Centrum voor Onderzoek in Diergeneeskunde en Agrochemie
Centre d'Etude et des Recherches Vétérinaires et Agrochimiques

Antimicrobial resistance

in *Salmonella* species

from poultry in 2016 in Belgium

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in methicillin-resistant *Staphylococcus aureus*

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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.

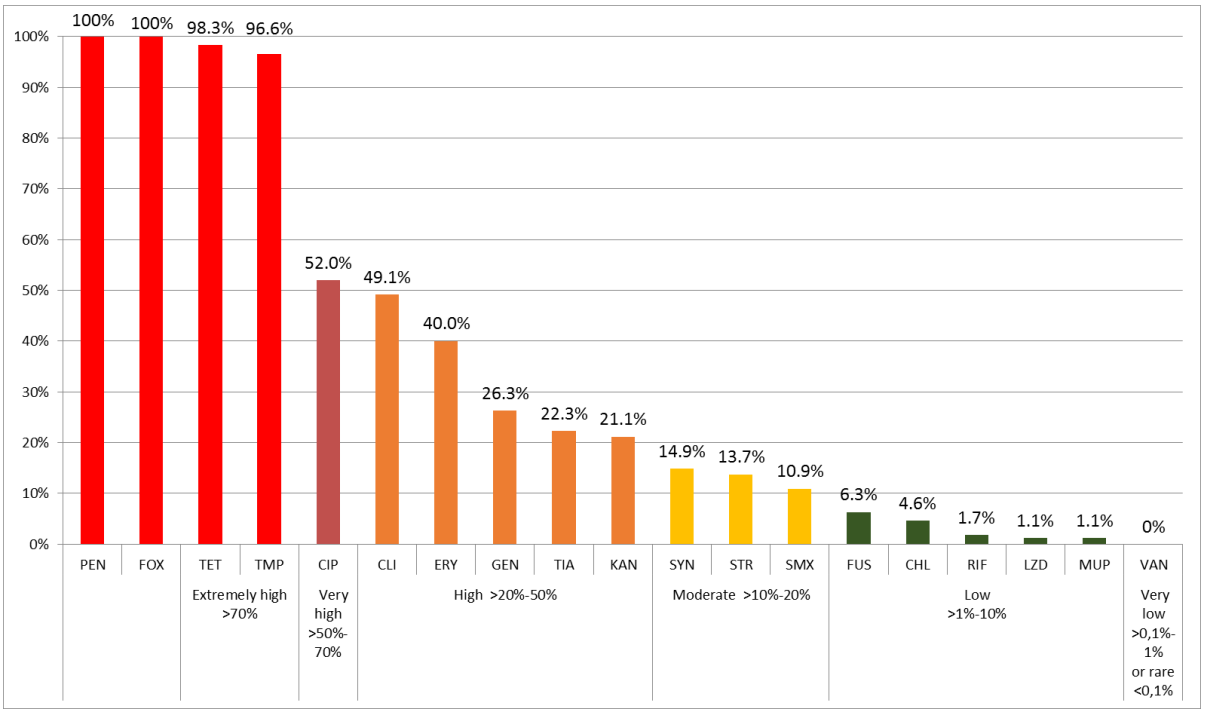

Figure 1 : Antimicrobial resistance prevalence for methicillin resistant *Staphylococcus aureus* (n= 175), isolated from pigs at the farm, based on epidemiological cut-off's, according to the European Committee on Antimicrobial Susceptibility (EUCAST) for cefoxitin (FOX), penicillin (PEN), clindamycin (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).

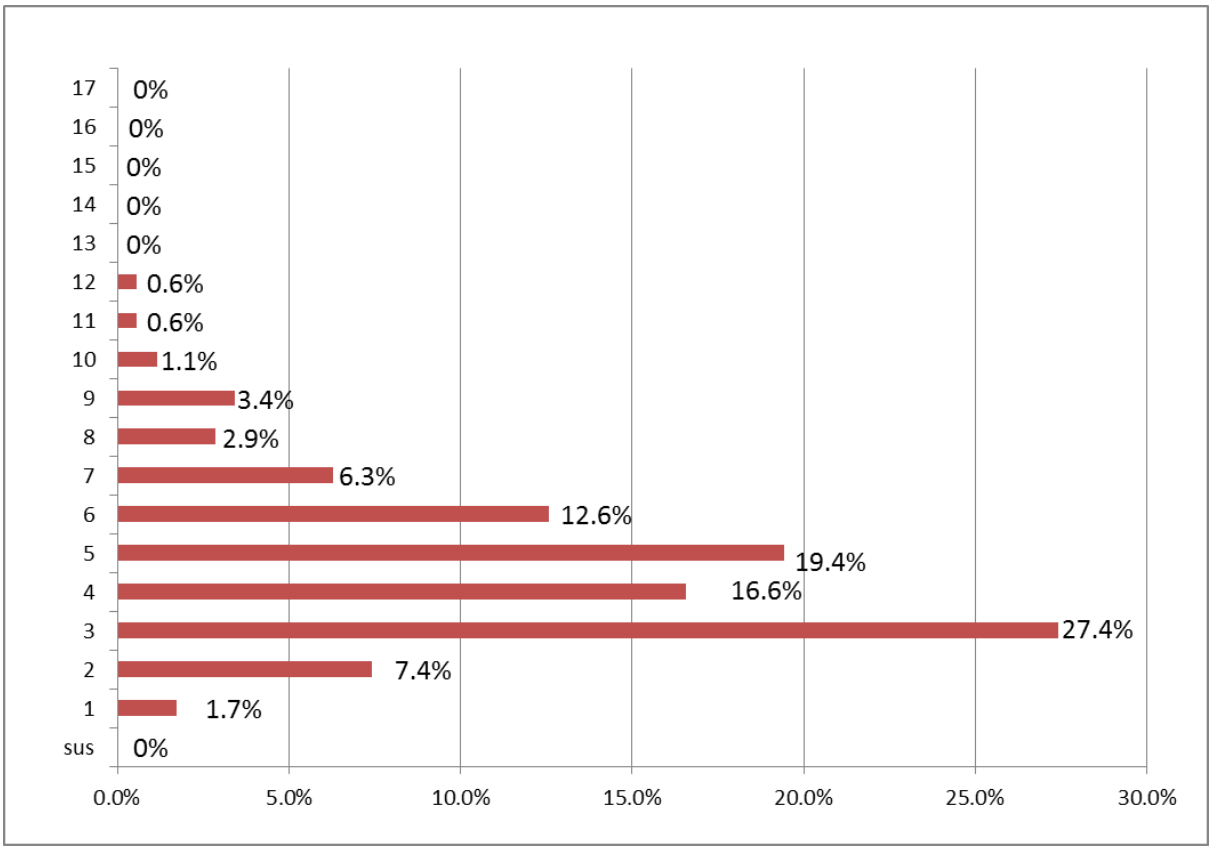

Figure 4 : Percentages of Methicillin Resistant *Staphylococcus aureus* from pigs (n= 175) showing full susceptibility (sus) or resistance to at least 1 antimicrobial. Resistance to cefoxitin and penicillin are not included.

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	-	-	-	-	-	-

References

AMCRA, 2014. Formularium voor verantwoord gebruik van antibacteriële middelen in voedselproducerende dieren. Eerste Editie versie 1.1.

Callens, B., Persoons, D., Maes, D., Laanen, M., Postma, M., Boyen, F., Haesebrouck, F., Butaye, P., Catry, B., Dewulf, J., 2012. Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. Prev Vet Med 106, 53-62.

Coates, T., Bax, R., Coates, A., 2009. Nasal decolonization of *Staphylococcus aureus* with mupirocin: Strengths, weaknesses and future prospects. J. Antimicrob. Chemother. 64, 9-15.

Crombé, F., Willems, G., Dispas, M., Hallin, M., Denis, O., Suetens, C., Gordts, B., Struelens, M., Butaye, P., 2012. Prevalence and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* among pigs in Belgium. Microb. Drug Resist. 18, 125–31.

Crombé, F., Argudín, M.A., Vanderhaeghen, W., Hermans, K., Haesebrouck, F. and Butaye, P. 2013. Transmission dynamics of methicillin-resistant *Staphylococcus aureus* in pigs. Frontiers in Microbiology, 4-57.

CODA-CERVA, 2012. MRSA surveillance 2012: Bovines. http://www.coda-cerva.be/images/stories/publications/Report%20MRSA%20cattle%20data%202012.pdf

CODA-CERVA, 2013. MRSA surveillance 2013: Bovines. http://www.coda-cerva.be/images/stories/publications/Report%20MRSA%20cattle%20data%202012.pdf

CODA-CERVA, 2013. MRSA surveillance 2013: Pigs. http://www.coda-cerva.be/images/pdf/MRSA%20surveillance%202013.pdf

CODA-CERVA, 2014. MRSA surveillance 2014: Poultry. http://www.coda-cerva.be/images/pdf/Rapport%20MRSA%202014.pdf

CODA-CERVA, 2015. Report on the occurrence of antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* from cattle in 2015 in Belgium.

Cookson, B.D., 1998. The emergence of mupirocin resistance: a challenge to infection control and antibiotic prescribing practice. J Antimicrob Chemother 41:11.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2016. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. EFSA Journal 2016;14(2):4380, 207 pp. doi:10.2903/j.efsa.2016.4380

Fluit, A.C., 2012. Livestock-associated *Staphylococcus aureus*. Clin. Microbiol. Infect. 18, 735-744.

Kadlec, K., Feßler, A.T., Hauschild, T., Schwarz, S., 2012. Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect. 18, 745-755.

Kahl, B.C., Mellmann, A., Deiwick, S., Peters, G., Harmsen, D., 2005. Variation of the Polymorphic Region X of the Protein A Gene during Persistent Airway Infection of Cystic Fibrosis Patients Reflects Two Independent Mechanisms of Genetic Change in *Staphylococcus aureus*. J. Clinical Microbiol. 43 (1), 502–505.

McNamee, P.T., Smyth, J.A., 2000. Bacterial chondronecrosis with osteomyelitis ('femoral head necrosis') of broiler chickens: a review. Avian Pathol. 29, 477–495.

Meemken, D., Blaha, T., Tegeler, R., Tenhagen, B.A., Guerra, B., Hammerl, J.A., Hertwig, S., Käsbohrer, A., Appel, B., Fetsch, A., 2010. Livestock associated methicillin-resistant *Staphylococcus aureus* (LaMRSA) isolated from lesions of pigs at necropsy in Northwest Germany Between 2004 and 2007. Zoonoses Public Health 57, e143-e148.

Smith, T.C., 2015. Livestock-Associated *Staphylococcus aureus*: The United States Experience. PLoS Pathog 11(2): e1004564.

Stegger M., Andersen P.S., Kearns A., Pichon B., Holmes M.A., Edwards G., Laurent F., Teale C., Skov R., Larsen A.R. 2011. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue *mecA*LGA251. Clinical Microbiology and Infections. 49, 732-734.

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., Butaye, P., 2010a. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet. Microbiol. 144, 166–171.

Verhegghe M., Crombé F., Luyckx K., Haesebrouck F., Butaye P., Herman L., Heyndrickx M., Rasschaert G. 2016. Prevalence and genetic diversity of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* on Belgian Pork. J Food Prot. 79 (1): 82-89.

WIV-ISP. Wetenschappelijk Instituut Volksgezondheid. Surveillance van antibioticaresistente bacteriën In Belgische ziekenhuizen: Jaarrapport 2014. http://www.nsih.be/download/MRSA/MRSA_ESBL_CPE_Y2014/RAPPORT_AMR_Y2014_NL.pdf

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Antimicrobial resistance

in *Salmonella* species

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Report on the occurrence of antimicrobial resistance in *Salmonella* species from poultry in 2016 in Belgium.

Summary

Salmonella spp. isolates were obtained in the context of the national *Salmonella* control programme in the primary production in Belgium. *Salmonella* serovar-specific data displayed large variability at the antimicrobial resistance level, with some serovars exhibiting greater resistance to certain antimicrobials or expressing multidrug resistance to a higher degree than other serovars. In *Salmonella* spp. from laying hens, antimicrobial resistance was absent, whereas in 2015, resistance to colistin was associated with *S.* Enteritidis. In broiler chickens however, antimicrobial resistance against sulfamethoxazole, ciprofloxacin, nalidixic acid, tetracycline, ampicillin and trimethoprim increased as compared to 2015. Antimicrobial resistance to colistin declined. Random sampling from non-selective culture plates didn't detect any *Salmonella spp.* resistant to cefotaxime and ceftazidime. In broiler chickens, *S.* Infantis was predominantly present and its presence even increased as compared to previous year, whereas in laying hens, *S.* Enteritidis was by far the most predominant serovar isolated. Levels of resistance were generally highest for *S.* Infantis, followed by *S.* Typhimurium isolated from broiler chickens. Multi-resistance, defined as resistance to at least three different antimicrobial classes, was seen in 50% of the *S.* Infantis strains. Co-resistance to ciprofloxacin, nalidixic acid and to sulfamethoxazole was a frequently recurring phenotypic resistance pattern. The horizontally transferable colistin resistance gene *mcr-1* or *-2* was not detected in phenotypically resistant *Salmonella* Enteritidis. No carbapenemase producing *Salmonella* were found. Clinically relevant levels of tigecycline resistance were present in 2015 in *Salmonella* from broiler chickens, but were not present this year.

Introduction

Salmonella spp. is one of the most important bacterial zoonotic agents. In spite of a decrease in the number of *Salmonella* spp. related infections, salmonellosis continues to be the second most commonly reported zoonotic disease in Belgium, as well as in the entire European Union (EU) (EFSA and ECDC, 2015). In Belgium, for instance, 3119 human cases of salmonellosis were reported in 2015 (FAVV, personal communication). Belgium has made efforts to reduce the prevalence of *Salmonella* spp. in flocks of breeder and broiler chickens, laying hens and meat turkeys by the implementation of a national control and monitoring programme. In view of its zoonotic aspect, antimicrobial susceptibility surveillance of *Salmonella* spp. is of great importance, as acquired resistance can hamper treatment of infected humans. Also, antimicrobial resistance in *Salmonella* spp. may be located on transferable elements and therefore take part in the spread of resistance to commensal and pathogenic human- and animal-related pathogens. The monitoring of zoonotic agents and its related antimicrobial resistance became mandatory for EU member states by the implementation of Directive 2003/99/EC and Regulation (EC) No 2160/2003, assuring comparability of data. The specific monitoring of AMR of isolates from layers, broilers and meat turkeys in the framework of the national *Salmonella* control programmes is laid down in Commission decision 2013/652/EU.

In this report, antimicrobial susceptibility data are presented for *Salmonella* spp. isolated from food-producing animals, more precisely from broiler chickens and laying hens. Antimicrobial susceptibility data on *Salmonella* spp. isolated from animal species other than poultry were not included in the monitoring program for 2016. Antimicrobial resistance data of *Salmonella* spp. isolated from food products from animals, as potential sources for distribution to humans via the food chain, are reported by the Institute for Public Health (WIV-ISP).

Materials and methods

Sampling

All *Salmonella* spp. isolates were obtained in the context of the national *Salmonella* control programmes organised by the Federal Agency for the Safety of the Food Chain FASFC - www.favv.be) and were analysed at CODA-CERVA (Veterinary and Agrochemical Research center, the National Reference Laboratory for antimicrobial resistance in animal productions). In addition, most *Salmonella* spp. that were isolated for diagnostic reasons or strains obtained during field research were also sent to the reference laboratory for serotyping. Most *Salmonella* spp. isolates were sent in by the regional laboratories (*Dierengezondheidszorg Vlaanderen* [www.dgz.be] and *Association Régionale de Santé et d'Identification Animales* [www.arsia.be]) and by other veterinary laboratories recognized by the FASFC which are involved in the official monitoring programmes.

Isolation and identification

Salmonella spp. was isolated at several laboratories (DGZ, ARSIA, laboratories of the Federal Food Agency, Lavetan, …) using the ISO 6579:2002/Amd1:2007 Annex D method (ISO, 2007). Serotyping was performed at CODA-CERVA (Veterinary and Agrochemical Research center, the National Reference Laboratory for antimicrobial resistance), according to the Kauffman-White-Le Minor scheme (Grimont and Weill, 2007; Guibordenche et al., 2010).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *Salmonella* spp. strains was tested using a micro broth dilution method (Trek Diagnostics). To this end, 1 to 3 colonies were suspended in sterile physiological water to an optical density of 0.5 McFarland. Ten microliter of this suspension is inoculated in 11 ml cation adjusted Mueller Hinton broth with TES buffer.

Fifty microliter of the Mueller-Hinton broth with bacteria was brought on a micro-titer plate with the antimicrobials lyophilised, produced by Trek Diagnostics, using the auto-inoculating system of Trek Diagnostics. The antimicrobial substances incorporated in the antimicrobial susceptibility testing were recommended by the European Food Safety Agency (EFSA) and included in the decision 2013/652/EU of the Commission. They were selected based on their public health relevance and as representatives of different antimicrobial classes (EFSA, 2012). Table 1 shows the antimicrobial substances tested, their abbreviations, the dilutions used and 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) (EUCAST, 2017).

Plates were incubated 18-24 hours at 35°C and read. The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration by which no visible growth could be detected. MICs were semi-automatically recorded by the Trek Vision system using the SWIN software. Results were automatically exported to an Excel file.

Table 1 : Panel of antimicrobial substances included in antimicrobial susceptibility testing, EUCAST epidemiological cut-off's (ECOFFs), and concentration ranges tested in *Salmonella* spp.

Antimicrobial (Abbreviation)	Concentration range, mg/l	*Salmonella* EUCAST ECOFF
Ampicillin (AMP)	**1–64**	**> 8**
Cefotaxime (FOT)	**0.25–4**	**> 0.5**
Ceftazidime (TAZ)	**0.5–8**	**> 2**
Meropenem (MERO)	**0.03–16**	**> 0.125**
Nalidixic acid (NAL)	**4–128**	**> 16**
Ciprofloxacin (CIP)	**0.015–8**	**> 0.064**
Tetracycline (TET)	**2–64**	**> 8**
Colistin (COL)	**1–16**	**> 2**
Gentamicin (GEN)	**0.5–32**	**> 2**
Trimethoprim (TMP)	**0.25–32**	**> 2**
Sulfamethoxazole (SMX)	**8–1024**	**NA[(a)]**
Chloramphenicol (CHL)	**8–128**	**> 16**
Azithromycin (AZI)	**2–64**	**NA[(b)]**
Tigecycline (TGC)	**0.25–8**	**> 1**

EUCAST: European Committee on Antimicrobial Susceptibility Testing

NA: not available.

(a): > 256 mg/l was used

(b): > 16 mg/l was used

The co-resistance patterns

In *Salmonella* spp. isolates, co-resistance to cefotaxime (FOT) and ciprofloxacin (CIP) was estimated, as these two antimicrobials are of particular interest in human medicine. Co-resistance was addressed using both ECOFFs (FOT > 0.5 mg/l and CIP > 0.064 mg/l) and CBPs (FOT > 2 mg/l and CIP > 0.06 mg/l) for *Salmonella* spp., established by EUCAST or as defined by the EU reference laboratory for antimicrobial resistance (DTU) (EUCAST, 2017).

Data description

In this report, an overview of antimicrobial resistance prevalence data is given for all isolated *Salmonella* serovars in broiler chickens and laying hens. Particular attention is given to the occurrence of antimicrobial resistance for selected *Salmonella* serovars of public health importance, based on the prevalence data of the Scientific Institute of Public Health (*S.* Typhimurium and its monophasic variants, *S.* Enteritidis, *S.* Kentucky, *S.* Infantis, *S.* Derby, *S.* Brandenburg, *S.* Virchow, *S.* Oranienburg, *S.* Agona and *S.* Stanley) (WIV-ISP, 2013).

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 %'.

A multi-resistant isolate is defined as resistant to at least three different antimicrobial substances, belonging to antimicrobial classes represented by the antimicrobials included in the analysis (Table 1). Resistance to nalidixic acid and resistance to ciprofloxacin, as well as the resistance cefotaxime and ceftazidime are respectively addressed together when considering multi-resistance. The frequency and percentage of isolates exhibiting multi-resistance were determined for the most prevalent *Salmonella* serovars in broiler chickens and laying hens, as well as for *Salmonella* serovars of public health importance (*S.* Typhimurium and its monophasic variants, *S.* Enteritidis, *S.* Kentucky, *S.* Infantis, *S.* Derby, *S.* Brandenburg, *S.* Virchow, *S.* Oranienburg, *S.* Agona and *S.* Stanley).

Results

Overall, 167 *Salmonella* spp. were isolated from broiler chickens (n= 127) and laying hens (n= 39). A summary of the *Salmonella* serotyping results is presented for broiler chickens (Table 2) and laying hens (Figure 4). Antimicrobial resistance profiles varied considerably among animal categories and among recovered *Salmonella* serovars (Table 2).

Broiler chickens

Antimicrobial resistance

Antimicrobial resistance prevalences for *Salmonella* spp. from broiler chickens (n= 127) are represented in Figure 1. Highest levels of resistance were reported for sulfamethoxazole, ciprofloxacin, nalidixic acid, tetracycline, ampicillin and trimethoprim. Antimicrobial resistance to these antimicrobials increased compared to 2015. Only low levels were seen for chloramphenicol, gentamicin and colistin. Antimicrobial resistance to colistin clearly declined compared to previous year. *Salmonella* spp. was fully susceptible to azithromycin, cefotaxime, meropenem, ceftazidime and tigecycline.

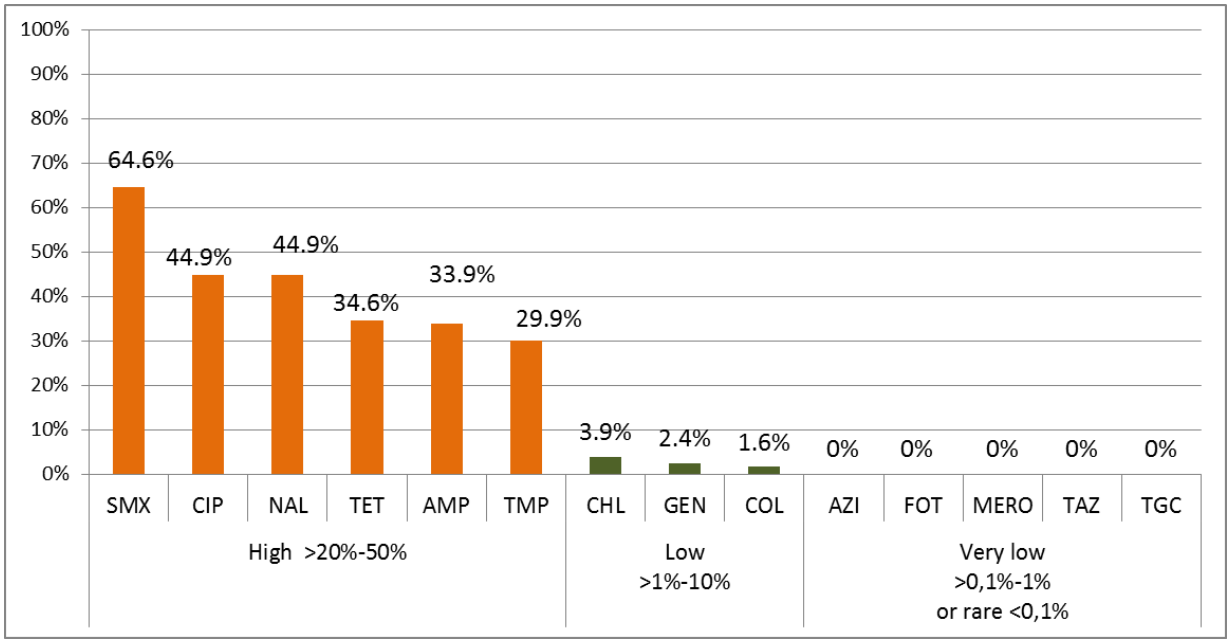


Figure 1 : Antimicrobial resistance prevalence for *Salmonella* spp. (n= 127), isolated from broiler chickens, based on epidemiological cut-off's, according to the European Committee on Antimicrobial Susceptibility (EUCAST) for ampicillin (AMP), azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), cefotaxime (FOT), gentamicin (GEN), meropenem (MERO), nalidixic acid (NAL), sulfamethoxazole (SMX), ceftazidime (TAZ), tetracycline (TET), tigecycline (TGC) and trimethoprim (TMP).

In broiler chickens, 26.0% of *Salmonella* spp. were fully susceptible and 40.9% showed multi-resistance (resistance to at least three different antimicrobial classes). There is clearly less full susceptibility and more multi-resistance than the previous year (47.0% full susceptibility and 20.9% multi-resistance). Resistance to 2 antimicrobials was most frequently observed (26.0%), followed by multi-resistance to 3 different antimicrobial classes (17.3%) (Figures 2 and 3). Resistance to 4 or 5 antimicrobial classes was present in 13.4% and 10.2% respectively, represented by *S.* Infantis, *S.* Typhimurium O5- and O5+, *S*. Paratyphi B, and *S.* 4,12:i:-.

The relative contribution of different *Salmonella* serovars in the total number of *Salmonella* spp. strains isolated from broiler chickens and their antimicrobial resistance prevalence can be found in Table 2. *S.* Infantis was predominantly present in broiler chickens (n= 52, 40.9%). Its presence has strongly increased since previous year (n= 27, 20.1%). *S.* Infantis was only fully susceptible in 3 out of the 52 strains (5.8%). Antimicrobial resistance to ampicillin, tetracycline and trimethoprim was higher than in 2015. Tigecycline resistance was no longer present, whereas 3 *S.* Infantis strains were

resistant to tigecycline in 2015. Antimicrobial resistance to 2 different antimicrobial classes occurred most frequently (n= 22, 42.3%). Co-resistance to ciprofloxacin, nalidixic acid and to sulfamethoxazole was a recurring phenotypic resistance pattern (22 out of 52 *S.* Infantis strains) and resistance to these antimicrobials was higher than previous year. Multi-resistance to 3, 4 or 5 different classes was seen in 19.2%, 7.7% and 23.1% of the *S.* Infantis strains (Figures 2 and 3). Co-resistance to sulfamethoxazole, tetracycline, quinolones, trimethoprim and ampicillin was present.

S. Typhimurium O5- and O5+ (n= 17, 13.4%) and *S.* Gaminara (n= 14, 11.0%) were also frequently isolated (Table 2). Other *Salmonella* serovars of public health importance were prevalent as follows: *S.* Typhimurium O5+ (n= 6, 4.7%) and its monophasic variant (n= 5, 3.9%), *S.* Agona (n=2, 1.6%), *S.* Enteritidis (n= 2, 1.6%) and *S.* Derby (n= 3, 2.4%). *S.* Brandenburg, *S.* Kentucky, *S.* Oranienburg, *S.* Stanley and *S.* Virchow were not isolated.

Table 2 : Antimicrobial resistance prevalence for *Salmonella* serovars isolated from broiler chickens (n= 127), based on epidemiological cut-off's, according to the European Committee on Antimicrobial Susceptibility (EUCAST) for ampicillin (AMP), azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), cefotaxime (FOT), gentamicin (GEN), meropenem (MERO), nalidixic acid (NAL), sulfamethoxazole (SMX), ceftazidime (TAZ), tetracycline (TET), tigecycline (TGC) and trimethoprim (TMP).

Salmonella spp. n= 127 (%)	AMP	AZI	CHL	CIP	COL	FOT	GEN	MERO	NAL	SMX	TAZ	TET	TGC	TMP
***S.* Infantis 52 (40.9%)**	15 28.9%	0 0%	0 0.0%	48 92.3%	0 0%	0 0%	0 0.0%	0 0.0%	48 92.3%	48 92.3%	0 0%	24 46.2%	0 0%	16 30.8%
***S.* Typhimurim O5- and O5+ 23 (18.1%)**	18 78.3%	0 0.0%	5 21.7%	6 26.1%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	6 26.1%	14 60.9%	0 0.0%	13 56.5%	0 0.0%	10 52.2%
***S.* Gaminara 14 (11.0%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 7.1%	0 0.0%	0 0.0%	2 14.3%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Paratyphi B 6 (4.7%)**	1 16.7%	0 0.0%	0 0.0%	1 16.7%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 16.7%	6 100%	0 0.0%	0 0.0%	0 0.0%	6 100%
***S.* Livingstone 5 (3.9%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* 4,5,12:i:- 5 (3.9%)**	5 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	5 100%	0 0.0%	5 100%	0 0.0%	0 0.0%
***S.* Mbandaka 4 (3.1%)**	0 0.0%	0 0.0%	0 0.0%	1 25.0%	0 0.0%	0 0.0%	2 50.0%	0 0.0%	1 25.0%	2 50.0%	0 0.0%	1 25.0%	0 0.0%	0 0.0%
***S.* Derby 3 (2.4%)**	3 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	3 100%	0 0.0%	0 0.0%	0 0.0%	3 100%
***S.* Agona 2 (1.6%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 50.0%	0 0.0%	1 50.0%	0 0.0%	0 0.0%
***S.* Enteritidis 2 (1.6%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	2 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Rissen 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Senftenberg 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	1 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Colorado 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Idikan 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Indiana 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Kottbus 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* Llandof 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%
***S.* 4,12:i:- 1 (0.8%)**	1 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 100%	0 0.0%	1 100%	0 0.0%	1 100%
***S.* 6,7:z29r:- 1 (0.8%)**	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%

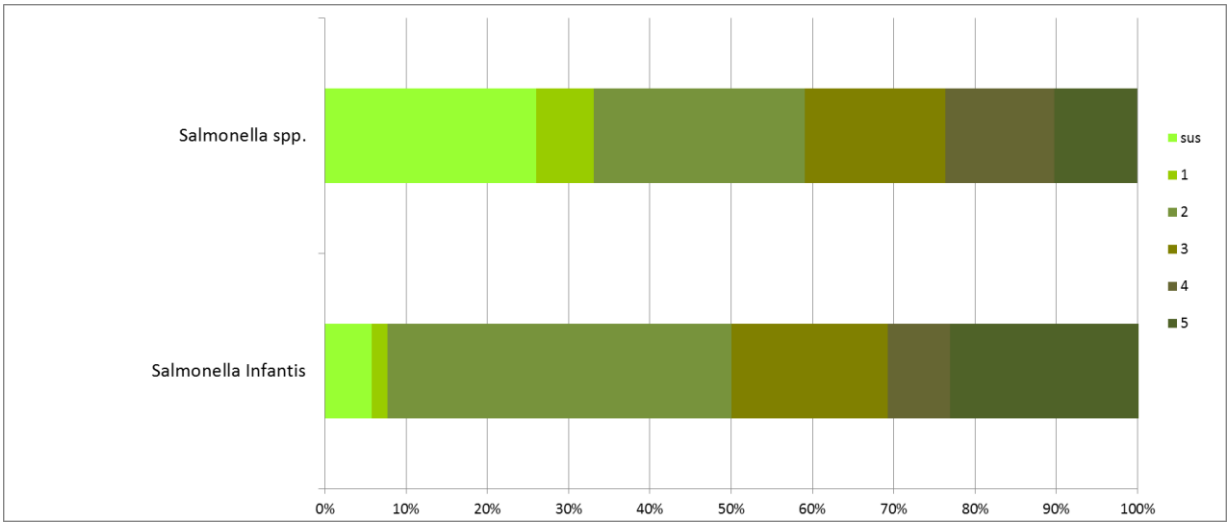


Figure 2 : Percentages of *Salmonella* serovars (n= 127) and *S.* Infantis (n= 52) from broiler chickens showing full susceptibility ("sus") or resistance to at least 1 antimicrobial. Resistance to nalidixic acid and resistance to ciprofloxacin, as well as the resistance to cefotaxime and ceftazidime are respectively addressed together.

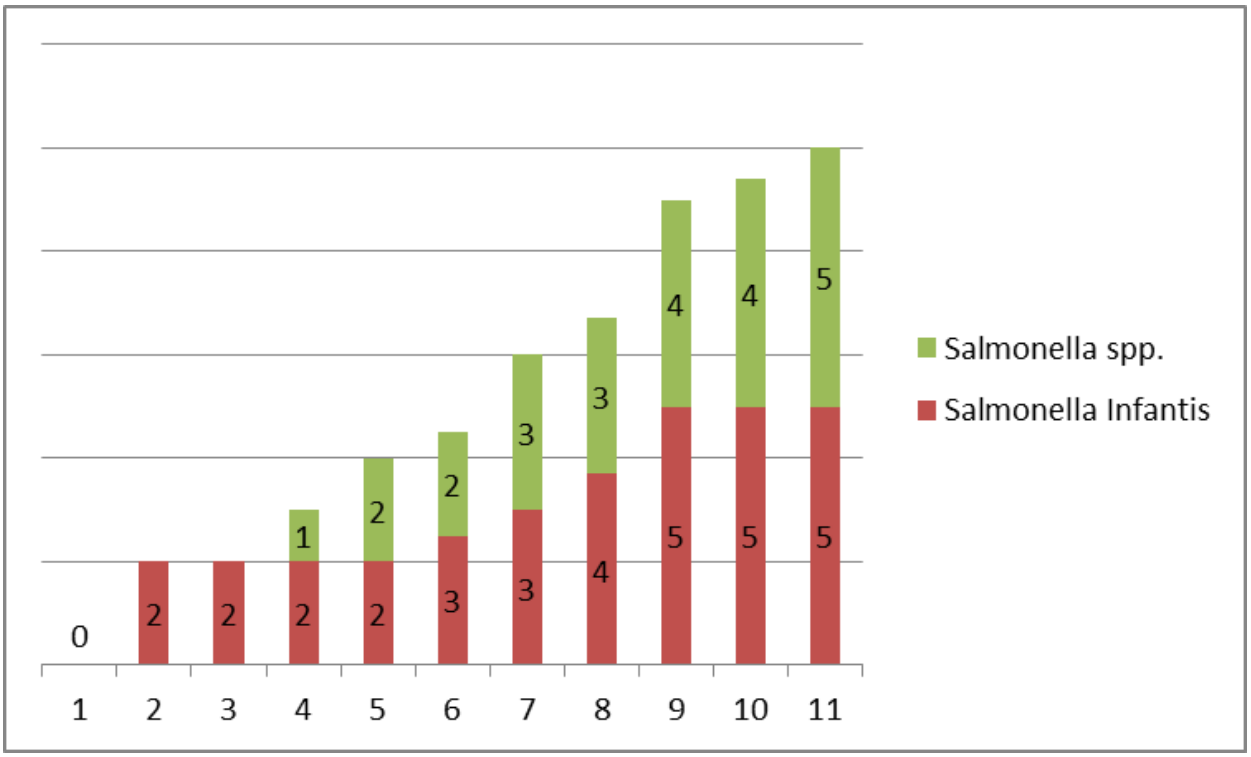


Figure 3 : Percentiles of all *Salmonella* serovars (n= 127) and *S.* Infantis (n= 52) from broiler chickens showing full susceptibility ("sus") or resistance to at least 1 antimicrobial. Resistance to nalidixic acid and resistance to ciprofloxacin, as well as the resistance to cefotaxime and ceftazidime are respectively addressed together.

Co-resistance to cefotaxime and ciprofloxacin in* Salmonella *spp.

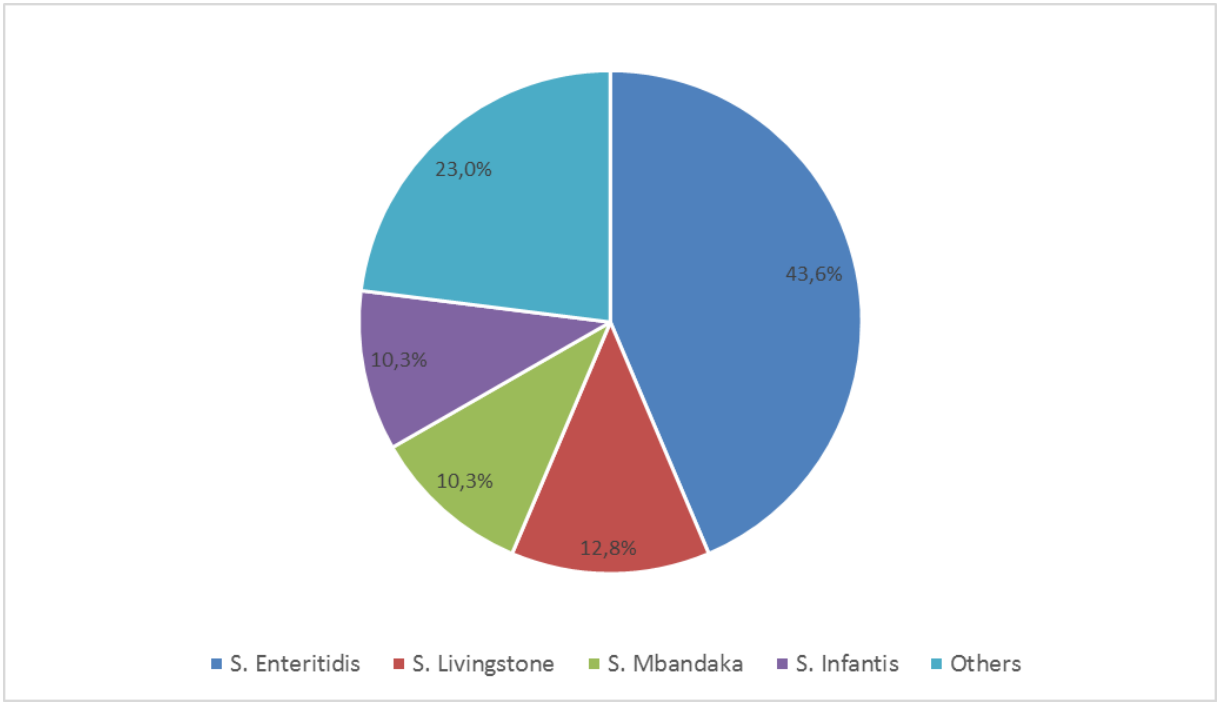
None of the *Salmonella* spp. strains showed co-resistance to cefotaxime and ciprofloxacin, based on epidemiological cut-off values or clinical breakpoints.

Laying hens

Antimicrobial resistance

In laying hens, all 39 *Salmonella* spp. strains were fully susceptible. In 2015, still 40.0% of the *Salmonella* isolates showed colistin resistance, which could almost entirely be attributed to *Salmonella* Enteritidis. Antimicrobial resistance to ciprofloxacin and nalidixic acid was found in one strain in 2015, but was also absent this year.

The relative contribution of different *Salmonella* serovars in the total number of *Salmonella* spp. strains isolated from laying hens can be found in Figure 4. *S.* Enteritidis was by far the most predominant serovar isolated from laying hens (n= 17, 43.6%), whereas other serovars were less frequently isolated. Other *Salmonella* serovars of public health importance were prevalent as follows: *S.* Infantis (n= 4, 10.3%). *S.* Agona, *S.* Brandenburg, *S.* Derby, *S.* Typhimurium and its monophasic variant, *S.* Kentucky, *S.* Oranienburg, *S.* Stanley and *S.* Virchow were not isolated.



Figuur 4 : The relative contribution of the *Salmonella* serovars isolated from laying hens (total number of strains= 39).

Co-resistance to cefotaxime and ciprofloxacin in *Salmonella* spp.

None of the *Salmonella* spp. strains showed co-resistance to cefotaxime and ciprofloxacin, based on epidemiological cut-off values or clinical breakpoints.

Discussion

The monitoring of *Salmonella* spp. prevalence in food-producing animals, potential sources of human salmonellosis, is a mandatory programme established by the European Commission. Temporal trends in the occurrence of *Salmonella* spp. in food-producing animals can be consulted in the annual reports of CODA-CERVA.

Salmonella serovar-specific data displayed large variability at the antimicrobial resistance level, with some serovars exhibiting greater resistance to certain antimicrobials or expressing multidrug resistance to a higher degree than other serovars. For some serovars only low numbers were isolated and serovar-specific prevalence of antibiotic resistance should therefore be nuanced.

Antimicrobial resistance in *Salmonella* spp. showed higher levels for broiler chickens compared to 2015, whereas for laying hens, all *Salmonella* spp. strains were fully susceptible. This is remarkable as for the laying hens, in 2015, considerable resistance to colistin in *S.* Enteritidis was present. *S.* Enteritidis has been reported with an intrinsic lower susceptibility towards colistin of an unknown genetic nature (Agersø et al., 2012). Indeed, in 2015, *S.* Enteritidis from broiler chickens and laying hens showed increased MIC values of 4 or 8 µg/ml (epidemiological cut-off value = 2 µg/ml) (CODA-CERVA, 2016). Also, this year, the two *S.* Enteritidis strains from broiler chickens displayed decreased susceptibility to colistin (MIC= 4 µg/ml), but MIC values ≥ 4 µg/ml for colistin were absent in *S.* Enteritidis from laying hens. No other serovars displayed colistin resistance. Until recently, colistin resistance was only described as the consequence of chromosomally located mutations. The existence of horizontally transferable resistance genes (*mcr-1* and *mcr-2*) have now globally been reported in bacteria from animals, food and humans (Arcilla et al., 2015; Hasman et al., 2015; Liu et al., 2015; Webb et al., 2015, Xavier et al., 2016). In Belgium, both *mcr-1* and *mcr-2* have been found in commensal and pathogenic *Escherichia coli* isolated from food-producing animals (Callens et al., 2016; Malhotra-Kumar et al., 2016; Xavier et al., 2016; CODA-CERVA, 2017), and in *Salmonella enterica* strains from chicken meat (Nadine Botteldoorn, WIV-ISP, personal communication). Although human salmonellosis is most frequently treated with fluoroquinolones, the presence of transferable colistin resistance mechanisms in some *Salmonella* spp. might represent a risk as this resistance might be transferred to other commensal or pathogenic bacteria in humans.

A limited antimicrobial usage in laying hens has been reported (van Hoorebeke et al., 2011). Antimicrobial resistance data from indicator bacterium *E. coli* isolated from laying hens can confirm the low antimicrobial selection pressure in the laying hen sector, yet no data are available for Belgium. In broiler chickens, however, antimicrobial resistance levels in *E. coli* are yearly monitored as part of the national monitoring program in food-producing animals. In 2016, only 8.4% of the strains were fully susceptible (CODA-CERVA, 2017). It has been estimated that on average, in broiler chickens, antimicrobials are being administered during 12% of their life (Persoons et al., 2012). Highest levels of antimicrobial resistance are seen for sulfamethoxazole, ampicillin, trimethoprim, tetracycline, ciprofloxacin and nalidixic acid in both *Salmonella* spp. and *E. coli* from broiler chickens (CODA-CERVA, 2017). Even higher levels of antimicrobial resistance to sulfamethoxazole, ampicillin, trimethoprim, tetracycline are present compared to previous year (CODA-CERVA, 2016). The

abovementioned antimicrobials are frequently listed as first and second choice in the treatment of poultry-associated diseases (AMCRA, 2014). Also, these antimicrobials belong to the most used classes of antimicrobials in animals in 2014 (BelVet-SAC, 2015). Results from the national data collection system for food-producing animals, i.e. poultry, mandatory from the 27th of February 2017, will provide insight in the antimicrobial usage patterns in broiler chicken and laying hen farms. The relation between antimicrobial use and resistance will therefore be accurately investigated in the coming years.

Resistance to ciprofloxacin and nalidixic acid, both quinolones, is frequently present in *Salmonella* spp. isolated from broiler chickens (44.9% and 44.9% respectively), and almost doubled compared to previous year. It should be noted that *S.* Infantis, the serovar highly responsible for the presence of quinolone resistance, was more frequently isolated than previous year (20.1% and 40.9% in 2015 and 2016 respectively) (CODA-CERVA, 2016). Nevertheless, in 2016, more *S.* Infantis strains were found resistant to ciprofloxacin and nalidixic acid (85.2% and 92.3% in 2015 and 2016 respectively). In *E. coli* from broiler chickens, quinolone resistance is also very high (57.5% and 48.5% to ciprofloxacin and nalidixic acid respectively), but the levels of antimicrobial resistance decreased as compared to 2015 (CODA-CERVA, 2017). Development of resistance to fluoroquinolones occurs mainly by mutations, yet, plasmid mediated quinolone resistance (pMQR) has emerged. Overall, ciprofloxacin resistance coincided with nalidixic acid resistance in the tested *Salmonella* spp. strains, indicating absence of pMQR in these strains (Strahilevitz et al., 2009). In Belgium, fluoroquinolones, and more precisely enrofloxacine and flumequine, are being widely used in poultry for the treatment of several infections, e.g. colibacillosis, and mycoplasmosis (Persoons et al., 2012). From the 21st of July 2016, a new royal decree drastically restricts the use of fluoroquinolones and 3rd and 4th generation cephalosporins in food-producing animals in Belgium. Its implementation should discourage the use of fluoroquinolones in food-producing animals in Belgium and will hopefully result in a decreased selection and spread of quinolone resistant strains. In the Netherlands, antimicrobial resistance to fluoroquinolones has dramatically decreased since their restricted use (Dorado-Garcia et al., 2016). Resistance to ciprofloxacin and nalidixic acid was observed in many multi-resistance patterns, most frequently in *S.* Infantis and in combination with sulfamethoxazole, indicating clonal expansion of some particular *S.* Infantis lineages.

Other serovars, such as *S.* Enteritidis, *S.* Typhimurium, *S.* Derby and *S.* Paratyphi B variant Java, serovars possibly involved in human salmonellosis, were also found resistant to various extents. Yet, given the relative low number of these serovars, correct estimation of the quinolone resistance prevalence in these serovars is elusive. Fluoroquinolones are widely regarded as the treatment of choice for severe salmonellosis in humans. Fluoroquinolone resistance is therefore a serious threat for the successful treatment of salmonellosis. No high-level resistance (MIC> 4 mg/l) was seen in *Salmonella* spp. from poultry. Yet, the European Committee on Antimicrobial Susceptibility (EUCAST) recommends a clinical breakpoint of 0.06 mg/l as there is clinical evidence for a poor response to ciprofloxacin in systemic infections caused by *Salmonella* spp. displaying low levels of resistance (MIC> 0.06 mg/l) (EUCAST, 2016). Ciprofloxacin-resistant *Salmonella* spp. strains from poultry would therefore be difficult to treat with fluoroquinolones when infecting humans.

As for fluoroquinolones, 3rd generation cephalosporins are effective and critically important for treating human salmonellosis. In poultry, cephalosporins are not licensed, although ceftiofur has been used off-label in one-day-old chickens at the hatchery, resulting in high ceftiofur resistance in commensal *E. coli* from broiler chickens (Persoons et al., 2010). In general, ESBLs and/or AmpC are

less prevalent in *Salmonella* spp. than in *E. coli*. Yet, cephalosporin resistance has been reported to increase in *S.* Parathypi B variant Java from broilers in Belgium, the Netherlands and Germany (Doublet et al., 2014). The specific detection of ESBL- and/or AmpC-producing *Salmonella* spp. was not in the scope of the national monitoring study on antimicrobial resistance of *Salmonella* spp. The random sampling from non-selective culture plates didn't detect any *Salmonella spp.* resistant to cefotaxime and ceftazidime in broiler chickens and laying hens. In 2015, only one *S.* Typhimurium was found cephalosporin resistant (CODA-CERVA, 2016). In *Salmonella* spp. from poultry meat cephalosporin resistance was not detected in 2015, yet 7 ESBL-producing *Salmonella* spp. out of 176 (3.97%) *Salmonella* strains were found on chicken carcasses in 2016 (Garcia-Graells, Cristina, WIV-ISP, personal communication). Different outcomes in antimicrobial resistance in bacteria from living animals and carcasses are indicative for cross-contamination with human sources or slaughterhouse equipment.

For the third year in a row, antimicrobials considered as last resort for treatment of extremely antimicrobial resistant isolates in humans, were tested for their antibacterial activity on *Salmonella* spp., i.e. azithromycin, meropenem and tigecycline.

Absence of meropenem resistance indicated that carbapenemase producers were not present in the tested *Salmonella* isolates. Carbapenems is a class of antimicrobials not used in food-producing animals, but reserved for humans. Yet, carbapenemase-producing *S. enterica* have been found in broiler chicken farms in Germany (Fisher et al., 2013; Poirel et al., 2012). In Belgium, carbapenem resistance has been reported in *Acinetobacter* spp. from horses (Smet et al., 2012) and in one commensal *E. coli* strain from pig meat (EFSA and ECDC, 2017).

Tigecycline, structurally related to tetracycline, but with a broader spectrum of activity, is used as a last resort molecule in the treatment of ESBL-infected patients in human medicine. It has no veterinary equivalent; and in contrast to previous year, clinically relevant resistance (clinical breakpoint is 2 mg/l), was not detected in *Salmonella* spp. from broiler chickens.

Supplementary data

Table 1: Minimum Inhibitory Concentrations for *Salmonella* spp. strains (n= 127), isolated from broiler chickens, using non-selective media for ampicillin (AMP), azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), cefotaxime (FOT), gentamicin (GEN), meropenem (MERO), nalidixic acid (NAL), sulfomethoxazole (SMX), ceftazidime (TAZ), tetracycline (TET), tigecycline (TGC) and trimethoprim (TMP). Epidemiological cut-off's (ECOFFs) are indicated as straight lines (|).

	<=0.008	<=0.015	<=0.03	<=0.06	<=0.12	<=0.25	<=0.5	<=1	<=2	<=4	<=8	16	32	64	128	256	512	1024	2048
AMP	-	-	-	-	-	-	-	34	39	11	0	0	0	43	-	-	-	-	-
AZI	-	-	-	-	-	-	-	-	4	46	62	15	0	0	0	-	-	-	-
CHL	-	-	-	-	-	-	-	-	-	-	100	22	0	0	0	5	-	-	-
CIP	-	54	16	0	5	17	24	10	1	0	0	0	-	-	-	-	-	-	-
COL	-	-	-	-	-	-	-	125	0	2	0	-	-	-	-	-	-	-	-
FOT	-	-	-	-	-	125	2	0	0	0	0	-	-	-	-	-	-	-	-
GEN	-	-	-	-	-	-	123	0	1	0	0	2	1	0	-	-	-	-	-
MERO	-	-	123	4	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-
NAL	-	-	-	-	-	-	-	-	-	70	0	0	0	0	13	44	-	-	-
SMX	-	-	-	-	-	-	-	-	-	-	2	1	15	25	2	0	0	0	82
TAZ	-	-	-	-	-	-	121	6	0	0	0	0	-	-	-	-	-	-	-
TET	-	-	-	-	-	-	-	-	75	8	0	0		3	41	-	-	-	-
TGC	-	-	-	-	-	69	31	27	0	0	0	-	-	-	-	-	-	-	-
TMP	-	-	-	-	-	59	29	1	0	0	0	0	0	38	-	-	-	-	-

Table 2: Minimum Inhibitory Concentrations for *Salmonella* spp. strains (n= 39), isolated from laying hens, using non-selective media for ampicillin (AMP), azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), cefotaxime (FOT), gentamicin (GEN), meropenem (MERO), nalidixic acid (NAL), sulfomethoxazole (SMX), ceftazidime (TAZ), tetracycline (TET), tigecycline (TGC) and trimethoprim (TMP). Epidemiological cut-off's (ECOFFs) are indicated as straight lines (|).

	<=0.008	<=0.015	<=0.03	<=0.06	<=0.12	<=0.25	<=0.5	<=1	<=2	<=4	<=8	16	32	64	128	256	512	1024	2048
AMP	-	-	-	-	-	-	-	32	7	0	0	0	0	0	-	-	-	-	-
AZI	-	-	-	-	-	-	-	-	1	26	12	0	0	0	0	-	-	-	-
CHL	-	-	-	-	-	-	-	-	-	-	39	0	0	0	0	0	-	-	-
CIP	-	31	7	1	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-
COL	-	-	-	-	-	-	-	34	5	0	0	-	-	-	-	-	-	-	-
FOT	-	-	-	-	-	39	0	0	0	0	0	-	-	-	-	-	-	-	-
GEN	-	-	-	-	-	-	38	1	0	0	0	0	0	0	-	-	-	-	-
MERO	-	-	38	1	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-
NAL	-	-	-	-	-	-	-	-	-	39	0	0	0	0	0	0	-	-	-
SMX	-	-	-	-	-	-	-	-	-	-	0	0	4	26	9	0	0	0	0
TAZ	-	-	-	-	-	-	39	0	0	0	0	0	-	-	-	-	-	-	-
TET	-	-	-	-	-	-	-	-	39	0	0	0	0	0	0	-	-	-	-
TGC	-	-	-	-	-	38	1	0	0	0	0	-	-	-	-	-	-	-	-
TMP	-	-	-	-	-	33	6	0	0	0	0	0	0	0	-	-	-	-	-

References

Agersø, Y., Torpdahl, M., Zachariasen, C., Seyfarth, A., Hammerum, A.M., Nielsen, E.M., 2012. Tentative colistin epidemiological cut-off value for *Salmonella* spp. Foodborne Pathog. Dis. 9, 367–9.

AMCRA, 2014. Formularium voor verantwoord gebruik van antibacteriële middelen in voedselproducerende dieren. Eerste Editie versie 1.1.

BelVet-SAC. Belgian Veterinary Surveillance of Antibacterial Consumption, 2015. National consumption report 2014. Available online: http://www.belvetsac.ugent.be/pages/home/BelvetSAC_report_2014%20finaal.pdf

Callens, B., Haesebrouck, F., Dewulf, J., Boyen, F., Butaye, P., Catr, B., Wattiau, P., De Graef, E., 2016. Risk of colistin resistance on the rise. Vlaams Diergeneeskundig Tijdschrift 85, 36-40.

CODA-CERVA, 2016. Report on the occurrence of antimicrobial resistance in *Salmonella* species from poultry in 2015 in Belgium. http://www.coda-cerva.be/images/pdf/Report%20on%20the%20occurrence%20of%20antimicrobial%20resistance%20in%20Salmonella%20species%20from%20poultry%20in%202015%20in%20Belgium%20-%20finaal.pdf

CODA-CERVA, 2017. Report on the occurrence of antimicrobial resistance in Escherichia coli from food-prouding animals in 2016. http://www.coda-cerva.be/index.php?option=com_content&view=article&id=121&Itemid=286&lang=nl

Dorado-Garcıa, A., Mevius, D.J., Jacobs, J.J.H., Van Geijlswijk, I.M., Mouton, J.W., Wagenaar, J.A., Heederik, D.J., 2016. Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. J Antimicrob Chemother. 71(12): 3607-3619.

Doublet, B., Praud, K., Nguyen-Ho-Bao, T., Argud??n, M.A., Bertrand, S., Butaye, P., Cloeckaert, A., 2014. Extended spectrum β-lactamase-and ampc β-lactamase-producing D-tartrate-positive *Salmonella enterica* serovar Paratyphi B from broilers and human patients in Belgium, 2008-10. J. Antimicrob. Chemother. 69, 1257–1264.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2015. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic agents and Food-borne Outbreaks in 2014. EFSA Journal 2015;13(12):4329, 191 pp., doi:10.2903/j.efsa.2015.4329

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2017. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015. EFSA Journal 2017;15(2):4694, 212 pp. doi:10.2903/j.efsa.2017.4694

EUCAST. European Committee on Antimicrobial Susceptibility Testing, 2017. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, 2017. http://www.eucast.org

Fischer, J., Rodrìguez, I., Schmoger, S., Friese, A., Roesler, U., Helmuth, R., Guerra, B., 2013. *Salmonella enterica* subsp. *enterica* producing VIM-1 carbapenemase isolated from livestock farms. J. Antimicrob. Chemother.

Guibourdenche M., Roggentin P., Mikoleit M., Fields P.I., Bockemuhl J., Grimont P.A. and Weill F.X. (2010) Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. Research in Microbiology. 161(1):26-29.

ISO (International Organization for Standardization) (2007) Detection of *Salmonella* spp. in animal feces and in environmental samples from the primary production stage. ISO 6579:2002/Amd1:2007, References 163 Annex D. Available online at http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=42109

Malhotra-Kumar, S., Xavier, B.B., Das, A.J., Lammens, C., Butaye, P., Goossens, H., 2016. Colistin resistance gene *mcr-1* harboured on a multidrug resistant plasmid. Lancet Infect. Dis. 3099, 7–8.

Persoons, D., Dewulf, J., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Butaye, P., Haesebrouck, F., 2010. Prevalence and persistence of antimicrobial resistance in broiler indicator bacteria. Microb. Drug Resist. 16, 67–74.

Persoons, D., Dewulf, J., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Butaye, P., Haesebrouck, F., 2012. Antimicrobial use in Belgian broiler production. Prev. Vet. Med. 105, 320–325.

Poirel, L., Berçot, B., Millemann, Y., Bonnin, R. a., Pannaux, G., Nordmann, and P., 2012. Carbapenemase- producing *Acinetobacter spp* . in Cattle , France. Emerg. Infect. Dis. 18, 523–525.

Smet, A., Boyen, F., Pasmans, F., Butaye, P., Martens, A., Nemec, A., Deschaght, P., Vaneechoutte, M., Haesebrouck, F., 2012. OXA-23-producing *Acinetobacter* species from horses: A public health hazard? J. Antimicrob. Chemother. 67, 3009–3010.

Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 22, 664-689.

van Hoorebeke, S., van Immerseel, F., Berge, A.C., Persoons, D., Schulz, J., Hartung, J., Harisberger, M., Regula, G., Barco, L., Ricci, A., de Vylder, J., Ducatelle, R., Haesebrouck, F., Dewulf, J., 2011. Antimicrobial resistance of *Escherichia coli* and *Enterococcus faecalis* in housed laying-hen flocks in Europe. Epidemiol. Infect. 139, 1610–20.

WIV-ISP. 2013. Jaarverslag. *Salmonella* en *Shigella* stammen gerapporteerd tijdens 2013 in België. http://bacterio.iph.fgov.be/reporting/reportspdf/2013-rapport%20Salmonella%20NL-versfinalcourverture.pdf

Xavier, B.B., Lammens, C., Ruhal, R., Kumar-Singh, S., Butaye, P., Goossens, H., Malhotra-Kumar, S. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr- 2*, in *Escherichia coli*, Belgium, June 2016. Euro Surveill. 2016;21(27):pii=30280.