MICROBIAL DRUG RESISTANCE
Volume 00, Number 0, 2013

DOI: 10.1089/mdr.2012.0131

Clinical Resistance and Decreased Susceptibility in *Streptococcus suis* Isolates from Clinically Healthy Fattening Pigs

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Streptococcus suis (*S. suis*) has often been reported as an important swine pathogen and is considered as a new emerging zoonotic agent. Consequently, it is important to be informed on its susceptibility to antimicrobial agents. In the current study, the Minimum Inhibitory Concentration (MIC) population distribution of nine antimicrobial agents has been determined for nasal *S. suis* strains, isolated from healthy pigs at the end of the fattening period from 50 closed or semiclosed pig herds. The aim of the study was to report resistance based on both clinical breakpoints (clinical resistance percentage) and epidemiological cutoff values (non-wild-type percentage). Non-wild-type percentages were high for tetracycline (98%), lincomycin (92%), tilmicosin (72%), erythromycin (70%), tylosin (66%), and low for florfenicol (0%) and enrofloxacin (0.3%). Clinical resistance percentages were high for tetracycline (95%), erythromycin (66%), tylosin (66%), and low for florfenicol (0.3%) and enrofloxacin (0.3%). For tiamulin, for which no clinical breakpoint is available, 57% of the isolates did not belong to the wild-type population. Clinical resistance and non-wild-type percentages differed substantially for penicillin. Only 1% of the tested *S. suis* strains was considered as clinically resistant, whereas 47% of the strains showed acquired resistance when epidemiological cutoff values were used. In conclusion, MIC values for penicillin are gradually increasing, compared to previous reports, although pigs infected with strains showing higher MICs may still respond to treatment with penicillin. The high rate of acquired resistance against tiamulin has not been reported before. Results from this study clearly demonstrate that the use of different interpretive criteria contributes to the extent of differences in reported antimicrobial resistance results. The early detection of small changes in the MIC population distribution of isolates, while clinical failure may not yet be observed, provides the opportunity to implement appropriate risk management steps.

Introduction

Streptococcus suis (*S. suis*) is an important swine pathogen affecting pigs of different ages, although susceptibility to the disease decreases with age after weaning.[4,36] It is known to cause meningitis, arthritis, septicemia, endocarditis, polyserositis, bronchopneumonia, and abortion,[4,23,36] but can also be found in the upper respiratory, alimentary, and urogenital tract of healthy pigs.[4,22] *S. suis* has also been implicated in disease in humans, especially among people in close contact with swine and pork.[20,27] Moreover, *S. suis* has recently been reported as an emerging zoonotic pathogen evidenced by a few large-scale outbreaks of severe *S. suis* epidemics in Asia.[28,41,42]

The most frequently applied treatment for pigs with clinical signs of *S. suis* infection is feed medication with antimicrobials, particularly, broad-spectrum penicillins.[9,19,37] Currently, no effective commercial vaccine is available. Prevention is based on the optimization of management, autogenous vaccines, and primarily the strategic administration of antimicrobial agents at periods with the highest risk, for example, weaning.[21,40] High levels of resistance to tetracyclines,[25,30] macrolides, and lincosamides[30] have been reported.

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Different methods are often applied for interpreting the results of antimicrobial susceptibility testing. In most studies, clinical breakpoints have been used resulting in the categorization of the tested isolates in susceptible, intermediate, or resistant against the tested antimicrobials (clinical resistance).[12] The use of clinical interpretive criteria may be sufficient from the point of view of the clinician as it predicts the antimicrobial effect of the drug in the patient at the prescribed dose.[14,35,38] However, these breakpoints can vary over time and between countries,[24] making comparisons between different studies and evolution of antimicrobial resistance patterns in *S. suis* over time hard. Moreover, this categorization precludes the detection of small changes in the population distribution that may indicate the acquisition of new resistance mechanisms of which the clinical implications are not yet clear, as has been noted for fluoroquinolones and Gram-negative bacteria.[13] For such changes to be noticed, epidemiological cutoff values are very valuable. These cutoff values are based on the differentiation between the wild-type and the non-wild-type population.[12,16,24] They enable to detect strains with a decreased susceptibility, which are isolates with Minimum Inhibitory Concentrations (MICs) that are non-wild type, but less than or equal to the susceptible clinical breakpoint.[12,35] However, only few studies report resistance results as MIC population distributions, necessary for setting the epidemiological cutoff values. Finally, *S. suis* from diseased animals have been tested more often[29,32,40] than *S. suis* from clinically healthy animals. This could lead to biased results, since isolates from diseased animals may represent a different population[3] and since they have often been exposed to an antimicrobial selection pressure shortly before sampling.[34]

This study aimed to report the level of resistance in *S. suis* isolates from clinically healthy fattening pigs at slaughter age. Resistance percentages were calculated based on both clinical breakpoints and epidemiological cutoff values.

Materials and Methods

Study design, sample, and data collection

For the isolation of *S. suis*, nasal swabs were taken from clinically healthy fattening pigs from 50 different pig herds in Belgium. A list of 140 pig herds that fulfilled the selection criteria were randomly selected from the Belgian farm-animal identification and registration database (SANITEL, 2010). The sampling frame consisted of all farrow-to-finish herds that used a closed or semiclosed production system and held at least 150 sows and 600 fattening pigs. The sample was stratified by province ($n = 5$), proportional to the number of pig herds per province. A random selection was performed using a computer-generated list (Toolbox, Cameron, 1999). All selected herds were contacted by telephone and the first 50 herds that were willing to cooperate in the study were visited between January and October 2010.

The pigs were sampled $\sim$2 weeks before the slaughter age. The average age of the pigs was 182 days (minimum 156 days; maximum 220 days). In each herd, 20 fattening pigs were randomly sampled.

Bacterial isolation

Swabs were plated on Columbia agar plates with 5% defibrinated sheep blood, supplemented with colistin and nalidixic acid (CNA; Oxoid, Basingstoke, United Kingdom) within 24 hr after collection and cultured at 35°C in a 5% CO_2-enriched atmosphere for 24 hr. Colonies showing alpha-hemolysis were purified for further identification.[1,26] Isolates showing a positive amylase reaction, a negative catalase reaction, and a negative Vogues-Proskauer test were considered to be *S. suis*.[1,22,23] The identity of 28 randomly chosen *S. suis* isolates was confirmed by sequencing the 16s rRNA gene as described before.[6] *S. suis* isolates were stored at -80°C until antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all isolates using the agar dilution method according to the standardized methods described by the Clinical and Laboratory Standards Institute.[11]

Inocula were prepared suspending colonies in sterile 0.9% NaCl to a turbidity equivalent of 0.5 Mac Farland and diluted 1/10. Using a Steers inoculum applicator, the suspensions were inoculated on the Muller-Hinton II agar (BBL; Cockeysville, MD) supplemented with 5% sheep blood and containing doubling concentrations, ranging from 0.03 μg/ml to 128 μg/ml of the following antimicrobial agents: enrofloxacin, erythromycin, florfenicol, lincomycin, penicillin, tetracycline, tiamulin, tilmicosin, and tylosin. The plates were incubated at 35°C in 5% CO_2-enriched atmosphere for 24 hr. The MIC was defined as the lowest concentration producing no visible growth. *S. aureus* ATCC®29213, *Enterococcus faecalis* ATCC®29212, and *S. pneumoniae* ATCC®49619 were included as quality control (QC) strains. Interpretation of the MIC values was done using both clinical breakpoints[11] and epidemiological interpretative criteria.[38] For enrofloxacin, lincomycin, tiamulin, tilmicosin, and tylosin, no clinical breakpoint for *S. suis* is available.[11] For florfenicol and tetracycline, the clinical breakpoint for swine respiratory disease caused by *S. suis* was used.[11] For erythromycin and penicillin, clinical breakpoints were used, as described by the Clinical and Laboratory Standards Institute (CLSI) for veterinary pathogens, but which were based on CLSI breakpoints for human *Streptococci*.[11] Since no epidemiological cutoff values are available for *S. suis* from EUCAST,[17] acquired resistance was assumed when MIC values showed a bimodal or multimodal distribution or tailing.[8,15] Isolates in the higher range of MICs were considered not to belong to the wild-type population. For antimicrobials for which no clear bimodal distribution was present, epidemiological cutoff values were used available from a previous study carried out in the same laboratory using identical test conditions. This was done for the following antimicrobials: penicillin, tilmicosin, erythromycin, lincomycin, tiamulin, and tetracycline.[30] The MIC_{50} and MIC_{90} were calculated and presented the lowest MIC at which at least 50% and 90% of the isolates in a test population are inhibited, respectively.

Results

In the current study, *S. suis* was recovered in 33.2% of all nasal samples (332/1000). The number of isolates obtained per herd was normally distributed, with on average, 6.6 isolates recovered from one herd (minimum number of isolates per herd equaled 5 isolates; maximum equaled 8 isolates; median equaled 7 isolates). The MIC values of 10

antimicrobial agents were determined for 332 *S. suis* isolates. Yet, a number of *S. suis* isolates showed poor growth under the prescribed conditions, as has been observed before[40] and their MIC could not be determined. Therefore, in this report, MIC data have been reported for a variable number of *S. suis* isolates (Table 1). The MIC values for QC strains were within the acceptable QC ranges when available.[11] For lincomycin, QC strains *S. aureus* ATCC®29213 and *E. faecalis* ATCC®29212 had similar MIC values as described earlier.[29,32]

In Table 1, the MIC distribution for all tested *S. suis* isolates is shown. A bimodal distribution was seen for enrofloxacin. A monomodal distribution was seen for florfenicol. For penicillin, a distribution with tailing toward higher MIC values was noted. No clear bimodal distribution was seen for erythromycin, lincomycin, tylosin, tilmicosin, tiamulin, and tetracycline.

In Table 2, the clinical breakpoints[11] and the epidemiological cutoff values for the different antimicrobials tested are shown. Based upon clinical breakpoints, percentage of susceptible, intermediate, and resistant *S. suis* strains are shown. Equally, based upon epidemiological cutoff values, % of wild-type and non-wild-type strains are presented.

No or very low percentages of clinical resistance were found against florfenicol (0.3%), and penicillin (1%). High to very high-resistance percentages were observed against erythromycin (66%) and tetracycline (95%).

Using the epidemiological cutoff values, low percentages of non-wild-type isolates were seen to enrofloxacin and florfenicol (0.3% and 0%, respectively). Acquired resistance was observed for penicillin (percentage of non-wild-type isolates equals 47%), tiamulin (57%), erythromycin (70%), tylosin (66–67%), tetracycline (98%), tilmicosin (72%), and lincomycin (92%).

Discussion

The choice of the epidemiological cutoff value, based on the distinction between the wild-type and the non-wild-type population within a bacterial population, should be fixed for one antimicrobial agent within a bacterial species, independent of time. Moreover, given that wild-type MIC distributions of bacteria of human and animal origin coincide, the same epidemiological cutoff value can be used for monitoring resistance in humans and in different animals.[2] Yet, discrepancies between antimicrobial susceptibility test protocols may result in the establishment of a different epidemiological cutoff value between studies within one bacterial species for one antimicrobial agent.[7,34] Nevertheless, the preferred method for reporting MIC results is to present all data in a distribution table, containing the quantitative data[39] to allow the reader to interpret the data with changing interpretive criteria over time (clinically or epidemiologically).

The high percentages of non-wild-type *S. suis* isolates for erythromycin, lincomycin, tilmicosin, tylosin, and tetracycline are in accordance with other studies reporting percentages of non-wild-type *S. suis* isolates for macrolides, lincosamides, and tetracyclines.[30,40]

Despite differences in interpretive criteria (clinical breakpoints or epidemiological cutoff values), susceptibility testing methods (disk diffusion, microdilution, and agar dilution), sampled animals (clinically healthy or diseased pigs, sows or fattening pigs), and geographical location, there seems to be a similarity concerning results on clinical resistance percentages, when available, and percentages of non-wild-type *S. suis* isolates for those antimicrobials, which in some studies have been supported by the identification of genotypic resistance mechanisms.[30,31]

In the farms included in the current study, macrolides were frequently used during the farrowing and battery period.[9] Genes encoding cross resistance to pmacrolides, lincosamides, and streptogramin B are widespread among *S. suis* isolates.[30] As a result, the administration of macrolides may select for resistance against these antimicrobials.

Similarities between the current study results and others have equally been found for the low-resistance percentages against florfenicol[40] and enrofloxacin.[30,40]

For tiamulin, a high percentage of *S. suis* isolates did not belong to the wild-type population, defined as having an MIC of $\leq 4\,\mu g/ml$, demonstrating acquired resistance in these isolates against this antibiotic. For tiamulin, no clinical breakpoints are available for *S. suis* and epidemiological cutoff values do not necessarily predict how a patient will respond to therapy. However, for 49% of the isolates, the MIC of tiamulin varied between 32 and $>128\,\mu g/ml$, being at least 8 to more than 32 times higher than for isolates belonging to the wild-type population. Although it has not yet been tested, the likelihood that pigs infected with isolates demonstrating the higher MIC values of tiamulin will

Table 1. Minimum Inhibitory Concentration Distribution for *Streptococcus suis* Isolates Obtained from Clinically Healthy Fattening Pigs on 50 Closed or Semiclosed Pig Herds

Antimicrobial agent	*Number of strains with MIC (μg/ml)*														*Number of isolates tested*
	≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	
Erythromycin	24	40	33	8	6	8	7	16	22	9	7	2	8	136	326
Lincomycin	2	0	3	6	3	9	24	11	10	3	7	7	7	214	306
Tylosin	3	6	1	1	27	63	8	2	3	1	1	4	13	197	330
Tilmicosin	6	0	0	1	2	13	15	20	9	15	9	14	10	174	288
Tiamulin	3	1	5	9	12	25	57	30	10	16	33	34	43	54	332
Tetracycline	0	0	4	2	4	5	9	6	19	40	115	112	14	0	330
Penicillin	22	32	48	71	86	54	13	1	2	0	0	0	0	0	329
Florfenicol	0	0	2	0	11	97	218	2	1	0	0	0	0	0	331
Enrofloxacin	1	9	22	129	122	17	0	0	1	0	0	0	0	0	301

MIC, minimum inhibitory concentration.

Table 2. MIC, CBP, and ECV of 10 Antimicrobial Agents for *Streptococcus suis* Isolates Obtained from Clinically Healthy Fattening Pigs on 50 Closed Or Semiclosed Pig Herds

	Epidemiological Interpretive Criteria			*Clinical Interpretive Criteria*							
				CBP[c]							
Antimicrobial agent	*ECV*	*Wild type*[a] %	*Non-wild type*[b] %	*S*	*I*	*R*	%R	%I	%S	MIC_{50}[d]	MIC_{90}[e]
Erythromycin[g]	0.12	30	70	0.25	0.5	1	66	2	32	16	>128
Lincomycin[f,g]	1	8	92	-	-	-	-	-	-	>128	>128
Tylosin[f]	2–4	33–34	66–67	-	-	-	-	-	-	>128	>128
Tilmicosin[f,g]	16	23	72	-		-	-	-	-	>128	>128
Tiamulin[f,g]	4	43	57	-	-	-	-	-	-	32	>128
Tetracycline[g]	0.25	2	98	0.5	1	2	95	2	3	64	128
Penicillin[g]	0.25	53	47	0.12	0.25–2	4	1	68	31	0.5	2
Florfenicol	8	99,7	0	2	4	8	0.3	0.6	99.1	4	4
Enrofloxacin[f]	1	99,7	0,3	-	-	-	-	-	-	0.5	1

The percentage of resistant (%R), intermediate (I%), and susceptible (%S) strains is provided based on clinical breakpoints. The percentage of wild type (WT) and non-wild type (non-WT) is provided based on epidemiological cutoff values.

[a]Wild type describes isolates with MICs below the epidemiological cutoff value (WT $\leq$X μg/ml).

[b]Non-wild type describes isolates with MICs above the epidemiological cutoff value (non-WT >X μg/ml).

[c]Clinical breakpoints were obtained from Clinical and Laboratory Standards Institute standards.[12] S: Susceptible, I: Intermediate, R: Resistant (R $\geq$Y μg/ml; Z μg/ml<I<Y μg/ml; S$\leq$Z μg/ml).

[d,e]MIC_{50} and MIC_{90} are the lowest MIC at which at least 50% and 90% of the isolates in a test population are inhibited in their growth.

[f]For enrofloxacin, lincomycin, tiamulin, tilmicosin, and tylosin, no clinical breakpoint is available.[11]

[g]Epidemiological cutoff value was based on study from Martel *et al*.[30]

CBP, clinical breakpoints; ECV, epidemiological cutoff values.

respond well to treatment with this antibiotic should be considered to be low.[34] For evaluation of tiamulin resistance in *S. suis* isolates, Zhang *et al*.[43] used the clinical breakpoint reported by CLSI[11] for *Actinobacillus* spp. causing respiratory tract disease in pigs (32 μg/ml) and reported that 34.4% of their isolates were resistant. Although this clinical breakpoint cannot be extrapolated as such to other bacterial species or disease conditions,[33] the percentage of isolates with a MIC of $\geq$32 μg/ml was clearly higher in this study. Also based on MIC determinations from *S. suis* isolates recovered between 1999 and 2000 from clinically diseased pigs, carried out in the same laboratory using identical test conditions,[30] a clear shift toward higher MIC values was observed in this study. The sampled pigs from this study did not receive tiamulin for prophylactic or metaphylactic reasons.[9] Yet, the use of tiamulin as a therapeutic antimicrobial agent against *Brachyspira* spp. and *Mycoplasma hyopneumoniae* infections is common[18] and cannot be ruled out for this study.

Broad-spectrum penicillins were the most frequently used antimicrobial class in pigs from this study, as described in a former study conducted in the same pig herds.[9] Based on the clinical breakpoint for penicillin[11] in this study, only one isolate could be categorized as resistant. Yet, when considering isolates with MICs beyond the wild-type cutoff value, a high number of isolates showed a decreased susceptibility. Penicillin resistance in streptococci is the result of the acquisition of stepwise mutations in genes encoding penicillin binding proteins.[2] A single-point mutation results in isolates with a modest increase in MIC, and infections due to these isolates may still be treatable with penicillins, but they are of great concern as they represent an introductory step to full resistance.[5] Isolates showing higher values of MICs are associated with additional mutations and most likely lead to therapy failure.[10] Additionally, these mutations are selected by the use of β-lactam antimicrobials.[10] As a result, reporting a decreased susceptibility based on epidemiological cutoff values is important as it can act as an early warning for an emerging clinical problem.[2,34]

Conclusions

The current study on *S. suis* isolates from healthy carrier pigs confirms the high level of acquired resistance to macrolides, lincosamides, and tetracycline. MIC values for penicillin are gradually increasing, compared to previous reports,[30] as has been seen for *S. pneumoniae* in humans, although pigs infected with strains showing higher MICs may still respond to treatment with this antibiotic. The high rate of acquired resistance against tiamulin has not been reported before.

Acknowledgments

This work was supported by a grant of the Federal Public Service of Health, Food Chain Safety and Environment (Grant number RT-07/9-ABRESZOON). The authors thank the farmers for participation in the study, last-year DVM students, H. Vereecke and A. Van de Kerckhove for technical assistance, and C. Davidsen for linguistic support.

Author Disclosure Statement

No competing financial interests exist.

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