011	VIII	Gen, Kan, Tet, Tob, Tri
D	R	4	33-56-36-7-3	1 (I)	V	t011	VI	Ery, Lin, Tet, Tob, Tri, Tyl
	Ba	6	33-56-36-7-3	**1 (I)**	**V**	**t011**	**VIII**	Tet, Tri
	Ba	2	33-57-37-7-3	3 (II)	V	t011	X	Tet, Tri
	Ba	5	33-57-37-7-3	*3 (II)*	*V*	*t011*	*VIII*	Cip, Tet, Tri
	Ba	1	33-57-37-7-3	*3 (II)*	*V*	*t011*	*VIII*	Cip, Tet
	Ba	3	35-61-36-7-3	41 (III)	V	t011	VIII	Cip, Ery, Lin, Tet, Tri, Tyl

[a] Mm, minced meat; R, rib; Fo, forelimb; C, chop; E, ear; Ba, bacon.
[b] Overall genotype is indicated in bold; related overall genotype is indicated in italics.
[c] Gen, gentamicin; Kan, kanamycin; Tet, tetracycline; Tob, tobramycin; Tri, trimethoprim; Cip, ciprofloxacin; Ery, erythromycin; Lin, lincomycin; Tyl, tylosin; Clr, chloramphenicol; Syn, quinupristin-dalfopristin.

studies indicate that a single standard protocol for pork may be needed. The high MRSA prevalence found in the present study also could be an accurate reflection of the high prevalence in Belgian pork compared with pork in other countries, but more research on that topic is needed. However, the fact that MRSA was isolated from three samples before enrichment but not after enrichment might indicate that these three samples contained MRSA strains that were unable to grow in salt-enriched (6.5%) broth, as was reported by Pang et al. *(19)*.

One of the aims of this study was to investigate the genetic diversity of the MRSA isolates obtained from these pork products. The majority of the isolates belonged to CC398, similar to most recent European studies on pork *(1, 2, 5)*. Four non-CC398 isolates also were isolated. One of these isolates belonged to *spa* type t011, which has been previously only associated with MRSA CC398. Two of these isolates found in two butcheries belonged to s*pa* type t127, which has been associated with ST1, a human MRSA sequence type (Ridom Bioinformatics, http://spaserver.ridom.de). One of the t127 strains was susceptible to all tested antimicrobial agents and the other was resistant to seven of these agents.

After molecular typing, two overall genotypes appeared dominant on all meat type samples collected in the four butcheries, which could be an indication of a common source for these isolates. The four butchers may have received pork from the same slaughterhouse or same meat distributor, but no source information was available. Another possibility, already suggested by Verhegghe et al. *(29),* is that a relatively small number of MRSA ST398 strains circulate within the Belgian pig population, resulting in a constant flow-through to slaughterhouses, workers, transport trucks, and butcheries, which implies that this production chain and more specifically a butchery could be considered a possible MRSA source for the human population. Finding the same and/or very closely related genotypes (same pulsotype, SCC*mec* type, and *spa* type but

TABLE 3. *Overview of the different antimicrobial agent susceptibility profiles and associated CC398 and non-CC398 MRSA isolates*

Susceptibility profile[a]	No. of isolates		
	CC398	Non-CC398	Total
None	20	2	22
Cip, Tet, Tri	19	0	19
Gen, Kan, Tet, Tob, Tri	19	0	19
Tet, Tri	13	0	13
Cip, Ery, Lin, Tet, Tri, Tyl	8	0	8
Cip, Lin, Tet, Tri	7	0	7
Tet	7	0	7
Ery, Gen, Kan, Lin, Tet, Tob, Tri, Tyl	6	0	6
Cip, Tet	4	0	4
Cip, Lin, Syn, Tet, Tri	4	0	4
Ery, Lin, Tet, Tri, Tyl	4	0	4
Lin, Tet, Tri	4	0	4
Mup	4	0	4
Ery, Lin, Tet, Tyl	3	0	3
Tet, Tob, Tri	3	0	3
Clr, Cip, Lin, Syn, Tet, Tri	2	0	2
Ery, Lin, Syn, Tet, Tri, Tyl	2	0	2
Ery, Lin, Tet, Tob, Tri, Tyl	2	0	2
Lin, Tet	2	0	2
Lin, Syn, Tet	2	0	2
Cip, Gen, Tet, Tri	1	0	1
Cip, Ery, Lin, Tet, Tri	1	0	1
Clr, Cip, Lin, Tet, Tri	1	0	1
Clr, Cip, Lin, Syn, Tet	1	0	1
Clr, Cip, Lin, Syn, Tet, Tyl	1	0	1
Clr, Cip, Gen, Kan, Lin, Syn, Tet, Tob	1	0	1
Ery, Lin, Tet	1	0	1
Ery, Fuc, Lin, Tob, Tri	0	1	1
Ery, Kan, Lin, Tet, Tob, Tri, Tyl	0	1	1
Gen, Kan, Tet, Tob, Tri, Tyl	1	0	1

[a] Cip, ciprofloxacin; Tet, tetracycline; Tri, trimethoprim; Gen, gentamicin; Kan, kanamycin; Tob, tobramycin; Ery, erythromycin; Lin, lincomycin; Tyl, tylosin; Syn, quinupristin-dalfopristin; Mup, mupirocin; Clr, chloramphenicol; Fuc, fucidin.

closely related MLVA type) throughout the pork production chain supports this hypothesis. The dominant genotypes entered the chain through colonized pigs. Certain MRSA strains are able to spread throughout the slaughterhouse, resulting in contaminated carcasses *(8, 26)*. Cross-contamination of carcasses also might occur during transport. Isolation of human MRSA indicates that within the pork production chain carcass and meat handling could spread MRSA. A genetically diverse population of MRSA isolates was found within one butchery, and isolates of the same (overall) genotype were variable in their antimicrobial resistance. This diversity could be due to import from carcasses (pig, poultry, and cattle), meat (pork, poultry, and beef), and persons handling these products. One hypothesis is that in the pork food chain, from slaughterhouse to retail, MRSA has become resident, leading to frequent cross-contamination of pork products. The meat sold to the consumer can become contaminated with several MRSA types derived from those present in the rearing pigs and/or those present in the production environment. Hence, the pork production chain can be considered a source of multiple MRSA types that could, by cross-contamination of the meat, be transmitted to the human population.

The high MRSA prevalence in this food chain indicates the need for control measures. Because of the high reported prevalence in pigs and the fact that disinfection strategies have only a temporal effect on lowering the MRSA prevalence of sows and piglets, control measures at the farm level will be difficult to implement *(21, 29)*. Control measures at the slaughterhouse and butchery are needed, such as thorough disinfection of slaughterhouses and butcheries, good hand hygiene of slaughterhouse workers and butchers, and adequate meat storage and handling practices to inhibit bacterial growth.

The CC398 isolates of pig origin are all resistant to tetracyclines, and nearly all are resistant to trimethoprim. However, fewer pork isolates are resistant to these antimicrobial agents *(27),* and these isolate are more variable than the pig isolates in their antimicrobial resistance *(29)*. Twenty of the MRSA CC398 isolates from two butcheries and different meat types (apparently one genetic strain: nontypeable with MLVA, SCC*mec* type V, and pulsotype II) were sensitive to all antimicrobial agents tested except the β-lactam antibiotics. One explanation could be that these isolates lost their antimicrobial resistance when moving through the food chain, as has been noted for community-acquired MRSA strains, or they might have a nonpig origin *(3)*. Although these 20 isolates still carried the tetracycline resistance genes *tetM* and *tetK,* these genes may be suppressed or expressed at a lower level. The observed sensitivity might be associated with the fact that only a single concentration of each antimicrobial agent was tested.

In conclusion, the MRSA prevalence on Belgian pork samples was 8% after direct plating, with the highest isolation rates from chops, ears, and forelimbs. Much higher prevalence (72%) was found after enrichment. The pork isolates appeared less resistant to tetracycline and trimethoprim than the pig isolates. This report is the first description of MRSA CC398 isolates susceptible to all tested antimicrobial agents except β-lactams. These isolates were susceptible to tetracycline, although the tetracycline resistance genes were present. A genetically diverse population of isolates was obtained from the butcheries, but these isolates had only a few apparent overall genotypes. Comparisons with isolates from the pork production chain support the hypothesis that some genotypes are maintained and spread throughout the chain, resulting in contaminated pork at the butchery level, which can allow MRSA transmission to the general human population.

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REFERENCES

1. Agersø, Y., H. Hasman, L. M. Cavaco, K. Pedersen, and F. M. Aarestrup. 2012. Study of methicillin resistant *Staphylococcus aureus*

(MRSA) in Danish pigs at slaughter and in imported retail meat reveals a novel MRSA type in slaughter pigs. *Vet. Microbiol*. 157:246–250.
2. Beneke, B., S. Klees, B. Stuhrenberg, A. Fetsch, B. Kraushaar, and B. A. Tenhagen. 2011. Prevalence of methicillin-resistant *Staphylococcus aureus* in a fresh meat pork production chain. *J. Food Prot*. 74:126–129.
3. Chambers, H. F. 2001. The changing epidemiology of *Staphylococcus aureus*? *Emerg. Infect. Dis*. 7:178–182.
4. Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Clinical and Laboratory Standards Institute, Wayne, PA.
5. de Boer, E., J. T. M. Zwartkruis-Nahuis, B. Wit, X. W. Huijsdens, A. J. de Neeling, T. Bosch, R. A. A. van Oosteroom, A. Vila, and A. E. Heuvelink. 2009. Prevalence of methicillin-resistant *Staphylococcus aureus* in meat. *Int. J. Food Microbiol*. 134:52–56.
6. de Jonge, R., J. E. Verdier, and A. H. Havelaar. 2010. Prevalence of meticillin-resistant *Staphylococcus aureus* amongst professional meat handlers in the Netherlands, March–July 2008. *Euro Surveill*. 15(46):pii=19712.
7. Doyle, E. M. 2015. Multidrug-resistant pathogens in the food supply. *Foodborne Pathog. Dis*. 12:261–279. doi:10.1089/fpd.2014.1865.
8. Gilbert, M. J., M. E. H. Bos, B. Duim, B. A. P. Urlings, L. Heres, J. A. Wagenaar, and D. J. J. Heederik. 2012. Livestock-associated MRSA ST398 carriage in pig slaughterhouse workers related to quantitative environmental exposure. *Occup. Environ. Med*. 69:472–478.
9. Graveland, H., E. van Duijkeren, A. van Nes, A. Schoormans, M. Broekhuizen-Stins, I. Oosting-van Schothorst, D. Heederik, and J. A. Wagenaar. 2009. Evaluation of isolation procedures and chromogenic agar media for the detection of MRSA in nasal swabs from pigs and veal calves. *Vet. Microbiol*. 139:121–125.
10. Hanson, B. M., A. E. Dressler, A. L. Harper, R. P. Scheibel, S. E. Wardyn, L. K. Roberts, J. S. Kroeger, and T. C. Smith. 2011. Prevalence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) on retail meat in Iowa. *J. Infect. Public Health* 4:169–174.
11. Huber, H., S. Koller, N. Giezendanner, R. Stephan, and C. Zweifel. 2010. Prevalence and characteristics of meticillin-resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin, Switzerland, 2009. *Euro Surveill*. 15(16):pii=19542.
12. Jackson, C. R., J. A. Davis, and J. B. Barrett. 2013. Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* isolates from retail meat and humans in Georgia. *J. Clin. Microbiol*. 51:1199–1207.
13. Maes, N., J. Magdalena, S. Rottiers, Y. De Gheldre, and M. J. Struelens. 2002. Evaluation of a triplex PCR assay to discriminate *Staphylococcus aureus* from coagulase-negative staphylococci and determine methicillin resistance from blood cultures. *J. Clin. Microbiol*. 40:1514–1517.
14. Milheiriço, C., D. C. Oliveira, and H. de Lencastre. 2007. Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: "SCC*mec* IV multiplex." *J. Antimicrob. Chemother*. 60:42–48.
15. Ng, L.-K., I. Martin, M. Alfa, and M. Mulvey. 2001. Multiplex PCR for the detection of tetracycline resistant genes. *Mol. Cell. Probes* 15:209–215.
16. Nunan, C., and R. Young. 2007. MRSA in farm animals and meat: a new threat to human health. Soil Association, Bristol, UK.
17. O'Brien, A. M., B. M. Hanson, S. A. Farina, J. Y. Wu, J. E. Simmering, S. E. Wardyn, B. M. Forshey, M. E. Kulick, D. B. Wallinga, and T. C. Smith. 2012. MRSA in conventional and alternative retail pork products. *PloS One* 7:e30092.
18. Oliveira, D. C., and H. de Lencastre. 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. 46:2155–2161.
19. Pang, L., Y. Luo, Y. Gu, X. Xu, J. Xu, F. Zhang, and S. Cui. 2015. Recovery method development of sodium chloride–susceptible methicillin-resistant *Staphylococcus aureus* isolates from ground pork samples. *Microb. Drug Resist*. 21:1–6.
20. Pletinckx, L. J., Y. De Bleecker, J. Dewulf, G. Rasschaert, B. Goddeeris, and I. De Man. 2012. Evaluation of salt concentrations, chromogenic media and anatomical sampling sites for detection of methicillin-resistant *Staphylococcus aureus* in pigs. *Vet. Microbiol*. 154:363–368.
21. Pletinckx, L. J., J. Dewulf, Y. De Bleecker, G. Rasschaert, B. Goddeeris, and I. De Man. 2013. Effect of a disinfection strategy on the methicillin-resistant *Staphylococcus aureus* CC398 prevalence of sows, their piglets and the barn environment. *J. Appl. Microbiol*. 114:1634–1641.
22. Rasschaert, G., W. Vanderhaeghen, I. Dewaele, N. Janez, X. Huijsdens, P. Butaye, and M. Heyndrickx. 2009. Comparison of fingerprinting methods for typing methicillin-resistant *Staphylococcus aureus* sequence type 398. *J. Clin. Microbiol*. 47:3313–3322.
23. Schijffelen, M. J., C. H. E. Boel, J. A. G. van Strijp, and A. C. Fluit. 2010. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. *BMC Genet*. 11:1–10.
24. Stegger, M., J. A. Lindsay, A. Moodley, R. Skov, E. M. Broens, and L. Guardabassi. 2011. Rapid PCR detection of *Staphylococcus aureus* clonal complex 398 by targeting the restriction-modification system carrying *sau1-hsdS1*. *J. Clin. Microbiol*. 49:732–734.
25. Strandén, A., R. Frei, and A. F. Widmer. 2003. Molecular typing of methicillin-resistant *Staphylococcus aureus*: can PCR replace pulsed-field gel electrophoresis? *J. Clin. Microbiol*. 41:3181–3186.
26. Van Cleef, B. A. G. L., E. M. Broens, A. Voss, X. W. Huijsdens, L. Zuchner, B. H. B. van Benthem, J. A. J. W. Kluytmans, M. N. Mulders, and A. W. Van de Giessen. 2010. High prevalence of nasal MRSA carriage in slaughterhouse workers in contact with live pigs in The Netherlands. *Epidemiol. Infect*. 138:756–763.
27. Vanderhaeghen, W., K. Hermans, F. Haesebrouck, and P. Butaye. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. *Epidemiol. Infect*. 138:606–625.
28. Van Loo, I. H. M., B. M. W. Diederen, P. H. M. Savelkoul, J. H. C. Woudenberg, R. Roosendaal, A. van Belkum, N. L. D. Toom, C. Verhulst, P. H. J. van Keulen, and J. A. J. W. Kluytmans. 2007. Methicillin-resistant *Staphylococcus aureus* in meat products, the Netherlands. *Emerg. Infect. Dis*. 13:1753–1755.
29. Verhegghe, M., F. Crombé, L. J. Pletinckx, F. Haesebrouck, P. Butaye, L. Herman, M. Heyndrickx, and G. Rasschaert. 2014. Genetic diversity of livestock-associated MRSA isolates obtained from piglets from farrowing until slaughter age on four farrow-to-finish farms. *Vet. Res*. 45:89.
30. Verhegghe, M., L. Herman, F. Haesebrouck, P. Butaye, M. Heyndrickx, and G. Rasschaert. 2015. Preliminary evaluation of good sampling locations on a pig carcass for livestock-associated MRSA isolation. *Int. J. Food Contam*. doi:10.1186/s40550-015-0013-3.
31. Voss, A., F. Loeffen, J. Bakker, C. Klaassen, and M. Wulf. 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerg. Infect. Dis*. 11:1965–1966.
32. Weese, J. S. 2010. Methicillin-resistant *Staphylococcus aureus* in animals. *ILAR J*. 51:233–244.
33. Weese, J. S., B. P. Avery, and R. J. Reid-Smith. 2010. Detection and quantification of methicillin-resistant *Staphylococcus aureus* (MRSA) clones in retail meat products. *Lett. Appl. Microbiol*. 51:338–342.
34. Zhang, K. Y., J. A. McClure, S. Elsayed, T. Louie, and J. M. Conly. 2005. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol*. 43:5026–5033.