

Antibiotic resistance and genetic diversity in *Staphylococcus aureus* from slaughter pigs in Switzerland

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Summary

Nasal carriage of *Staphylococcus aureus* was evaluated in pigs at slaughterhouse. The nasal cavities of 304 pigs from 54 herds were screened. Eighty-nine percent of the farms harbored pigs that were colonized with *S. aureus*. Among them, no MRSA were found, indicating a low prevalence. However, pigs were found to harbor *S. aureus*, which displayed resistance to penicillin (*blaZ*) (62.5%), tetracycline [*tet*(M)] (33.3%), streptomycin (*str_{PS194}*) (27%), clindamycin [*erm*(B)] (4.1%), erythromycin [*erm*(B)] (4.1%), kanamycin (4.1%), chloramphenicol (*cat_{PC194}*) (2%) and gentamicin [*aac*(6′)-*Ie-aph*(2′)-*Ia*] (2%). The *S. aureus* isolates mainly belong to Ridom *spa* type *t034* (31.3%), *t208* (14.6%) and *t899* (12.5%). These pig-associated *spa* types have not yet been detected in hospitalized human patients in Switzerland. Surveillance programs are now necessary at both inland and import levels to rapidly detect and suppress the emergence of MRSA in pigs in Switzerland.

Schlüsselwörter: *Staphylococcus aureus*, Schwein, Antibiotikaresistenz, *spa* Typisierung

Antibiotika-Resistenz und genetische Diversität bei *Staphylococcus aureus* aus Schlachtschweinen in der Schweiz

Im Schlachthof wurden 304 Schweine aus 54 verschiedenen Betrieben mittels Nasentupfer auf das Vorkommen von *Staphylococcus aureus* untersucht. Schweine, die mit *S. aureus* infiziert waren, wurden in 89% der Betriebe gefunden. Es wurden keine MRSA nachgewiesen, was auf eine tiefe Prävalenz hindeutet. Die *S. aureus* Isolate zeigten Resistenzen gegen Penicillin (*blaZ*) (62.5%), Tetrazyklin [*tet*(M)] (33.3%), Streptomycin (*str_{PS194}*) (27%), Clindamycin [*erm*(B)] (4.1%), Erythromycin [*erm*(B)] (4.1%), Kanamycin (4.1%), Chloramphenicol (*cat_{PC194}*) (2%) und Gentamicin [*aac*(6′)-*Ie-aph*(2′)-*Ia*] (2%). Die *S. aureus* Isolate gehörten hauptsächlich zu den Ridom *spa* Typen *t034* (31.3%), *t208* (14.6%) und *t899* (12.5%). Solche spezifischen Schweine-assoziierten Klone wurden bis heute bei hospitalisierten Menschen in der Schweiz noch nicht nachgewiesen. Bei der inländischen Schweinehaltung aber auch beim Import von Schweinen sollten Überwachungsprogramme eingeführt werden, um einen frühzeitigen Nachweis von MRSA zu ermöglichen und eine rasche Verbreitung in der Schweinpopulation der Schweiz zu verhindern.

Keywords: *Staphylococcus aureus*, pigs, antibiotic resistance, *spa* typing

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is characterized by the *mecA* gene which confers resistance to all β -lactam antibiotics through a penicillin binding protein PBP2a. It is also often resistant to other commonly used antibiotics (Deurenberg et al., 2007), and represents a challenge for antibiotic therapy. It has been spreading worldwide becoming one of the major causes of nosocomial and community-acquired infections in humans (de Lencastre et al., 2007). The concern rose when pigs examined at slaughterhouse were found to act as a reservoir for MRSA in different EU countries (The Netherlands, Denmark, Belgium, Germany) and Canada (de Neeling et al., 2007; Guardabassi et al., 2007; Willems et al., 2007;

Schwarz et al., 2008; Khanna et al., 2008). It turned out that a new MRSA clone ST398 belonging mainly to Ridom *spa* types *t034*, *t011*, *t108* has been selected in animals and is now also spreading to humans (Wulf et al., 2008). People working with pigs also became carriers of this specific pig clone (Voss et al., 2005; Armand-Lefevre et al., 2005; van Loo et al., 2007a). It has also been found in pork meat at retail outlets in the Netherlands (van Loo et al., 2007b), and has already reached hospitals in that country (van Rijen et al., 2008) and in Denmark (Lewis et al., 2008).

Surveillance guidelines were elaborated to determine the situation of MRSA in pig husbandry in different EU countries and limit the spread of multidrug-resistant *S. aureus* in animals and in the community (European Food

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Safety Authority, 2008). Based on these guidelines, the prevalence of nasal carriage of *S. aureus*, including MRSA, in pigs in Switzerland was determined. The strains were tested for antibiotic resistance and the resistance genes were identified. The genetic relatedness between *S. aureus* isolates has been analyzed to determine whether specific pig clones are widespread in Swiss hospitals (Fenner et al., 2008a; Fenner et al., 2008b). This study is also the basis for molecular characterization of *S. aureus* in pigs for further surveillance programs.

Animals, Material and Methods

Nasal swabs were collected from 304 pigs of 54 different farms. The pig selection is a representation for the largest cantons producing pigs in Switzerland (Fig. 1) with 193 pigs (64%) from the canton of Berne (38 farms), 42 (14%) from Lucerne (7 farms), 16 (5%) from Zurich (2 farms), 16 (5%) from St. Gallen (2 farms), 12 (4%) from Aargau (2 farms), 10 (3%) from Nidwalden (1 farm), 10 (3%) from Thurgau (1 farm) and 5 (2%) from Zug (1 farm) tested.

The sampling criteria were based on previous studies from other countries where MRSA were found (Canada: 285 pigs, 20 farms (Khanna et al., 2008); Denmark: 100 pigs, 3 farms (Guardabassi et al., 2007); The Netherlands: 310 pigs, 35 farms (van Duijkeren et al., 2008). Preliminary results generated by screening 10 pigs from 10 herds showed that at least half of the pigs were colonized with *S. aureus* within one herd. Based on these results, only 5 pigs per herd were then screened for the presence of *S. aureus* and MRSA. The nasal cavities of 304 pigs from 54 different herds from different cantons in Switzerland were screened during January and February 2008 at slaughterhouse for the presence of MRSA. Nasal swabs were analysed using methods recommended by EU-guidelines (European Food Safety Authority 2008), but

using an enrichment broth without aztreonam and ceftizoxime in order to enrich both MSSA and MRSA. The swabs were placed directly into tubes containing Mueller Hinton Broth supplemented with 6.5% NaCl and inoculated at 37 °C for 24h under agitation. From there, a loop-full was spread onto MRSA selective agar plates (BBL™ CHROMagar™ MRSA; Becton Dickinson, Franklin Lakes, NJ) and onto tryptone soy agar plates containing 5% sheep blood (TSA-SB) (Oxoid Ltd., Basingstoke, England). The plates were incubated at 37 °C for 24h. All strains displaying an α - or α - β -hemolysis on TSA-SB plates were spread onto chromID™ *S. aureus* agar (bioMérieux, Marcy l'Etoile, France), a selective agar which allows to distinguish *S. aureus* from other staphylococci. On this agar, *S. aureus* colonies appear green (Perry et al., 2003). Green colonies were Gram-stained and tested for catalase activity. *S. aureus* were confirmed by Ridom *spa* typing as described previously (Harmsen et al., 2003). The *spa* types were determined using the software Ridom StaphType (RidomStaphType, Ridom GmbH, Würzburg, Germany). MRSA strain *S. aureus* As5 t034 was used as a positive control (kindly received from P. Butaye).

Minimal inhibitory concentrations (MICs) of 18 antibiotics were determined in Mueller-Hinton broth by use of custom Sensititre susceptibility plates NLV57 (Trek Diagnostics System, East Grinstead, England, and MCS Diagnostics, BV, Swalmen, The Netherlands) according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2006). Antibiotic resistance genes were detected by PCR using *taq* polymerase (FIREPole® BioDyne, Tartu, Estonia) according to manufacturer's instructions and using specific primers and annealing temperatures (Tab. 1). The production of β -lactamase was tested on BBL™ DrySlide™ Nitrocefin (Becton Dickinson, Franklin Lakes, NJ) using colonies grown on Mueller-Hinton agar for 18h at 37 °C with 0.05 μ g penicillin per ml to induce β -lactamase production (Schnellmann et al., 2006).

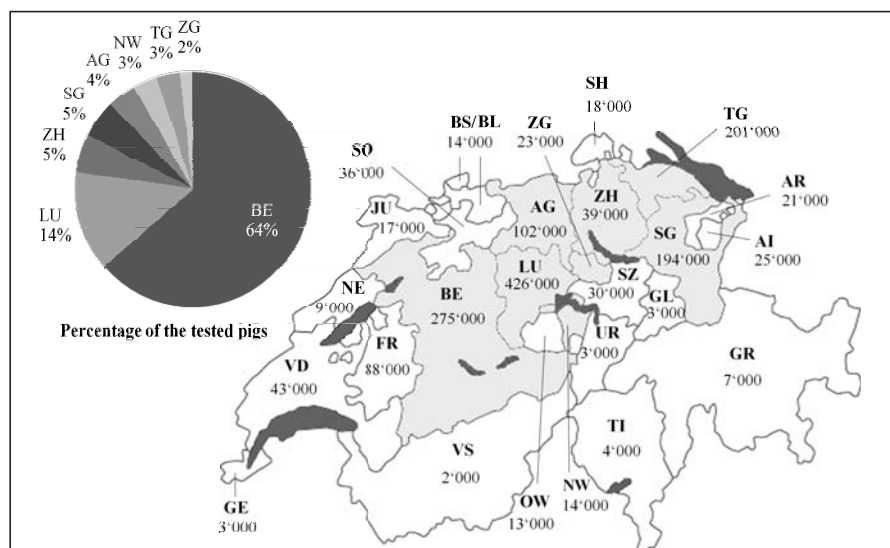


Figure 1: Geographical distribution of pigs in the different cantons of Switzerland, and respective percentage of pigs tested for *S. aureus* nasal carriage. Cantons of origin of the tested pigs appear in grey. AG, Aargau; AI, Appenzell Innerrhoden; AR, Appenzell Ausserrhoden; BL, Basel-Land; BS, Basel-Stadt; BE, Bern; FR, Fribourg; GE, Genève; GL, Glarus; GR, Graubünden; JU, Jura; LU, Luzern; NE, Neuchâtel; SG, St. Gallen; SH, Schaffhausen; SZ, Schwyz; SO, Solothurn; TG, Thurgau; TI, Ticino; UR, Uri; VD, Vaud; VS, Valais; ZG, Zug; ZH, Zürich.

Table 1: Oligonucleotide used for PCR analysis for detection of antibiotic resistance genes and *spa* typing.

Gene	Primer name	Sequence (5'→3')	Annealing-temperature	Primer design, reference or source
<i>aac(6')-Ie-aph(2')-Ia</i>	aac(6')-aph(2')-F	CAGAGCCTTGGGAAGATGAAG	55 °C	(Vakulenko et al., 2003)
	aac(6')-aph(2')-R	CCTCGTGTAAATTCATGTTCTGGC		
<i>ant(4')</i>	ant4-Ia-F	TGGGGATGATGTTAAGGC	50 °C	This study
	ant4-Ia-R	GCGTTTTGACACATCCAC		
<i>aph(3')-IIIa</i>	aph3-III-F	CCGCTGCGTAAAAGATAC	50 °C	(Perreten et al., 2005)
	aph3-III-R	GTCATACCACTTGTCGGC		
<i>blaZ</i>	blaZ-F	CAGTTCACATGCCAAAGAG	45 °C	This study
	blaZ-R1	CAGCAGGTGTTGAAGTATC		
<i>cat_{pC194}</i>	catpC194-F	CGACTTTTAGTATAACCACAGA	50 °C	(Schnellmann et al., 2006)
	catpC194-R	GCCAGTCATTAGCCTAT		
<i>erm(B)</i>	erm(B)F	AGTAACGGTACTTAAATTGTTTAC	54 °C	(Perreten et al., 2005)
	erm(B)R	GAAAAGGTACTCAACCAAATA		
<i>mecA</i>	mecA-F1	GATGATACMTTCGTTCCAC	54 °C	(Schnellmann et al., 2006)
	mecA-R1	CGAGTGCTACTCTAGCAA		
<i>spa</i>	spa-1095F	AGACGATCCTTCGGTGAGC	55 °C	(Harmsen et al., 2003)
	spa-1517R	GCTTTTGCAATGTCATTTACTG		
<i>str</i>	str-pS194-F1	GTGATTCTGATGGTCTTG	50 °C	(Schnellmann et al., 2006)
	str-pS194-R1	GCTACATACGTTGAGACA		
<i>tet(M)</i>	tet(M)-F	TCATAGACACGCCAGGAC	50 °C	This study
	tet(M)-R	CATCCGAAAATCTGCTGG		
<i>vga(A)</i>	<i>vga(A-Av)</i> -F ^{a)}	TGGTGGTGAAGTAAACACG	52 °C	This study
	<i>vga(A)</i> -R	AAGTTCGTTTCICTTTTCGACG		
<i>vga(A)v</i>	<i>vga(A-Av)</i> -F ^{a)}	TGGTGGTGAAGTAAACACG	52 °C	This study
	<i>vga(A)v</i> -R	CTTTTAGCCTTGCTTCCG		
<i>vga(B)</i>	<i>vga(B)</i> -F	GAATAAGGCGCAAGGAATG	52 °C	This study
	<i>vga(B)</i> -R	CATCTTGAATGGAGGTAGAC		
<i>vgb(A)</i>	<i>vgb(A)</i> -F	TACAGAGTACCCACTACC	52 °C	(Perreten et al., 2005)
	<i>vgb(A)</i> -R	CTTGTCACACTCCATTGC		
<i>vgb(B)</i>	<i>vgb(B)</i> -F	GTCTATTCCTCGATTCCAGG	54 °C	(Perreten et al., 2005)
	<i>vgb(B)</i> -R	TGCAAACCATACGGATCC		

a) *vga(A-Av)*-F anneals in both *vga(A)* and *vga(A)v*.

Results

No MRSA were found among the 304 pigs tested. In order to determine the resistance profile of the methicillin-susceptible *S. aureus* (MSSA), one *S. aureus* isolate per herd (one from each farm, which was *S. aureus* positive) was randomly chosen and tested for antibiotic resistance and genotype. Out of 54 herds, 6 harbor pigs free of *S. aureus* and 48 harbor at least one pig colonized with *S. aureus* (n = 48). Among these 48 *S. aureus* isolates, 13 different *spa* types were identified. The most frequent *spa* types were *t034* (31.3%), *t208* (14.6%), *t899* (12.5%), whereas *spa* types *t337*, *t1939*, *t1333*, *t4048*, *t4049*, *t4358*, *t4472*, *t4473*, *t4474*, *t4475*, were found sporadically (Tab. 2). Twenty-seven percent (n = 13) of the *S. aureus* isolates

were susceptible to all antibiotics and 73% (n = 35) were resistant to at least one antibiotic (Tab. 2). Strains displaying resistance to more than 3 antibiotics belonged to *spa* type *t034* (Tab. 2). Penicillin resistance due to the expression of the β -lactamase gene *blaZ*, was the most common resistance trait (62.5%), followed by resistance to tetracycline (33.3%) [*tet(M)*], streptomycin (*str_{pS194}*) (27%), erythromycin and clindamycin [*erm(B)*] (4.1%), gentamicin [*aac(6')-Ie-aph(2')-Ia*] (2%), chloramphenicol (*cat_{pC194}*) (2%) and kanamycin (4.1%). Kanamycin-resistant strains did not contain either the aminoglycoside phosphotransferase genes *aph(3')-IIIa* and *aph(2')-Ia* or the adenyltransferase gene *ant(4')-Ia*. The MIC distribution of each antibiotic is presented in Table 3. Eleven strains showing decreased susceptibility (MIC \geq 1 μ g/ml)

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Table 2: Antibiotic resistance pattern and *spa* types of *S. aureus* from nasal cavities of pigs in Switzerland.

Antibiotic resistance phenotype and genotype		n	%	<i>spa</i> types (n)
PEN	<i>blaZ</i>	9	18.7	<i>t337</i> (2), <i>t208</i> (5), <i>t4049</i> (2)
PEN	<i>blaZ</i> TET <i>tet</i> (M)	8	16.6	<i>t034</i> (7), <i>t4358</i> (1)
PEN	<i>blaZ</i> STR <i>str</i>	5	10.4	<i>t208</i> (1), <i>t899</i> (1), <i>t4049</i> (1), <i>t4473</i> (1), <i>t4474</i> (1)
PEN	<i>blaZ</i> KAN unknown	1	2.0	<i>t337</i> (1)
PEN	<i>blaZ</i> TET <i>tet</i> (M) STR <i>str</i>	5	10.4	<i>t034</i> (5)
PEN	<i>blaZ</i> TET <i>tet</i> (M) ERY <i>erm</i> (B) CLI <i>erm</i> (B)	1	2.0	<i>t034</i> (1)
PEN	<i>blaZ</i> TET <i>tet</i> (M) ERY <i>erm</i> (B) CLI <i>erm</i> (B) GEN <i>aac</i> (6')- <i>aph</i> (2') CHL <i>cat</i> _{pc194}	1	2.0	<i>t034</i> (1)
STR	<i>str</i>	3	6.3	<i>t1939</i> (1), <i>t4049</i> (1), <i>t4358</i> (1)
TET	<i>tet</i> (M)	1	2.0	<i>t034</i> (1)
KAN	unknown	1	2.0	<i>t208</i> (1)
Total antibiotic resistant		35	73.0	<i>t034</i> (15), <i>t899</i> (1), <i>t1939</i> (1), <i>t377</i> (3), <i>t208</i> (7), <i>t4049</i> (4), <i>t4474</i> (1), <i>t4473</i> (1), <i>t4475</i> (1), <i>t4358</i> (1)
Total antibiotic susceptible		13	27.0	<i>t1939</i> (2), <i>t1333</i> (1), <i>t899</i> (5), <i>t4048</i> (4), <i>t4472</i> (1)

Antibiotic abbreviations and resistance breakpoints in parentheses: CHL, chloramphenicol (MIC \geq 32 μ g/ml); CLI, clindamycin (MIC 4 μ g/ml); ERY, erythromycin (MIC \geq 8 μ g/ml); GEN, gentamicin (MIC \geq 16 μ g/ml); KAN, kanamycin (MIC \geq 64 μ g/ml); PEN, penicillin (MIC \geq 0.25 μ g/ml); STR, streptomycin (MIC \geq 32 μ g/ml); TET, tetracycline (MIC \geq 16 μ g/ml).

Table 3: Minimum inhibitory concentrations (MICs) of 18 antimicrobial agents for 48 *S. aureus* isolates from pigs.

Antimicrobials	Number of strains with MIC (μ g/ml) of													
	≤ 0.12	0.12	0.25	0.5	1	2	4	8	16	32	64	128	≤ 1000	≥ 1000
Amikacin								48						
Amoxicillin/clavulanic acid ^{a)}					48									
Cephalotin					47		1							
Chloramphenicol						2		17	28		1			
Clindamycin			46				2							
Enrofloxacin		46		1	1									
Erythromycin		4		34	8				2					
Gentamicin					46		1			1				
Kanamycin ^{a)}													46	2
Linezolid			1		1	20	25	1						
Nitrofurantoin								46		2				
Oxacillin		18		22	7	1								
Penicillin G	18		2	11	13	3	1							
Quinopristin-dalfopristin			37		9	2								
Streptomycin						12			22	1				13
Tetracycline				31		1					16			
Trimethoprim/sulfamethoxazole ^{b)}			48											
Vancomycin				48										

The dilution ranges tested for each antibiotic are those contained within the white area (Sensititre custom plate NLV57). Values situated above or below this range indicate MIC values higher than the highest concentration tested respectively values smaller or equal to the lowest concentration tested. Resistance breakpoints are indicated with vertical black lines. They are those recommended for *S. aureus* in the CLSI supplement M100-S18 (Clinical and Laboratory Standards Institute, 2008), except for streptomycin, for which breakpoint from the French Society for Microbiology (www.sfm.asso.fr) was used.

a) The strains were only tested for high level resistance to kanamycin.

b) Concentration given for amoxicillin (ratio of amoxicillin/clavulanic acid 2:1) and trimethoprim (ratio of trimethoprim/sulfamethoxazole 1:19).

to the combination quinopristin-dalfopristin were tested for the presence of *vga(A)v*, *vga(A)*, *vga(B)*, *vgb(A)* and *vgb(B)* genes. Five of them contained a *vga(A)v* gene. All the strains were susceptible to oxacillin and did not harbor the *mecA* gene.

Discussion and Conclusion

In Switzerland, the number of farms harboring pigs with *S. aureus* was high (89%). However, MRSA were not detected in the nasal cavities of the tested pigs indicating that the prevalence of MRSA in pigs is very low compared to The Netherlands and Ontario, Canada (de Neeling et al., 2007; Khanna et al., 2008). A previous study also reported the absence of MRSA on pig carcasses at slaughterhouse in Switzerland (Nitzsche et al., 2007). In our study, most of the isolated methicillin-susceptible *S. aureus* (MSSA) strains displayed resistance to antibiotics that are often used in pig husbandry in Switzerland, such as penicillin, streptomycin and tetracycline. A few strains showed a multidrug resistance pattern. They belong to the genetic lineage *spa* type *t034*, which is the dominant *S. aureus* clone in pigs in Switzerland. The *t034 spa* type is also predominant among pigs in Denmark (Guardabassi et al., 2007; Lewis et al., 2008), whereas most pigs in the Netherlands carry MRSA belonging to *spa* types *t011*

and *t108* (de Neeling et al., 2007). Besides *spa* type *t034*, the pigs in Switzerland were found to harbor a variety of genetically different *S. aureus* in their nasal cavities. They belong to *spa* types which are different from those found in invasive MSSA and MRSA associated with infections in humans in Switzerland (Fenner et al., 2008a; Fenner et al., 2008b), suggesting no association between human infections and pig reservoir. However, the risk of transfer between pigs and to humans exists as demonstrated in other countries with MRSA (van Loo et al., 2007a, Khanna et al., 2008, van Duijkeren et al., 2008). *S. aureus* belonging to pig-associated *spa* type *t034*, respectively *t011* and *t108* are already circulating among pigs and humans in Denmark and in the Netherlands (Guardabassi et al., 2007; van Belkum et al., 2008; Lewis et al., 2008). Such MRSA may rapidly emerge and spread through the pig population in Switzerland if appropriate measures are not taken.

A continuous surveillance of pigs has been now implemented by the Swiss Federal Veterinary Office at the National Centre for Zoonoses, Bacterial Animal Diseases and Antimicrobial Resistance (ZOBA) of the Institute of Veterinary Bacteriology, University of Berne and should also be extended to imported animals at the border. Indeed, the pig production in Switzerland is mainly covered by own production (1.6 Mio pigs in 2006 (Schweizerische Eidgenossenschaft, 2008)) and importation of live

Antibiorésistance et diversité génétique de *Staphylococcus aureus* provenant de porcs abattus en Suisse

304 porcs provenant de 54 exploitations différentes ont été examinés à l'abattoir au moyen d'un écouvillon nasal quant à la présence de *Staphylococcus aureus*. Des porcs porteurs de *S. aureus* ont été trouvés dans 89 % des exploitations. On n'a pas trouvé de MRSA, ce qui indique une prévalence basse. Les *S. aureus* isolés présentaient des résistances à la pénicilline (*blaZ*) (62.5%), à la tétracycline [*tet(M)*] (33.3 %), à la streptomycine (*str_{ps194}*) (27 %), à la clindamycine [*erm(B)*] (4.1%), à l'érythromycine [*erm(B)*] (4.1%), à la kanamycine (4.1%), au chloramphénicol (*cat_{pC194}*) (2%) et à la gentamicine [*aac(6')-Ie-aph(2')-Ia*] (2%). Les *S. aureus* isolés appartenait principalement aux types Ridom *spa* *t034* (31,3%), *t208* (14,6%) et *t899* (12,5%). De tels clones spécifiquement associés aux porcs n'ont jusqu'à présent pas été mis en évidence chez des êtres humains hospitalisés en Suisse. Des programmes de surveillances doivent être introduits pour les porcs indigènes mais aussi lors d'importation de porcs pour permettre une identification précoce des MRSA et pour empêcher leur rapide propagation dans la population porcine suisse.

Resistenza agli antibiotici e diversità genetica dello *Staphylococcus aureus* nei suini da macello in Svizzera

Al macello sono stati esaminati via tamponi nasali la presenza di *Staphylococcus aureus* su 304 maiali provenienti da 54 aziende differenti. Maiali, colonizzati da *S. aureus* sono stati trovati nell'89% delle aziende. Non sono stati riscontrati MRSA cosa che indica una bassa prevalenza. Gli isolati di *S. aureus* hanno evidenziato resistenze alla penicillina (*blaZ*) (62.5%), tetraciclina [*tet(M)*] (33.3 %), streptomycina (*str_{ps194}*) (27 %), clindamicina [*erm(B)*] (4.1%), eritromicina [*erm(B)*] (4.1%), kanamicina (4.1%), cloramfenicolo (*cat_{pC194}*) (2%) e gentamicina [*aac(6')-Ie-aph(2')-Ia*] (2%). Gli isolati di *S. aureus* appartengono maggiormente al Ridom *spa* di tipo *t034* (31.3%), *t208* (14.6%) e *t899* (12.5%). Questi cloni specifici associati ai suini, non sono stati finora individuati in persone ricoverate in ospedale in Svizzera. Nella tenuta di maiali in Svizzera come pure per l'importazione di maiali bisognerebbe introdurre dei programmi di sorveglianza per rendere possibile una rapida individuazione di MRSA e per impedire una rapida propagazione nella popolazione suina in Svizzera.

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animals is low (1010 pigs in 2007; 0.06 %) (Swiss Federal Customs Administration, personal communication). The very low number of imported pigs allows the implementation of surveillance at the importation level. MRSA surveillance in both imported and local pigs will help to rapidly detect and then take appropriate measures to suppress the emergence of MRSA in Switzerland.

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