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## Heterogeneity among methicillin-resistant *Staphylococcus aureus* from Italian pig finishing holdings

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### Abstract:

A survey for methicillin-resistant *Staphylococcus aureus* (MRSA) in finishing pig holdings was carried out in Italy in 2008. MRSA isolates were characterised by spa-, MLST-, SCCmec- and antimicrobial susceptibility typing. A prevalence of 38% (45/118, 95% CI 29.4%-46.9%) positive holdings was observed. Eleven different spa-types were found among 102 MRSA isolates, clustering in lineages associated with farm animals (ST398, ST9, ST(CC)97 in 36 holdings) and humans (ST1, 7 holdings). Nine (7.6 %) holdings were positive for two, three or four different and unrelated spa-types in various combinations. ST398 was the most prevalent lineage (33 positive holdings). The most prevalent spa-type was t899 (ST398), detected in 22 positive holdings. Three novel spa-types (t4794 of ST9; t4795 of ST97; t4838 of ST398) were detected. Ten holdings were positive for spa-type t1730, that proved to be a new single-locus variant of ST97, within the CC97. The most prevalent SCCmec was Type V (79 isolates), while Type IVb was found in 10 isolates. None of the isolates was positive for Panton-Valentine Leukocidin, while most of t127 and t1730

isolates, one t4794, one t4795, and one t2922 were positive for *LukE-LukD* genes. All 64 antimicrobial susceptibility tested isolates were resistant to tetracyclines, with high resistance rates to trimethoprim (68.8%), erythromycin (60.9%), and ciprofloxacin (35.4%). All t127, ST1 isolates were resistant to tetracycline-ciprofloxacin-erythromycin. This survey provides the first report of MRSA ST1 and ST(CC)97 among pigs and the first report of MRSA ST9 from pigs in Europe. The presence of human-associated CA-MRSA (t127, ST1, SCCmec type V) in 6% holdings surveyed can represent an additional MRSA reservoir for infections in humans.

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of hospital-associated infections (Johnson et al., 2005) and has during the last decade emerged as a significant pathogen in the community (Vandenesch et al., 2003; Kluytmans-Vandenbergh & Kluytmans, 2007; ).

Community-acquired MRSA(CA-MRSA) clones have been increasingly detected in companion animals as dogs and horses (van Duijkeren et al., 2004; Weese et al., 2005) and also observed in livestock animals (Juhász-Kaszanyitzky et al., 2007, Khanna et al. 2008). Recently, isolates belonging to MRSA Clonal Complex (CC) 398 have emerged in pig farming, later spread to other farm animal species and it is now prevalent in farm animals in some countries of Central and Northern Europe (de Neeling et al., 2007; van Duijkeren et al., 2008) and North America (Khanna et al., 2008 ). MRSA CC398 of animal origin has also emerged as a cause of colonization and infection in humans and it is responsible for >20% of all MRSA in the Netherlands (van Loo et al., 2007). Recently, MRSA isolates of Sequence Type(ST) 9 were reported in pigs from China (Wagenaar et al., 2009).

At present, there is no available information in Europe on the possibility that pigs can represent a competent host and a reservoir for STs other than CC398 MRSA and other farm-associated clones. Lineages of human origin (spa-type t002, CC5, USA 100) have been isolated from pigs in 3/9 holdings investigated in Ontario, Canada, in a small-scale population study (Khanna et al., 2008).

The aim of our study was to estimate the herd prevalence of MRSA colonization in finishing pig holdings in Italy, to provide phenotypic and molecular characterisation of isolates for epidemiological purposes, and to gather information about their relationships and their possible origin.

## **Materials and Methods**

### **a. Survey at slaughter and data analysis**

Herds were selected by a random sampling procedure from the national registry of holdings rearing pigs for slaughter (farrow to finish, weaner to finisher, finisher holdings), at the base of the pig production pyramid. For among-herd prevalence estimates, a simple random sampling technique was used, with a minimum desired sample size (primary sample size) calculated based on a 20% expected prevalence, a 95% confidence level and 7.5% desired absolute precision (Thrusfield, 1995). Through this procedure, 118 holdings from 29 provinces and 10 different regions (Northern Central and Southern Italy, accounting for >90% of the Italian pig population) were enrolled for the survey. The study was conducted from January to June 2008 in several major slaughterhouses in Italy, located in Northern and Central Italy that received finishers from the selected holdings. From each holding, 60 individual nasal swabs were sampled (secondary sample size), in order to allow the detection of at least one positive animal with a within-herd expected prevalence of 5% and a 95% confidence level. The swabs were put in Amies with charcoal transport medium, stored at +4°C and sent to laboratory for cultures.

Prevalence estimates and multivariate analysis of data were performed with EpiInfo version 3.5.1 (CDC, Atlanta, United States). Outcomes examined included: the positive status of the holding and the positive status from a human-associated spa-type/sequence type. Information on independent variables included: province and region of holdings sampled, production technique (e. g. farrow to finish, weaner to finisher, finisher herds), holding size, slaughterhouse of sampling. For purposes of the analysis, a variety of summary variables were created: size category of holdings (small: 500-

3000, medium: 3001-9000, large: 9001+), macro-regions (north-east, north-west, central-south), putative origin of spa-type/sequence type detected in the holding (human, animal, human and animal). A multivariate analysis (logistic regression) was performed with initial model containing all the selected variables followed by a backward deletion procedure, and prevalence Odds Ratios (OR) with 95% Confidence Intervals (CI) and p-values were calculated.

#### b. Laboratory procedures

Pooled cultures of 10 nasal swabs (6 pools from each holding) were carried out in Müller-Hinton broth containing 6.5% NaCl and incubated at 37°C for 16-20 hours (pre-enrichment); One millilitre of the pre-enrichment culture was added to 9 ml of Phenol Red Mannitol Broth + 5 mg/L oxacillin and 75 mg/L aztreonam and incubated at 37°C for 16-20 hours (selective enrichment). The selective enrichment culture was then plated on Oxacillin Resistance Screen Agar (ORSA, Oxoid) and 5% sheep blood agar and incubated at 37°C for 48 hours. Suspected colonies were identified as *S. aureus* by means of standard techniques: colony morphology (blue colonies on ORSA), Gram staining, catalase, coagulase tube test and Staphytest Plus test kit (Oxoid). The identification at species level was further confirmed by Polymerase Chain Reaction assay (PCR) (Baron et al., 2004). The detection of the *mecA* gene by PCR was performed as described by Strommenger and colleagues (2003). MRSA isolates were genotyped by DNA sequencing of the X-region of the *Staphylococcus* Protein A (spa-typing, Harmsen et al., 2003), with repeats and *spa* types determined by Ridom StaphType software (Ridom GmbH, Würzburg, Germany) and Multi-Locus Sequence Typing (MLST) (Enright et al., 2000). Typing of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) was performed by PCR as previously described (Kondo et al., 2007). Isolates carrying *ccr* and *mec* gene combinations classified as Type IV SCC*mec* were subtyped according to Zhang and colleagues (2005).

MRSA isolates were screened for genetic determinants of the following toxins: Gamma-hemolysin components A, B, and C (*hlg*) and Gamma-hemolysin variant (*hlg-2*), Panton Valentine (PV)

leukocidin components S and F (*lukS-PV-lukF-PV*) according to McClure and colleagues (2006), LukM-LukF'-PV (*lukM*), and LukE-LukD (*lukE-lukD*) leukocidins, according to Jarraud and colleagues (2002), exfoliative toxins ETA (*eta*), ETB (*etb*) and ETD (*etd*), according to Yamaguchi and colleagues (2002), toxin shock syndrome toxin (TSST) according to Fueyo and colleagues (2005).

Susceptibility testing was performed on a representative collection of isolates, including at least one isolate for each spa-type (or when appropriate, for each spa-type and SCC*mec* combination) detected in positive holdings. Broth microdilution testing was performed in 96-well microtiter plates (Trek Diagnostic Systems, Westlake, OH) and interpreted according to the European Committee on Antibiotic Susceptibility Testing (EUCAST) epidemiological cut-offs. The following drugs were tested: erythromycin, ciprofloxacin, chloramphenicol, clindamycin, gentamicin, kanamycin, linezolid, quinopristin-dalfopristin, streptomycin, sulphonamides, tetracycline, trimethoprim, vancomycin.

## Results

A total of 45 out of 118 (38.1%, 95% CI 29.4%-46.9%) holdings had at least one MRSA-positive pool. A total of 98 (14%) positive pools were detected out of the 701 cultured. Nearly 50% (22/45) of positive holdings had only one positive pool. Two, three and four positive pools were found in 18%, 13% 11% of positive holdings, respectively, while five and six positive pools were found in 4% of positive holdings. Eleven different spa-types were detected, three of which have never been described before (t4794, t4795, t4838), and belonging to five different MLST profiles, ST398, ST1, ST9, ST97, ST1476 (Table 1). An estimated 7.6% (9/118; 95% CI 2.8-12.4%) of the holdings had their animals positive for 2, 3 or 4 unrelated spa-types belonging to ST1, ST97, ST1476 or ST398 in different combinations. Four holdings (3.4%, 95% CI 0.1-6.7%) were positive for both human- and animal-associated MRSA: t127 and t011, t034, t889, t2922, t1730, t4795 (Table 2). An estimated 28% (33/118; 95% CI 19.9-36.1%) of holdings were found positive for pig-associated

ST398 lineage and an estimated 5.9% (7/118; 95% CI 1.7-10.2%) were positive for human-associated MRSA (t127, ST1). Fourteen holdings were positive for other lineages, one for ST9 (t4794), three for t4795, ST97 and ten for t1730. The MLST performed on t1730 isolates showed that they are a single-locus variant (in the *gmk* gene) of ST97, now classified as ST1476, and thus they belong to CC97. The most prevalent spa-type was t899, ST398, detected in 22/45 (49%) positive holdings, with an overall holding prevalence of 18.6% (95% CI 11.6-25.7%). Among the 102 isolates from the 45 positive holdings, the most frequent SCC*mec* *ccr* and *mec* combination (Kondo et al. 2007) was 5C (i. e. Type V), detected in 79 isolates. Ten isolates carried the 2B combination (i. e. Type IV) and were also positive in the IVb subtype molecular assay. Thirteen isolates showed a positive result in the PCR assay for both *ccrA2-ccrB* and *ccrC* genes in a novel 5+2B combination (i. e. genetic elements described in the Type IV and Type V SCC*mec* cassettes). Among the novel spa-types, both t4794, ST9 and t4795, ST97 isolates carried the SCC*mec* 5C combination (Type V), and the t4838 the 5+2B combination.

None of the 102 isolates carried the Panton-Valentine Leukocidin (PVL) genes. Most of the t127 (10/12) and t1730 (11/13) along with one t4794, one t4795, and one t2922 were positive for *LukE-LukD* genes.

Antimicrobial susceptibility testing performed on 64 isolates (Table 3) showed that 100% were tetracycline-resistant. A high proportion of isolates were also resistant to trimethoprim (44/64, 68.8%) and to erythromycin (39/64, 60.9%). The overall resistance rate to ciprofloxacin was 35.9% (23/64). No resistance to linezolid, quinopristin-dalfopristin and vancomycin was observed.

All the t127, ST1 and most (9/10) t1730, CC97 isolates tested showed a common pattern of tetracycline, macrolide and fluoroquinolone resistance, while among the three t4795, ST97 isolates, two were resistant to ciprofloxacin and none to erythromycin. Conversely, among ST398 isolates, only 43% (12/28) of t899 and related spa-types were resistant to erythromycin (all t899 isolates), with only one isolate resistant to ciprofloxacin (t2922). Isolates of the related spa-types t011, t034

and t108 showed a high rates of resistance to erythromycin (12/16, 75%), but lower resistance rates to ciprofloxacin (4/16, 25%).

When analysing the data in a multivariate model for possible predictor variables, the only significant risk factor observed was holding size (“large” vs “small”) for the outcome variable “positive status of the holding” with a prevalence Odds Ratio (OR) 3.67 (95% CI 1.04-112.91). The best fitting model for the predictor variable holding size “large vs “small” and the outcome variable was that adjusted for “macro-region”, “production technique”, and “slaughterhouse of sampling” (likelihood ratio test 11.7; 2 d. f.; p-value 0.0028), as a significant difference with the reduced model without the variable “slaughterhouse of sampling” was found:  $-2 \ln(\text{likelihood M1}/\text{likelihood M2})$  chi square p-value < 0.05; 2 d. f.; where M1 is the reduced model and M2 is the extended model. No significant association between independent variables and the outcome “positive status of the holding from a human-associated spa-type/sequence type” were found.

## Discussion

This is the first description of MRSA among pig population in Italy, the first description of three novel spa-types (t4794, ST9; t4795, ST97; t4838, ST398), the first report of MRSA of ST1 ST1476 and ST97 lineages among pigs, and the first report of MRSA ST9 in Europe.

Pigs reared in Italy may represent a relevant reservoir for both farm- associated and human-associated MRSA clones, as animals of nearly 40% of Italian finishing holdings were found positive. The majority of holdings were positive for ST398 lineage, with two main spa-types groups detected (t899 and related t2922, t4838 spa-types, along with t011 and related t034, and t108 spa-types). Spa type t899 proved to be the most prevalent spa-type in Italian pig holdings, although it had been considered a rare type in pigs until recent description in the Netherlands (van Dujikeren et al., 2008). The presence of unrelated spa-types within the same holding (up to four spa-types), either belonging to ST398, CC97 or to ST1, suggests subsequent introduction of new strains over time as the most common pattern of colonization and diffusion among animals and holdings.

More than forty percent (19/45) of positive holdings had their animals colonised either by human-associated MRSA of the ST1 lineage, or by CC97 clones, and demonstrating that pigs may acquire and spread MRSA belonging to CCs other than CC398. ST1 belongs to CC1, a particularly successful lineage which includes MW2 CA-MRSA associated with deaths in children in the United States (Centers for Disease Control and Prevention, 1999) , and lukS-PV/luk-F-PV-positive CA-MRSA also known as USA400 (McDougal et al., 2003). Additionally, PVL-negative ST1 t127, SCC*mec* IVa, was among the most common CA-MRSA reported by researchers in the United Kingdom, closely associated with injecting drug-users and homeless people (Otter et al., 2009). One PVL-positive t127 CA-MRSA was recently reported in a Dutch patient in a study conducted in the Euregio Meuse-Rhine Region (Deurenberg et al., 2009). A cluster of t127, ST1 infections in humans and horses in a veterinary teaching hospital in Germany (Cuny et al., 2008) and a mastitis outbreak in cattle (Juhász-Kaszanyitzky et al., 2007) were also reported recently, suggesting the ability of this lineage to adaptation to new host species. As CC97 is concerned, one t1730 MRSA isolate had already been detected from an Italian participant at a conference of the International Veterinary Pig Society (Wulf et al., 2008), but at that time this finding was not included among the spa-types associated with risk factors related to pig-farming and thus not analyzed by MLST. Clonal Complex 97 is known at present to be one of the *S. aureus* lineages associated with cattle, and especially with bovine mastitis (Sung et al., 2008; Hasman et al., *in press*). The new spa-type t4795 differs from t1730 in a single deletion event (repeat 34) and shown to belong to ST97. Spa-type 4838 is closely related to t899 and differs only by a single point mutation in the first repeat (now repeat 283 instead of 07), while spa-type t4794, ST9 has a Ridom succession sequence of only two repeats (35-34), suggesting multiple deletion events in the X-region. The MSSA phenotype of lineage ST9 is known to be present in pigs in Europe (Kehrenberg et al., 2009; Hasman et al., *in press*) and MRSA isolates of this ST type, spa-type t899, were found recently in pigs in China (Wagenaar et al., 2009). In this respect, as all the t899 isolates from this study were classified as ST398 by MLST, the same Ridom sequence succession (spa-type t899) both in ST9 and ST398

could be explained with the occurrence of a horizontal transfer of the X-region between the two pig-associated lineages. Human- and cattle-associated spa-types and CCs (t127, t1730 and t4795) were also detected recently in Italian holdings of breeding pigs during an European baseline survey (European Commission Decision 2008/55/EC), which demonstrates the diffusion of these clones also at the top of the pyramid of the Italian pig population (Battisti, unpublished data). Additionally, this study shows that in Italy *mecA* mediated beta-lactam resistance in MRSA from pigs is carried in Type IV, V, or a combination (5+2B, Kondo, 2007) of the *ccr* and *mec* elements of the SCC*mec* cassette. None of the isolates, irrespective of their lineage, carried PVL genes or any of the other toxin genes considered for the study except for the Luke-LukD determinants, found in most of the t127, ST1 and t1730, ST1476 isolates and in three other spa-types of lineages known to be associated with animals (ST9, ST97, ST398). The *Luke-LukD* are bi-component leukotoxins located in a putative staphylococcal pathogenicity island (SapIn3/SapIm3) on the chromosome of *S. aureus* (Kuroda et al. 2001) and are found in most of human CA-MRSA as well as in HA-MRSA (Vandenesch et al., 2003) from France and USA. Those genes were also detected in a high proportion of avian and bovine isolates, but in none of the 72 isolates from pig tonsils in a study carried out in Japan (Yamada et al., 2005). All isolates tested for antimicrobial susceptibility proved to be resistant to tetracyclines, a feature consistent with the data reported from isolates of different spa-types and lineages in other European countries (van Duijkeren et al., 2008) where these drugs have as well been widely used for mass medication. Additionally, high resistance rates to trimethoprim, erythromycin and ciprofloxacin are evident from this survey, with a pattern of tetracycline, erythromycin and ciprofloxacin resistance that seems a common feature among isolates of the ST1 and ST(CC)97 lineages. We did not detect any significant differences in the macro-regional distribution of positive holdings, in the production technique, or in spa-types and STs associated with the positive status. This is probably because in Italy MRSA are also widespread in breeding holdings with an overall estimated prevalence that exceeds 30% (Battisti, unpublished data) and they act as a reservoir when colonised breeders are sold to holdings of farrow-to-finish

and of growing-finishing pigs. Larger farms (9000+ animals reared) were more likely to be MRSA-positive than small ones. The reason for this is not known, but might be because larger farms are more likely to have several trading contacts and thereby are more likely to be exposed to contamination. In conclusion, a heterogeneity of spa-types and STs (eleven spa-types clustering in five different STs) is evident in Italian pig finishing holdings. This population study also suggests that pigs may be not only competent hosts and a possible reservoir for MRSA of the cattle-associated CC97 lineages, but also for the human-associated ST1, thus representing a possible source of exposure for the community from a zoonotic pathogen that may need little or no adaptation to humans. Concerns arise also from the presence of different spa-types and STs in the same holdings, as it may favour the exchange and the spread of virulence determinants among different MRSA lineages. The antimicrobial resistance profile observed suggests that beside the use of beta-lactams, the amount and pattern of usage of other antimicrobials should be investigated as a further important predictor in the spread of MRSA within and among Italian pig holdings.

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**Table 1. Spa-types and MLST profiles among holdings surveyed**

<b>spa-type</b>	<b>ST</b>	<b>n holdings where it was detected</b>	<b>% among positive holdings</b>	<b>% holdings surveyed</b>
t011	398	4	8.9	3.4
t034	398	6	13.3	5.1
t108	398	4	8.9	3.4
t2510	398	1	2.2	0.8
t4838	398	2	4.4	1.7
t2922	398	4	8.9	3.4
t899	398	22	48.9	18.6
t4794	9	1	2.2	0.8
t4795	97	3	6.7	2.5
t127	1	7	15.6	5.9
t1730	1476	10	22.2	8.5

**Table 2: Holdings with more than one spa-type or ST detected**

holding code	n spa- types	spa-type		
		spa-types	relatedness	STs
1	2	t011, t108	related	398
2	3	t127, t899, t1730	unrelated	1, 398, 1476
3	3	t011, t899, t1730	unrelated	398, 1476
4	2	t108, t899	unrelated	398
5	2	t034, t899, t2510, t4795	unrelated	398, 97
6	2	t108, t2922	unrelated	398
7	3	t127, t899, t2922	unrelated	1, 398
8	2	t127, t2922	unrelated	1, 398
9	2	t127, t899	unrelated	1, 398
10	2	t1730, t4795	related	1476, 97
11	2	t1730, t034	unrelated	1476, 398

**Table 3. Distribution of non-beta-lactam antimicrobial resistance of 64 selected MRSA isolates of different Spa-types and Sequence Types from pig holdings, Italy 2008**

spa-type	ST	n tested	n (%) of resistant isolates <sup>a</sup>												
			CIP	CHL	CLI	ERY	KAN	GEN	LIN	STR	SUL	SYN	TET	TMP	VAN
t011	398	4	1 (25)	0 (0)	3 (75)	3 (75)	1 (25)	2 (50)	0 (0)	1 (25)	0 (0)	0 (0)	4 (100)	3 (75)	0 (0)
t034	398	7	2 (29)	4 (57)	6 (86)	6 (86)	4 (57)	4 (57)	0 (0)	5 (71)	4 (57)	0 (0)	7 (100)	7 (100)	0 (0)
t108	398	4	1 (25)	1 (25)	4 (100)	3 (75)	1 (25)	1 (25)	0 (0)	1 (25)	1 (25)	0 (0)	4 (100)	4 (100)	0 (0)
t2510	398	1	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
t4838	398	1	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)
t2922	398	4	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (75)	1 (25)	0 (0)	4 (100)	3 (75)	0 (0)
t899	398	23	0 (0)	6 (26)	15 (65)	12 (52)	7 (30)	6 (26)	0 (0)	7 (30)	11 (48)	0 (0)	23 (100)	15 (65)	0 (0)
t4794	9	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)
t4795	97	3	2 (67)	0 (0)	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)
t1730	1476	10	10 (100)	2 (20)	9 (90)	9 (90)	3 (30)	3 (30)	0 (0)	3 (30)	3 (30)	0 (0)	10 (100)	8 (80)	0 (0)
t127	1	6	6 (100)	0 (0)	6 (100)	6 (100)	3 (50)	3 (50)	0 (0)	3 (50)	1 (17)	0 (0)	6 (100)	3 (50)	0 (0)

<sup>a</sup> CIP, ciprofloxacin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; KAN, kanamycin; Gen, gentamicin; LIN, linezolid, STR, streptomycin, SUL, sulphonamides, SYN, quinopristin-dalfopristin; TET, tetracycline; TMP, trimethoprim; VAN, vancomycin