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CODA - CERVA

Antimicrobial resistance

in methicillin-resistant *Staphylococcus aureus*

from pigs in 2016 in Belgium

Report on the occurrence of antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* from pigs in 2016 in Belgium.

Summary

The overall MRSA prevalence in fattening pigs and sows in 2016 was 63.3% and 59.2% respectively. This level is very similar to the MRSA prevalence in pigs in 2013. MRSA ST398, mainly associated with livestock animals, was the predominant sequence type in sows and fattening pigs. The main *spa*-type was t011 and all were associated with MRSA ST398. A change in *spa*-types could be seen between 2013 and 2016, suggesting a changing profile according to adaptations of the animal host. Among MRSA strains from pigs, in 2016, resistance was detected for all antimicrobials tested, except for the glycopeptide antibiotic vancomycin. Antimicrobial resistance to linezolid and mupirocin was only present in two strains. One quarter of the MRSA strains showed resistance to three other antimicrobial classes in addition to the cefoxitin and penicillin resistance. Antimicrobial resistance to tetracycline, trimethoprim and ciprofloxacin was the predominant resistance pattern. Antimicrobial resistance decreased compared to 2013, except for tetracycline and trimethoprim.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognised as an important cause of infections in humans for decades. Strains of MRSA causing infections in humans can be divided into three broad categories, healthcare-associated (HA-), community-associated (CA-) and livestock-associated (LA-) MRSA. LA-MRSA has been detected in pigs, poultry, bovines, horses and dogs and LA-strains have been shown to be distinct from human-derived strains (Fluit, 2012). HA-MRSA and CA-MRSA include strains which predominantly affect humans, yet, there is also an exchange of strains between the reservoirs (Fluit, 2012). LA-MRSA may therefore also be harbored by humans and cause illness in humans. Pigs are often carriers of LA-MRSA, but are only rarely infected (Meemken et al., 2010). In chickens, several disease manifestations have been described (McNamee and Smyth, 2000). Staphylococcal mastitis has been reported in dairy industry (Vanderhaeghen et al., 2010a). In Belgium, in 2014, a 3-fold decrease in the incidence of nosocomial MRSA is seen since 2003 (WIV-ISP, 2015). Also, the proportion of MRSA strains out of the clinical *S. aureus* strains showed a decrease of 14% between 2003 and 2014 (WIV-ISP, 2015). At the European level, a significantly decreasing trend of human-derived MRSA was observed from 2011 to 2014. Yet, MRSA remains a human public health priority, as the percentage of MRSA remains above 25.0% in 7 out of 29 EU countries. However, in Belgium, a decreasing trend of 4% has been observed between 2011 and 2014 (EFSA and ECDC, 2016).

In the framework of the surveillance by Federal Agency for the Safety of the Food Chain (FASFC), a surveillance of MRSA is executed, in order to determine the prevalence and diversity of MRSA strains isolated from production animals. The surveillance consists of a cycle of three years. Poultry was monitored in 2014 and bovines in 2015. In this report, prevalence and antimicrobial susceptibility data are presented for MRSA isolated from pigs.

Materials and methods

Sampling

Three-hundred twenty-four farms were sampled. 10 nasal swabs per farm were taken.

Isolation and identification

Nasal swabs were pooled and incubated in Mueller-Hinton (MH) broth (Becton Dickinson) supplemented with NaCl (6.5%) at 37°C for 18-24h. One ml of this broth was added to Tryptic Soy Broth (TSB) supplemented with cefoxitin (3.5 mg/l) and aztreonam (75 mg/l) and incubated at 37°C for 18-24h. Ten microliter of this enrichment was plated on Brilliance MRSA 2 (Oxoid) and incubated 18-24h at 37°C. Presence of MRSA was suspected based on colony morphology and confirmed using a triplex real-time PCR method.

Confirmation by real-time PCR

Per sample, one to five suspected colonies were selected from the Brilliance MRSA 2 plate. DNA was extracted as described in SOP/BAC/ANA/18. MRSA confirmation was performed using a triplex real-time PCR method. This PCR allows detecting the Staphylococcal aureus specific gene, *nuc*, the presence of the *mecA* gene responsible for methicillin resistance and the variant *mecC* gene.

Genotyping

Spa typing

All MRSA isolates were *spa*-typed by sequencing the repetitive region of the *spa* gene encoding for the staphylococcal protein A. This method depicts the rapid evolution, since through recombination, the repeats may change fast. The protein A (*spa*) gene was amplified according to the Ridom StaphType standard protocol (**www.ridom.de/staphtype**) and the amplification was checked on a 2% agarose gel. Sequencing was performed with an ABI capillary instrument using standard protocols and sequences were compared with the international Ridom database.

CC398 PCR

CC398 PCR was performed on all MRSA following protocol described by Stegger *et al.* 2011. This method allows the rapid detection of the *S. aureus* sequence type ST398.

Antimicrobial susceptibility testing

Antimicrobial resistance was determined using the micro broth dilution method (Sensititre, Trek Diagnisis Systems, Magellan Biosciences) following the manufacturer's instructions (SOP/BAC/ANA/11) and using the epidemiological cut-off's (ECOFFs), established by the European Committee on Antimicrobial Susceptibility (EUCAST) or as defined by the EU reference laboratory on antimicrobial resistance (DTU) for *S. aureus*. Samples were first inoculated on a blood agar plate and incubated at 37°C for 24 hours. Three to five colonies from the agar plate were then added in 4 ml of sterile physiological water and adjusted to 0.5 McFarland. Ten microliter of this suspension was inoculated in a tube containing 11ml cation adjusted MuellerHinton broth with TES (Trek Diagnostics). Fifty µl of this inoculum was then inoculated per well using the AIM™ Automated Inoculation Delivery System and incubated at 37°C for 24 hours**.** Sensititre plates were read with Sensititre Vision System® for semi-automatic registration of the Minimum Inhibitory Concentration (MIC) of the different antimicrobials tested. The MIC was defined as the lowest concentration by which no visible growth could be detected.

Table 1 : Panel of antimicrobial substances included in antimicrobial susceptibility testing, concentration ranges tested and EUCAST epidemiological cut-off's (ECOFFs) for methicillin resistant *Staphylococcus aureus*

Antimicrobial (Abbreviation)	Concentration range, mg/l	EUCAST ECOFF
Chloramphenicol (CHL)	4-64	> 16
Ciprofloxacin (CIP)	0.25-8	> 1
Clindamycine (CLI)	0.12-4	> 0.25
Erythromycine (ERY)	0.25-8	> 1
Cefoxitin (FOX)	0.5-16	> 4
Fusidic acid (FUS)	0.5-4	> 0.5
Gentamicin (GEN)	1-16	> 2
Kanamycine (KAN)	4-64	> 8
Linezolid (LZD)	1-8	> 4
Mupirocin (MUP)	0.5-256	> 1
Penicillin (PEN)	0.12-2	> 0.12
Rifampicin (RIF)	0.016-0.5	> 0.03

Sulfamethoxazole (SMX)	64-512	> 128
Streptomycin (STR)	4-32	> 16
Quinupristin/dalfopristin (SYN)	0.5-4	> 1
Tetracycline (TET)	0.5-16	> 1
Tiamulin (TIA)	0.5-4	> 2
Trimethoprim (TMP)	2-32	> 2
Vancomycin (VAN)	1-16	> 2

EUCAST: European Committee on Antimicrobial Susceptibility Testing

Data analysis and description

Data from the Excel file generated by the software of the semi-automated susceptibility equipment (sensivision, Trek Diagnostics) were incorporated in the LIMS system at CODA-CERVA together with the metadata associated with the sampling. These files were validated for consistency.

Isolates with a MIC value higher than the ECOFF value were considered not to belong to the wild type population and percentages of isolates with a reduced susceptibility, i.e. non-wild type, were calculated. Throughout the report, isolates with a reduced susceptibility will be referred to as 'resistant isolates', whereas when the clinical interpretative criterion was used, the term 'clinical resistance' will be used.

The number of antimicrobials to which a strain was resistant was counted and cumulative percentages or percentiles were calculated. Graphical representations were prepared in Excel.

Throughout the report, terms used to describe the levels or occurrence of antimicrobial resistance are those proposed by EFSA. Rare: <0.1 %', 'very low: >0.1 % to 1.0 %', 'low: >1 % to 10.0 %', 'moderate: >10.0 % to 20.0 %', 'high: >20.0 % to 50.0 %', 'very high: >50.0 % to 70.0 %', 'extremely high: >70.0 %'. Although these terms are applied to all antimicrobials, the significance of a given level of resistance will depend on the particular antimicrobial and its importance in human and veterinary medicine.

A multi-resistant isolate is one defined as resistant to at least three different antimicrobial substances, included in the analysis (Table 1). It should be noted that all confirmed MRSA strains should show resistance to minimum 2 antibiotics, cefoxitin and penicillin.

Statistical analysis

The number of resistant strains was counted and resistance percentages were calculated. Exact confidence intervals for the binomial distribution were calculated using a VBA script in Excel. A 95% symmetrical two-sided confidence interval was used with p=0.025. The lower and upper bound of confidence interval for the population proportion was calculated. Based on the Pearsons chi-square test, and where appropriate the Fischer exact test, significance of the differences were calculated.

Results

Prevalence of Methicillin Resistant *Staphylococcus aureus* and the sequence type ST398

The presence of MRSA was confirmed for 199 strains out of the 324 analyzed samples (61.4%), based on real-time PCR. MRSA was present in both fattening pigs and sows (Table 2). Among 175 MRSA strains recovered, 141 (80.6%) were positive for the cc398 PCR and considered as MRSA sequence type ST398.

Table 2 : Prevalence of Methicillin Resistant *Staphylococcus aureus* and its 95% Confidence Interval sequence type ST398 in fattening pigs and sows

Animal category	Number of pooled samples	MRSA positive (%) 95% Confidence Interval
Fattening pigs	177	112 (**63.3%**) 55.7% - 70.0%
Sows	147	87 (**59.2%**) 50.8% - 67.0%
Total	324	199 (**61.4%**) 55.9% - 67.0%

Characterization of Methicillin Resistant *Staphylococcus aureus*

Out of the 199 MRSA strains, 175 were characterized by their genotype (*spa*-typing and CC398 PCR). Hundred forty-one strains were MRSA ST398. Nine different *spa*-types were found. The vast majority was however the commonly isolated t011 and all of them were associated with MRSA ST398. Amongst the ST398 strains, 6 different spa types were found. Thirty-four MRSA strains were different from MRSA ST398. Among these MRSA strains the following *spa*-types were found: t034, t037, t898, t1451, t1456, t1580 and t1985 (Table 3).

Table 3 : Total number of Methicillin Resistant *Staphylococcus aureus* in pigs corresponding to the different genotypes (n= 175)

***spa*-types**	**t011**	**t034**	**t037**	**t1456**	**t1985**	**t4659**	**Total**	
ST398	126	4	2	4	4	1	**141**	
***spa*-types**	**t034**	**t037**	**t898**	**t1451**	**t1456**	**t1580**	**t1985**	**Total**
ST398 negative	8	1	1	3	2	6	13	**34**

Antimicrobial resistance of Methicillin Resistant *Staphylococcus aureus*

Antimicrobial resistance occurrence for 175 tested MRSA strains is presented in figure 1.

As expected due to the presence of the *mecA* gene, all MRSA strains were resistant to cefoxitin and penicillin. Antimicrobial resistance was at extremely high levels for tetracycline and trimethoprim; at very high levels for ciprofloxacin; at high levels for clindamycine, erythromycin, gentamicin, tiamulin and kanamycin; and at moderate levels for quinupristin/dalfopristin, streptomycine and sulfamethoxazole. For fusidic acid, chloramphenicol, rifampicin, linezolid and mupirocin antimicrobial resistance levels remained low, whereas for vancomycin antimicrobial resistance remained undetected.

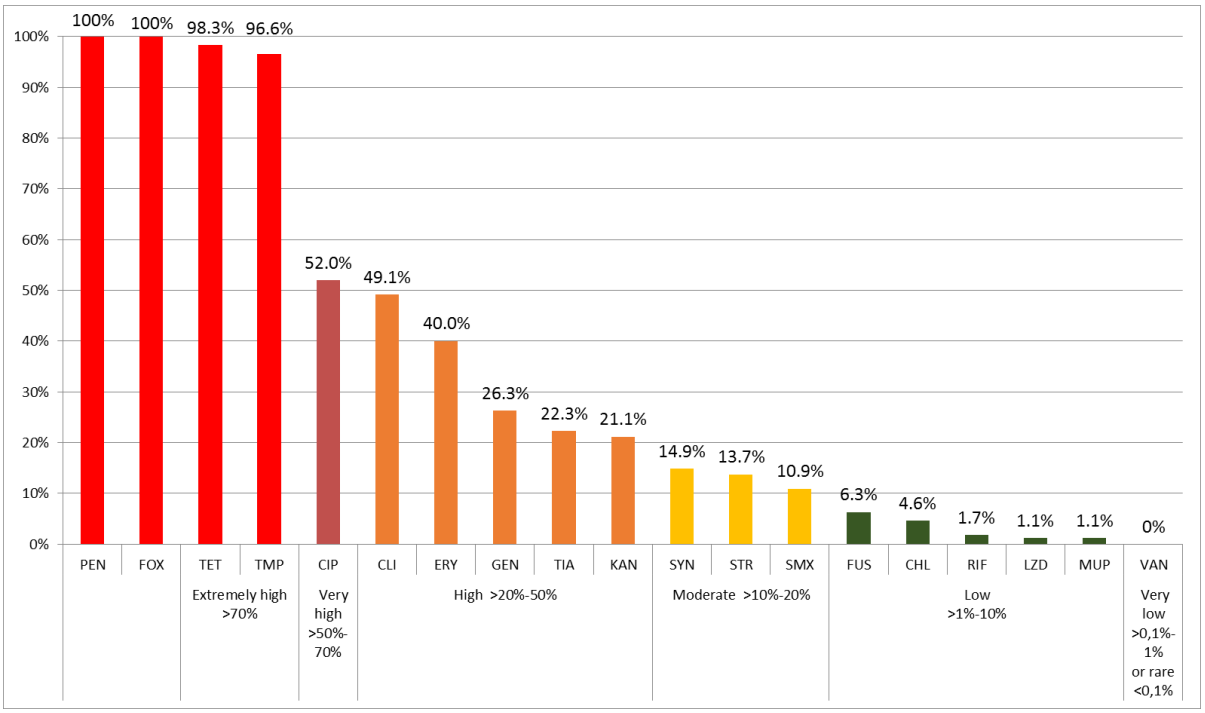

Figure 1 : Antimicrobial resistance prevalence for methicillin resistant *Staphylococcus aureus* (n= 175), isolated from pigs at the farm, based on epidemiological cut-off's, according to the European Committee on Antimicrobial Susceptibility (EUCAST) for cefoxitin (FOX), penicillin (PEN), clindamycin (CLI), tetracycline (TET), erythromycin (ERY), trimethoprim (TMP), kanamycin (KAN), gentamicin (GEN), ciprofloxacin (CIP), streptomycine (STR), quinupristin/dalfopristin (SYN), sulfamethoxazole (SMX), chloramphenicol (CHL), tiamulin (TIA), fusidic acid (FUS), rifampicin (RIF), linezolid (LIN), mupirocin (MUP), vancomycin (VAN).

Multiple antimicrobial resistance patterns of Methicillin Resistant *Staphylococcus aureus*

All confirmed MRSA strains showed resistance to minimum 2 antibiotics, cefoxitin and penicillin, and these resistances were not included in the multi-resistance patterns.

In pigs, MRSA strains showed resistance to a least 1 other antimicrobial (next to cefoxitin and penicillin) and were mainly resistant to 3 antimicrobial substances (27.4%). Antimicrobial resistance to trimethoprim, tetracycline and ciprofloxacin was the predominant resistance pattern. Two strains (ST398, t011) showed resistance to 10 antibiotics. Two strains showed resistance to 11 (ST398, t011) and 12 antimicrobials (different from ST398, t1985) (Figure 4).

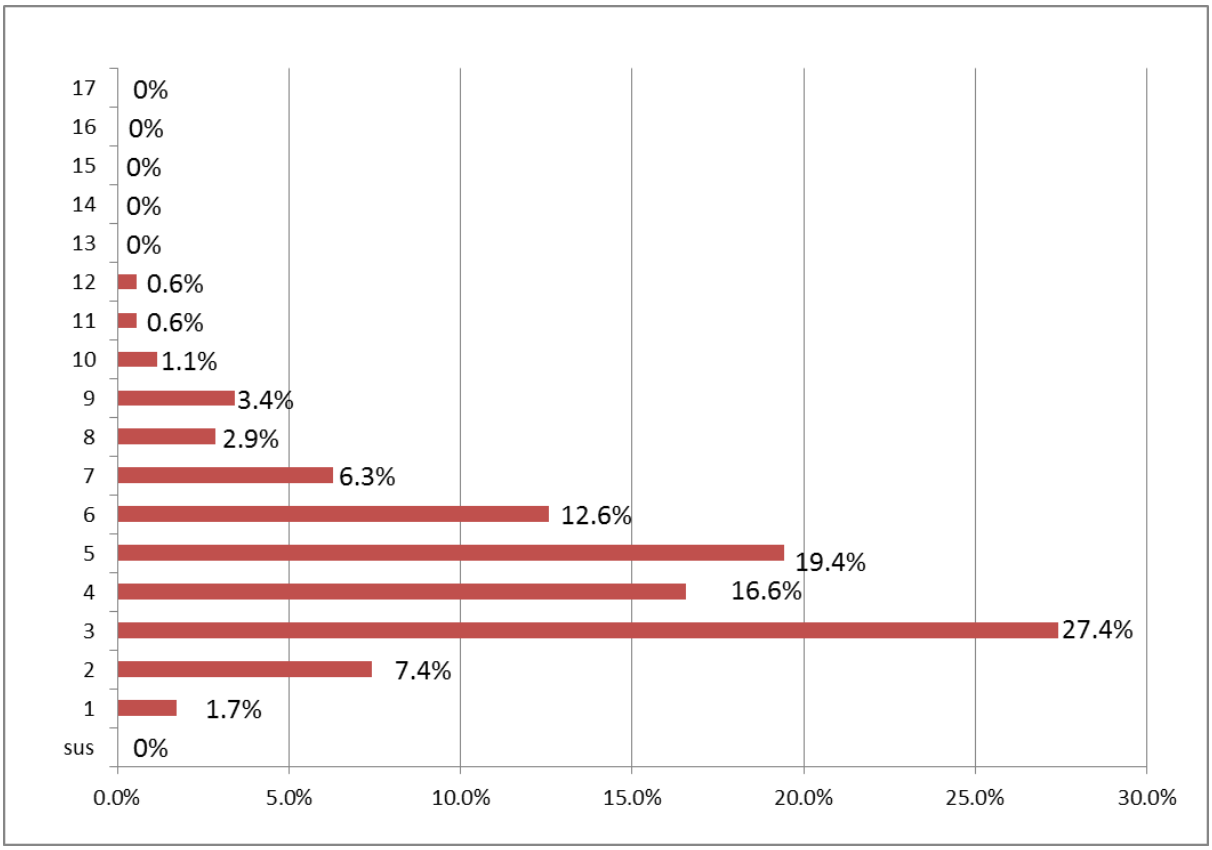

Figure 4 : Percentages of Methicillin Resistant *Staphylococcus aureus* from pigs (n= 175) showing full susceptibility (sus) or resistance to at least 1 antimicrobial. Resistance to cefoxitin and penicillin are not included.

Discussion

The MRSA prevalence in fattening pigs and sows in 2016 was 63.3% and 59.2% respectively, which is very similar to the prevalence in 2013 (overall MRSA prevalence of 65.6%) (CODA-CERVA, 2013). MRSA ST398, mainly associated with livestock animals, was the predominant sequence type. No further MLST subtyping was conducted. Therefore, sequence types classified among hospital-acquired (HA-) or community-acquired (CA-) MRSA, could not be identified. In view of reports on possible spreads of HA-MRSA to livestock, sequence typing is of critical relevance (Smith, 2015). As in MRSA collected from pigs in 2013, the main *spa*-type was t011 and all were associated with MRSA ST398. Five other less prevalent *spa*-types were recovered, associated with MRSA ST398, whereas in 2013 still 12 other spa-types were recovered from pigs. A change in *spa*-types reflects adaptations of MRSA to its host and might indicate that host adaptations are underway (Kahl et al., 2005). The new *spa*-types identified in pigs in 2016 (t037, t898 and t4659), were not solely associated to ST398. MRSA *spa*-type t037 has been shown to be associated to ST239, a dominant sequence type of HA-MRSA (http://spa.ridom.de/). This confirms the spread to livestock of MRSA originating from humans and an adaptation of the strains to an animal host.

MRSA prevalence in pigs is higher than in poultry, bovines for meat and dairy cattle (CODA-CERVA, 2014, 2015). The high level of MRSA in veal calves surpasses the MRSA presence in fattening pigs (78.2% out of 147 samples). Among MRSA isolates from pigs, antimicrobial resistance was detected for all antimicrobials tested, except for vancomycin. Antimicrobial resistance to tetracycline was common, with only three isolates susceptible to this antimicrobial. Tetracycline resistance is typically associated with LA-MRSA, belonging to sequence type ST398, and is due to the presence of the *tet*(M) gene on a chromosomally located transposon, often in combination with the plasmid-encoded *tet*(K) gene (Crombé et al., 2012; Crombé et al., 2013). Susceptibility to tetracycline in MRSA has previously been found, despite the presence of resistance genes (Verhegge et al., 2016). Antimicrobial resistance genes can be suppressed or expressed at a lower level, resulting in the absence of phenotypic (Verhegge et al., 2016). Likewise, resistance to trimethoprim is widespread by the presence of the *drf*K gene and trimethoprim susceptible strains are only very rarely found (Kadlec et al., 2012). In this monitoring study, 6 MRSA strains were found susceptible to trimethoprim. For all other antimicrobials tested, resistance of MRSA has clearly decreased compared to data from pigs in 2013 (CODA-CERVA, 2013). Although other risk factors have been described, antimicrobial use is recognized as the main selector for antimicrobial use. Cross-sectional studies estimating herd-level antimicrobial use in fattening pigs have revealed intensive antimicrobial use in these animals (Callens et al., 2012). The national data collection system Sanitel-MED, mandatory from 27th February, will provide a continued monitoring of antimicrobial usage. These data will allow to associate evolutions in antimicrobial resistance levels with antimicrobial usage patterns. For ciprofloxacin, a critically important antimicrobial for human and veterinary medicine, resistance was 52%, but decreased compared to 2013 (61.1%). Ciprofloxacin resistance was most often associated with resistance to tetracycline and trimethoprim, but co-resistance up to 12 antimicrobials was seen (β-lactam resistance of MRSA not included). Resistance to rifampicin, linezolid, mupirocin was only low and vancomycin resistance was not detected. Linezolid and vancomycin are both antimicrobials of last resort for treating *S. aureus* infections in humans and resistance to them is currently extremely rare. Mupirocin is not licensed in animals and is used for topical treatment and decolonization of MRSA in the nose of human patients (Coates et al., 2009). Cross-resistance with other antimicrobials does not occur, due to mupirocin's novel mechanism of action (Cookson, 1998), but the *Mup*A gene,

conferring mupirocin resistance, may co-transfer with other antibacterial resistance genes, i.e. tetracycline and trimethoprim (Dowling, 2013). MRSA isolated from pigs in 2014 still showed 10% resistance to mupirocin, whereas in this study resistance was only detected in two MRSA strains. Also in cattle, mupirocin resistance decreased by 10% between 2012 and 2015 (CODA-CERVA, 2015).

All MRSA strains were resistant to at least 1 other antimicrobial, in addition to the cefoxitin and penicillin resistance typically related to MRSA, and were mainly resistant to 3 antimicrobial substances. A maximum of resistance to 12 antimicrobials was seen in one MRSA strain. Antimicrobial resistance genes in LA-MRSA are often located on plasmids, resulting in multi-resistant LA-MRSA strains (Kadlec et al., 2012). The co-localization of these resistance genes with other resistance genes enables their co-selection and persistence. LA-MRSA can therefore act as a donor and a recipient of antimicrobial resistance genes within the Gram-positive gene pool.

Supplementary data

Table 4: Minimum Inhibitory Concentrations for methicillin-resistant *Staphylococcus aureus* strains (n= 175), isolated from pigs for chloramphenicol (CHL), ciprofloxacin (CIP), clindamycine (CLI), erythromycin (ERY), cefoxitin (FOX), fusidic acid (FUS), gentamicin (GEN), kanamycine (KAN), linezolid (LZD), mupirocin (MUP),penicillin (PEN), rifampicin (RIF), sulfamethoxazole (SMX), streptomycin (STR), quinupristin/dalfopristin (SYN), tetracycline (TET), tiamulin (TIA), trimethoprim (TMP) and vancomycin (VAN). Epidemiological cut-off's (ECOFFs) are indicated as straight (|) lines.

	<=0.016	<=0.03	<=0.06	<=0.12	<=0.25	<=0.5	<=1	<=2	<=4	<=8	16	32	64	128	256	512	1024	2048
CHL	-	-	-	-	-	-	-	-	7	140	20	0	6	2	-	-	-	-
CIP	-	-	-	-	47	18	19	2	8	54	27	-	-	-	-	-	-	-
CLI	-	-	-	80	9	3	1	1	0	81	-	-	-	-	-	-	-	-
ERY	-	-	-	-	31	73	1	0	0	0	70	-	-	-	-	-	-	-
FOX	-	-	-	-	-	0	0	0	9	52	92	22	-	-	-	-	-	-
FUS	-	-	-	-	-	164	4	4	33	0	-	-	-	-	-	-	-	-
GEN	-	-	-	-	-	-	126	3	11	5	12	18	-	-	-	-	-	-
KAN	-	-	-	-	-	-	-	-	128	10	2	0	6	29	-	-	-	-
LZD	-	-	-	-	-	-	58	114	1	2	0	-	-	-	-	-	-	-
MUP	-	-	-	-	-	170	3	2	0	0	0	0	0	0	0	0	-	-
PEN	-	-	-	0	0	1	0	8	166	-	-	-	-	-	-	-	-	-
RIF	171	1	1	1	1	0	0	-	-	-	-	-	-	-	-	-	-	-
SMX	-	-	-	-	-	-	-	-	-	-	-	-	150	6	2	0	17	-
STR	-	-	-	-	-	-	-	-	50	83	18	11	13	-	-	-	-	-
SYN	-	-	-	-	-	130	19	14	7	5	-	-	-	-	-	-	-	-
TET	-	-	-	-	-	3	0	0	0	0	0	172	-	-	-	-	-	-
TIA	-	-	-	-	-	124	12	0	1	38	-	-	-	-	-	-	-	-
TMP	-	-	-	-	-	-	-	6	0	0	0	0	169	-	-	-	-	-
VAN	-	-	-	-	-	-	175	0	0	0	0	0	-	-	-	-	-	-

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