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Antimicrobial susceptibility of nine udder pathogens recovered from bovine clinical mastitis milk in Europe 2015–2016: VetPath results

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ABSTRACT

VetPath is an ongoing pan-European antimicrobial susceptibility monitoring programme collecting pathogens from diseased cattle, pigs and poultry not recently treated with antibiotics. Non-duplicate isolates (n = 1244) were obtained from cows with acute clinical mastitis in eight countries during 2015–2016 for centrally antimicrobial susceptibility testing according CLSI standards.

Among *Escherichia coli* (n = 225), resistance was high to ampicillin and tetracycline, moderate to kanamycin and low to amoxicillin/clavulanic acid and cefazolin. The $MIC_{50/90}$ of danofloxacin, enrofloxacin and marbofloxacin were 0.03 and 0.06 µg/mL. For *Klebsiella* spp. (n = 70), similar results were noted, except for ampicillin and kanamycin. We detected 3.7 % (11/295) Enterobacteriaceae isolates carrying an ESBL/AmpC gene. *Staphylococcus aureus* (n = 247) and coagulase-negative staphylococci (CoNS; n = 189) isolates were susceptible to most antimicrobials tested except to penicillin (25.1 and 29.1 % resistance). Two *S. aureus* and thirteen CoNS isolates harboured *mecA* gene. *Streptococcus uberis* isolates (n = 208) were susceptible to β-lactam antibiotics (87.1–94.7 % susceptibility), 23.9 % were resistant to erythromycin and 37.5 % to tetracycline. Resistance to pirlimycin was moderate. For *Streptococcus dysgalactiae* (n = 132) the latter figures were 10.6 and 43.2 %; pirlimycin resistance was low. MIC values for *Streptococcus agalactiae, Trueperella pyogenes* and *Corynebacterium* spp. were generally low.

This current VetPath study shows that mastitis pathogens were susceptible to most antimicrobials with exceptions of staphylococci against penicillin and streptococci against erythromycin or tetracycline. For most antimicrobials, the percentage resistance and $MIC_{50/90}$ values among the major pathogens were comparable to that of the preceeding VetPath surveys. This work highlights the high need to set additional clinical breakpoints for antimicrobials frequently used to treat mastitis.

1. Introduction

Mastitis is a painful inflammation of the udder that affects the quality and the quantity of milk production leading to high economic losses. Bovine mastitis is considered to be one of the most costly diseases affecting dairy cattle worldwide and the most common reason for the use of antimicrobials in dairy cows (Barlow, 2011; Ruegg, 2017; Keane, 2019). The economic impact of mastitis consists of therapy

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costs, the cost of discarded milk, increased workload, reduced milk production, and culling and replacement costs. Because of the steady increase in milk production from cows, the importance of bovine mastitis is increasing worldwide (Ruegg, 2017; Martin et al., 2018). Antimicrobials have been used to treat mastitis for about sixty years, often prescribed without prior susceptibility testing, and are important parts of therapy of the disease, although not the solution for health management practices leading to poor udder health. In many dairy herds, mastitis control programmes have been implemented to try to reduce these losses including the antimicrobial therapy for cases of acute clinical mastitis. Enhanced milking hygiene including teat disinfection, correctly maintained equipment and personal hygiene are absolutely key to reduce the microbial load with contagious pathogens, and therefore the risk for new infections.

Acquired antimicrobial resistance in bacteria is a growing concern in both human and veterinary medicine. Monitoring of antimicrobial resistance, therefore, is important from both animal health and human health perspectives and is on many national and international agendas action plans. As any exposure of bacteria to antibiotics for therapy of mastitis may lead to selection of resistance and mastitis is a common reason for usage of antimicrobials, antimicrobial susceptibility testing of udder pathogens should regularly be conducted, a practice which is consistent with antimicrobial stewardship programmes. Monitoring antimicrobial resistance trends over time is important to ensure long-term efficacy of the antibacterial products. Access to recent repository of antimicrobial susceptibility data help guide the veterinarians in selecting the most appropriate antibiotic for treatment of mastitis, particularly given that mastitis therapy is commonly initiated before susceptibility testing of the pathogen because the infection cannot be left untreated. European susceptibility monitoring data of mastitis pathogens, however, is limited. Although there are a number of national, annual veterinary surveillance programmes for pathogens in place in Europe (*e.g.,* GE*RM*-Vet in Germany, RESAPATH in France, SVARM in Sweden, UK-VARSS in Great Britain), these lack harmonisation in relation to sampling schedules, methodology and interpretive criteria (GE*RM*-Vet, 2018; RESAPATH, 2019; SVARM, 2019; UK-VARSS, 2018). Only a few recent *ad hoc* studies are available (*e.g.,* Minst et al., 2012; Bengtsson et al., 2009; Overesch et al., 2013; Käppeli et al., 2019) and their methodologies including test methods (susceptibility testing either qualitative or quantitative) and breakpoints also are not harmonized (the RESAPATH and UK-VARSS surveys are based on disk diffusion methodology and apply national breakpoints).

To help address this problem, monitoring programmes are currently commissioned by the Executive Animal Health Study Centre (CEESA) investigating pathogens from both farm and companion animals (de Jong et al., 2013). CEESA's VetPath programme is dedicated to bacterial pathogens from several types of infections, including dairy mastitis, as well as other types of infections of diseased farm animals (cattle, pigs, poultry) not recently treated with antimicrobials across Europe. The VetPath programme is based on a protocol with harmonized methods of sampling, mastitis case/isolate enrolment and bacterial isolation. As the use of multiple laboratories to conduct susceptibility testing can potentially introduce bias into a surveillance study (Kahlmeter and Brown, 2002), a single central laboratory conducts the determination of minimal inhibitory concentrations (MICs) using a panel of approved antimicrobials commonly used in European veterinary medicine.

The first monitoring period (2002–2006) of three major mastitis pathogens (*Escherichia coli, Staphylococcus aureus, Streptococcus uberis*) was followed by the second period (2009–2012) with additionally including *Klebsiella* spp., coagulase-negative *Staphylococcus* spp. (CoNS) and *Streptococcus dysgalactiae* (Thomas et al., 2015; de Jong et al., 2018). Here we present the findings for isolates of the third monitoring period (2015–2016) recovered pre-treatment from cows with acute clinical mastitis across eight European countries, and additionally include data for the less frequent mastitis pathogens *Streptococcus agalactiae, Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) and *Corynebacterium* spp. To determine whether resistance has changed over time, the results of the percentage resistance observed were compared to those of the preceding VetPath studies.

2. Materials and methods

2.1. Animal criteria and sampling procedures

The design of the survey including the animal populations, clinical history and the sampling procedures were described previously (Thomas et al., 2015; de Jong et al., 2018). In short, in each of the eight countries included in the project (Belgium, Czech Republic, France, Germany, Italy, the Netherlands, Switzerland, the United Kingdom), milk samples were taken from cows with acute local or systemic clinical signs of mastitis and/or macroscopically abnormal milk secretions. In each country surveyed, a single national coordinator assumed responsibility for the collection of the samples and their processing according to uniform protocols for pathogen isolation. In an attempt to achieve similar numbers of isolates from the participating countries, equal numbers of isolates per bacterial species were indicated for each country. The numbers varied from 10 (low prevalence bacteria) up to 30 (high prevalence bacteria) isolates per bacterial species per country.

Records on standard case report forms of all samples indicated that 88.9 % of the sampled animals had not been exposed to antibacterial treatment for at least 3 weeks prior to sampling. The remaining 11.1 % of the samples were from animals with the treatment status characterized as "unknown". In all cases, only one sample per year was included from each herd sampled, to increase the likelihood of testing epidemiologically unrelated strains. If several cows with acute clinical mastitis were present in a herd, one cow was randomly selected. The isolates were identified to genus and species level by using conventional methods (NMC, 2017) such as colony morphology, coagulase and catalase tests, Gram staining and standard biochemical tests (API systems) before shipment to the central laboratory (IHMA Europe Sàrl, Monthey, Switzerland). If growth characteristics raised doubts on the identification or unusual susceptibility profiles were observed for the species, Matrix Assisted Laser Desorption Ionization – Time of Flight mass spectrometry (MALDI-TOF MS) using the Microflex LT system with the MALDI Biotyper 3.1.66 software and MBT library of 8468 main spectra (Bruker Daltonics, Bremen, Germany) was applied according to manufacturer's instructions to confirm the identity. In addition, the identity of all CoNS was confirmed by MALDI-ToF.

2.2. Antimicrobial susceptibility testing

At the central laboratory, MICs for all isolates were determined by broth microdilution in serial two-fold dilutions contained in 96-well microtitre plates (prepared at the central laboratory), in accordance with performance standards of the Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute (CLSI, 2018 and preceding version). Quality control strains (*E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *Streptococcus pneumoniae* ATCC 49619) were included on each day of testing, and, where reference ranges were available, MIC data was only accepted if MICs of the control strains were within the required reference ranges (Clinical and Laboratory Standards Institute (CLSI, 2018). The following antimicrobials/antimicrobial combinations (test ranges expressed as μg/mL), representing seven antimicrobial classes, were tested: amoxicillin (0.008–64), amoxicillin/clavulanic acid (AMC; 0.03/0.015-64/32), ampicillin (0.004–16), cefazolin (0.06 − 128), cephalexin (0.03–32), cephalonium (0.03–32), cephapirin (0.03–32), cefquinome (0.008–16), cloxacillin (0.03–8), penicillin G (0.004–8), danofloxacin (0.008–8), enrofloxacin (0.008–8), marbofloxacin (0.008–8), erythromycin (0.03–64), tylosin (0.03–64), kanamycin (0.12 − 128), kanamycin/cephalexin (0.12 − 128), neomycin (0.12 − 64), lincomycin (0.06 − 128),

Table 1
MIC distribution for *Escherichia coli* (n = 225) from acute mastitis in dairy cows (For interpretation of the references to color in this table note, the reader is referred to the web version of this article.).

Antimicrobial Agent	MIC (μg/ml)																	Susceptible		Intermediate		Resistant		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	[n]	[%]	[n]	[%]	[n]	[%]		
Amoxicillin									6	57	80	28			1	53		-	-	-	-	-	-	4	>64
Amoxicillin/clavulanate*									6	64	89	49	11	2	4			208	92.4	11	4.9	6	2.7	4	8
Ampicillin*									5	59	95	12		54				171	76.0	0	0.0	54	24.0	4	>16
Cefazolin*								2	104	86	17	4	1	1			10	192	85.3	17	7.6	16	7.1	2	4
Cephalexin										1	38	145	31	1	9			-	-	-	-	-	-	8	16
Cephalonium									1	74	100	26	9	5	10			-	-	-	-	-	-	4	16
Cephapirin							1	4	1	36	83	62	23	15	1			-	-	-	-	-	-	4	16
Cefquinome			1	51	142	19	2		2	1	1			6				-	-	-	-	-	-	0.06	0.12
Penicillin G													225					-	-	-	-	-	-	>8	>8
Danofloxacin			12	118	75	3	1	3			1		12					-	-	-	-	-	-	0.03	0.06
Enrofloxacin			20	153	34	1	2	2		1			12					-	-	-	-	-	-	0.03	0.06
Marbofloxacin		2	21	173	12			4	1			6	6					-	-	-	-	-	-	0.03	0.06
Kanamycin*									1	83	98	9	1	1	1	1	30	192	85.3	1	0.4	32	14.2	4	>128
Kanamycin/cephalexin									2	124	57	9	1	1	24	4	3	-	-	-	-	-	-	2	64
Neomycin									93	89	11	2		6	9	15		-	-	-	-	-	-	2	64
Lincomycin/spectinomycin												14	177	24	4	4	2	-	-	-	-	-	-	16	32
Penicillin/framycetin									27	145	19	4	1	4	18	7		-	-	-	-	-	-	2	64
Penicillin/streptomycin									1	27	119	23	3	52				-	-	-	-	-	-	4	>16
Rifaximin/cefacetrile									1	73	131	7	12	1				-	-	-	-	-	-	4	4
Tetracycline*								1	56	97	18		2		3	48		172	76.4	0	0.0	53	23.6	2	>64

Amoxicillin/clavulanic acid (2:1), concentration for amoxicillin is given; kanamycin/cephalexin (10:1), concentration for kanamycin is given; lincomycin/spectinomycin (1:2), concentration for lincomycin is given; penicillin/framycetin (2:1) and penicillin/streptomycin (1:2), concentration for penicillin is given; rifaximin/cefacetrile (1:2), concentration for rifaximin is given.
The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. Breakpoints are employed according to VET08. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines, respectively. A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available. *indicates that the breakpoint is based on human interpretive data included in VET08. Countries included (number of isolates in parentheses) are Belgium (34), Czech Republic (15), France (17), Germany (28), Italy (39), the Netherlands (29), Switzerland (30), United Kingdom (33).

lincomycin/spectinomycin (0.06 − 128), pirlimycin (0.06–64), penicillin/framycetin (0.008–64), penicillin/dihydrostreptomycin (0.004–16), rifaximin (0.03–64), rifaximin/cefacetrile (0.03–64), and tetracycline (0.06–64). The MICs of cloxacillin, lincomycin, pirlimycin, rifaximin, erythromycin and tylosin against *E. coli* and *Klebsiella* spp. are not reported because of intrinsic resistance (www.eucast.org).

2.3. ESBL/AmpC screening

The bimodal MIC frequency distribution was used to select isolates potentially producing ESBL/AmpC. *E. coli* isolates and *Klebsiella* spp. isolates with cefquinome MIC ≥ 1 μg/mL were selected for identifying the acquired determinants of resistance to β-lactams. Genomes were sequenced with Illumina NextSeq to obtain 150-bp reads with at least 90x coverage. Raw reads were trimmed with sickle (version 1.33), subsampled to 80x using home-made script and then assembled with SPAdes (version 3.12) using careful option and kmer size of '22,33,55,77'. Genomic data were explored to identify *in silico* the resistance determinants and the sequence types (STs) using the database from the Center for Genomic Epidemiology (both available on the www.genomicepidemiology.org website). Resistance determinants were identified and STs were determined using BLAT software (version 35) with ResFinder database and pyMLST (0.2), respectively.

2.4. mecA/mecC screening

To detect methicillin resistance in staphylococci, oxacillin MICs (range 0.015 − 16 μg/mL) were additionally assessed only for this genus. Subsequently oxacillin-resistant *S. aureus* and CoNS strains were examined for the presence of *mecA* gene by PCR according to Zhang et al. (2012). If negative, the isolates were further screened for the presence of the *mecC* gene (García-Álvarez et al., 2011). The oxacillin-resistant, *mecA*-positive *S. aureus* strain ATCC BAA-1556 and the *mecC*-positive *S. aureus* strain ATCC BAA-2312 were used for quality control. Negative control was *S. aureus* ATCC 29213.

2.5. Data analyses

MIC results are expressed as frequency distributions because internationally-endorsed breakpoints are not available for most veterinary antibiotics. The MIC_{50} and MIC_{90} values were determined for each organism-drug combination tested (Gram-negative organisms: 20 antibiotics/antibiotic combinations; Gram-positive organisms: 26 (or 27: staphylococci) antibiotics/antibiotic combinations). If breakpoints were available, results were also categorized and reported as susceptible, intermediate susceptible and resistant. Such categorizations for bovine mastitis pathogens based on veterinary-specific breakpoints are currently available only for ceftiofur, penicillin/novobiocin and pirlimycin of which the latter is the only veterinary antibiotic in our panel. The veterinary-specific breakpoints of pirlimycin (susceptibility (S) and resistance (R) breakpoints expressed as μg/mL) for *S. aureus* and streptococci are S ≤ 2 and R ≥ 4 (Clinical and Laboratory Standards Institute (CLSI, 2018). For a few other antibiotics tested that are also used in human medicine, human interpretive data taken from CLSI M100-S series (Clinical and Laboratory Standards Institute (CLSI, 2019) and adopted in the veterinary documents (Clinical and Laboratory Standards Institute (CLSI, 2017; 2018), were employed for AMC (S ≤ 8, R ≥ 32 for Enterobacteriaceae), ampicillin (S ≤ 8, R ≥ 32 for *E. coli*),

Table 2
MIC distribution for *Klebsiella oxytoca/pneumoniae* ($n = 70$) from acute mastitis in dairy cows (For interpretation of the references to color in this table note, the reader is referred to the web version of this article.).

Antimicrobial Agent	MIC (μg/ml)																Susceptible		Intermediate		Resistant		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	[n]	[%]	[n]	[%]	[n]	[%]		
Amoxicillin										2			4	12	31	21		-	-	-	-	-	-	64	>64
Amoxicillin/clavulanate*									12	47	5	3	2	1				67	95.7	2	2.9	1	1.4	2	4
Ampicillin*											2	1	12	55				3	4.3	12	17.1	55	78.6	>16	>16
Cefazolin*									43	17	4	2		1	1		2	60	85.7	4	5.7	6	8.6	1	4
Cephalexin										3	55	9	1		2			-	-	-	-	-	-	4	8
Cephalonium									1	48	16		1		4			-	-	-	-	-	-	2	4
Cephapirin									22	34	7	4			3			-	-	-	-	-	-	2	8
Cefquinome				19	36	7	2	2		2				2				-	-	-	-	-	-	0.06	0.25
Penicillin G												1	69					-	-	-	-	-	-	>8	>8
Danofloxacin				10	45	11	1	1	2									-	-	-	-	-	-	0.06	0.12
Enrofloxacin			2	23	41	2			2									-	-	-	-	-	-	0.06	0.06
Marbofloxacin			4	41	23			2										-	-	-	-	-	-	0.03	0.06
Kanamycin*									26	36	4	1	1	2				68	97.1	2	2.9	0	0.0	2	4
Kanamycin/cephalexin									40	24	3		2	1				-	-	-	-	-	-	1	4
Neomycin								51	18	1								-	-	-	-	-	-	0.5	1
Lincomycin/spectinomycin											1	55	9	2			3	-	-	-	-	-	-	8	16
Penicillin/framycetin								19	49	2								-	-	-	-	-	-	1	1
Penicillin/streptomycin									11	45	2	1		11				-	-	-	-	-	-	2	>16
Rifaximin/cefacetrile									7	43	14	2	1	3				-	-	-	-	-	-	2	4
Tetracycline*								4	34	18	3	1				10		59	84.3	1	1.4	10	14.3	1	>64

Amoxicillin/clavulanic acid (2:1), concentration for amoxicillin is given; kanamycin/cephalexin (10:1), concentration for kanamycin is given; lincomycin/spectinomycin (1:2), concentration for lincomycin is given; penicillin/framycetin (2:1) and penicillin/streptomycin (1:2), concentration for penicillin is given; rifaximin/cefacetrile (1:2), concentration for rifaximin is given.
The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. Breakpoints are employed according to VET08. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available. *indicates that the breakpoint is based on human interpretive data included in VET08. Countries included (number of isolates in parentheses) are Belgium (10), Czech Republic (5), Germany (23), Italy (4), the Netherlands (10), Switzerland (12) and United Kingdom (6).

cefazolin (S ≤ 2, R ≥ 8 for Enterobacteriaceae), oxacillin (S ≤ 2, R ≥ 4 for *S. aureus*; S ≤ 0.25, R ≥ 0.5 for CoNS), penicillin G (S ≤ 0.12, R ≥ 0.25 for staphylococci and streptococci; S ≤ 2, R ≥ 4 for *Corynebacterium* spp.), erythromycin (S ≤ 0.5, R ≥ 8 for staphylococci; S ≤ 0.25, R ≥ 1 for streptococci; S ≤ 0.5, R ≥ 2 for *Corynebacterium* spp.), kanamycin (S ≤ 16, R ≥ 64 for Enterobacteriaceae) and tetracycline (S ≤ 4, R ≥ 16; S ≤ 2, R ≥ 8, for streptococci; S ≤ 4, R ≥ 16 for *Corynebacterium* spp.). Since these interpretive criteria are not veterinary-specific, but are adopted from human medicine, their true value for veterinary pathogens, particularly mastitis pathogens, is unknown. CLSI S and R breakpoints, where available, are also indicated in Tables 1–6. For all other veterinary antibiotics/antibiotic combinations (14–26 antimicrobials per species), interpretive criteria are not available for mastitis isolates, and consequently no interpretation is performed.

Percentages of resistance were compared with the values observed in the preceding sampling period of VetPath 2002–2006 and VetPath 2009–2012 using the non-parametric chi-square test or Fisher Exact test (two-sided) for the six major pathogens for five antimicrobials/antimicrobial combinations with defined CLSI breakpoints tested in all periods. A *P* value of ≤ 0.05 was considered as a significant difference.

3. Results

Overall 1244 isolates were obtained from acute mastitis cases: 225 *E. coli,* 70 *Klebsiella* spp., 247 *S. aureus,* 189 CoNS, 208 *S. uberis,* 132 *S. dysgalactiae,* 44 *S. agalactiae,* 94 *T. pyogenes* and 35 *Corynebacterium* spp. The numbers of isolates from each country are detailed in the footnotes of Tables 1–7. MIC distributions, MIC_{50} and MIC_{90} values and, if applicable, % susceptible, intermediate and resistant isolates are presented for each bacterial species in Tables 1–6; Table 7 presents a summary for three species. The MICs of the quality control strains were always within the acceptable CLSI ranges.

3.1. E. coli (Table 1)

The MICs of the antibiotics tested showed a bimodal distribution, except for amoxicillin/clavulanic acid and cephapirin. Resistance varied from 2.7 % (AMC) to 24.0 % (ampicillin). For cefquinome (no breakpoints defined), few strains showed MICs in the upper concentrations of the dilution range corresponding to 4.4 % of the tested isolates (n = 10). Whole genome sequencing of these 10 isolates revealed the presence of ESBL-encoding genes (three $bla_{CTX\text{-}M\text{-}1}$, two $bla_{CTX\text{-}M\text{-}15}$, two $bla_{CTX\text{-}M\text{-}55}$, three $bla_{CMY\text{-}2}$) and narrow-spectrum β-lactamase encoding genes (four $bla_{OXA\text{-}1}$ and six $bla_{TEM\text{-}1}$) in different combinations. Sequence types (STs) were 58 (thrice), 88, 90, 131, 162, 398 (twice), and 617. *In vitro* activities of danofloxacin, enrofloxacin and marbofloxacin were high with MIC_{90} of 0.06 μg/mL and 5.3 % of the strains (n = 12) exhibiting a MIC higher than the range of concentrations tested. Resistance to kanamycin amounted to 14.2 %. For neomycin (no breakpoints) 13.3 % of the strains exhibited MIC values higher than those within what could be considered the wild type MIC distribution. Lincomycin/spectinomycin, penicillin/framycetin, penicillin/streptomycin and rifaximin/cefacetrile had MIC_{50} and MIC_{90} values of 2–16 and 4 − 64 μg/mL. Whereas 23.6 % of the strains were resistant to tetracycline, none of the isolates had intermediate MIC values.

Table 3
MIC distribution for *Staphylococcus aureus* (n = 247) from acute mastitis in dairy cows (For interpretation of the references to color in this table note, the reader is referred to the web version of this article.).

Antimicrobial Agent	MIC (μg/ml)																	Susceptible		Intermediate		Resistant		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	[n]	[%]	[n]	[%]	[n]	[%]		
Amoxicillin					11	101	64	9	9	32	12	6	2		1			-	-	-	-	-	-	0.25	2
Amoxicillin/clavulanate					5	106	65	31	37	1	1				1			-	-	-	-	-	-	0.25	1
Ampicillin				2	91	72	20	1	16	24	12	4	2	3				-	-	-	-	-	-	0.12	2
Cefazolin						36	102	94	12	1	1						1	-	-	-	-	-	-	0.25	0.5
Cephalexin				1	1		1	1	9	100	86	42	4		2			-	-	-	-	-	-	4	8
Cephalonium				2	83	121	39		1						1			-	-	-	-	-	-	0.12	0.25
Cephapirin				8	14	98	111	9	1		3	1		1	1			-	-	-	-	-	-	0.25	0.25
Cefquinome							29	116	91	5	3	2		1				-	-	-	-	-	-	0.5	1
Cloxacillin						23	147	68	6	1	1		1					-	-	-	-	-	-	0.25	0.5
Oxacillin*					4	35	130	67	8	1		1		1				245	99.2	-	-	2	0.8	0.25	0.5
Penicillin G*			18	101	57	8	1	3	9	21	14	9	6					184	74.5	-	-	63	25.5	0.06	4
Danofloxacin				2	47	124	70	2					2					-	-	-	-	-	-	0.12	0.25
Enrofloxacin				8	98	112	24	3			1		1					-	-	-	-	-	-	0.12	0.25
Marbofloxacin					2	57	140	45	1			1	1					-	-	-	-	-	-	0.25	0.5
Erythromycin*					4	99	130	5				1				8		238	96.4	0	0.0	9	3.6	0.25	0.25
Tylosin							2	59	127	52	2					5		-	-	-	-	-	-	1	2
Kanamycin						1	1	5	45	181	10			1			3	-	-	-	-	-	-	2	2
Kanamycin/cephalexin						1	2	8	97	133	2			1	2		1	-	-	-	-	-	-	2	2
Neomycin						7	73	148	15	1		1		1	1			-	-	-	-	-	-	0.5	0.5
Lincomycin							3	80	136	6	1	9	1	3	2	1	5	-	-	-	-	-	-	1	2
Lincomycin/spectinomycin								2	100	118	5	1	10	4	2	1	4	-	-	-	-	-	-	2	4
Pirlimycin					1	12	136	79	8	3	2	1	1			4		239	96.8	-	-	8	3.2	0.25	0.5
Penicillin/framycetin		1	15	103	61	7	13	44	1	1				1				-	-	-	-	-	-	0.06	0.5
Penicillin/streptomycin			11	91	64	16	4	7	36	16				2				-	-	-	-	-	-	0.06	1
Rifaximin				4	3	26	115	82	15	1						1		-	-	-	-	-	-	0.25	0.5
Rifaximin/cefacetrile				6	46	109	80	6										-	-	-	-	-	-	0.12	0.25
Tetracycline*						10	170	47	1			1	1	4	2	11		228	92.3	1	0.4	18	7.3	0.25	0.5

Amoxicillin/clavulanic acid (2:1), concentration for amoxicillin is given; kanamycin/cephalexin (10:1), concentration for kanamycin is given; lincomycin/spectinomycin (1:2), concentration for lincomycin is given; penicillin/framycetin (2:1) and penicillin/streptomycin (1:2), concentration for penicillin is given; rifaximin/cefacetrile (1:2), concentration for rifaximin is given.
Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. Breakpoints are employed according to VET08. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines respectively. For antibiotics without intermediate zone, a single green line is indicated. A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available. *indicates that the breakpoint is based on human interpretive data included in VET08. Countries included (number of isolates in parentheses) are Belgium (29), Czech Republic (15), France (28), Germany (32), Italy (51), the Netherlands (31), Switzerland (31), United Kingdom (30).

3.2. *Klebsiella spp.* (Table 2)

The occurrence of resistance to β-lactams varied from 1.4 % (AMC) to 78.6 % (ampicillin). The cefquinome MIC distribution was multimodal, with 4 isolates fulfilling the criteria for ESBL/AmpC screening. Of these four isolates, one carried the combination of ESBL-encoding $bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}110}$, another one carried the ESBL-encoding $bla_{CTX\text{-}M\text{-}15}$, the naturally-occurring $bla_{SHV\text{-}1}$, and the acquired $bla_{TEM\text{-}1}$ and $bla_{OXA\text{-}1}$, and the two other isolates harboured $bla_{SHV\text{-}61}$ or $bla_{SHV\text{-}1}$. STs were 13, 37, 1962 and a novel ST. MICs of fluoroquinolones (FQs) varied from 0.015 to 1 μg/mL; MIC_{90} values were 0.06 − 0.12 μg/mL with for each FQ 2 isolates with a deviating high MIC. Only for enrofloxacin an epidemiological cut-off value has been set (≤ 0.125 μg/mL; www.eucast.org), which results in 2 enrofloxacin non-wild type isolates. The MIC distribution of kanamycin and kanamycin/cephalexin seemed bimodal with MIC_{50} and MIC_{90} values of 1 − 2 and 4 μg/mL, respectively. Neomycin had MIC_{50} and MIC_{90} values of 0.5 and 1 μg/mL. Lincomycin/spectinomycin, penicillin/framycetin, penicillin/streptomycin and rifaximin/cefacetrile exhibited MIC_{50} and MIC_{90} values of 1 − 8 and 1- > 16 μg/mL. Resistance of *Klebsiella* spp. to tetracycline was moderate (14.3 %).

3.3. *S. aureus* (Table 3)

Penicillin G showed a wide, bimodal MIC distribution and a correspondingly high percentage of resistance (25.5 %). For the remaining β-lactams MIC_{50} and MIC_{90} values varied from 0.12 to 4 and 0.25–8 μg/mL, respectively, and MIC distribution was also bimodal. Two *S. aureus* isolates (0.8 %) recovered from samples of Czech Republic and Italy, were resistant to oxacillin, both positive for *mecA* gene. For each FQ, two isolates (0.8 %) displayed deviating high MICs, suggesting that acquired resistance was present among *S. aureus* isolates. For erythromycin, nine isolates were out of the normal range; this corresponded to 3.6 % resistance. Compared to erythromycin, tylosin displayed clearly higher MIC values resulting in MIC_{50} and MIC_{90} values of 1 and 2 μg/mL. $MIC_{50/90}$ of both kanamycin and kanamycin/cephalexin amounted to 2 μg/mL. Neomycin distribution of MICs was wide and multimodal with $MIC_{50/90}$ of 0.5 μg/mL. Resistance to pirlimycin was 3.2 %. MIC_{50} and MIC_{90} values for penicillin/framycetin, penicillin/streptomycin, rifaximin and rifaximin/cefacetrile were 0.06 − 0.25 and 0.25 − 1 μg/mL, respectively. Whilst there was one intermediate isolate for tetracycline, 7.3 % of the isolates exhibited resistance to tetracycline.

Table 4
MIC distribution for coagulase-negative *Staphylococcus* spp. (n = 189) from acute mastitis in dairy cows[a] (For interpretation of the references to color in this table note, the reader is referred to the web version of this article.).

Antimicrobial Agent	MIC (μg/ml)																	Susceptible		Intermediate		Resistant		MIC_{50}	MIC_{90}
	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	[n]	[%]	[n]	[%]	[n]	[%]	(μg/ml	(μg/ml)
Amoxicillin			2	1	36	45	56	21	6	7	5	5	3	2				-	-	-	-	-	-	0.25	2
Amoxicillin/clavulanate				3	23	62	66	22	12	1								-	-	-	-	-	-	0.25	0.5
Ampicillin			2	7	49	51	39	11	8	7	5	4	5	1				-	-	-	-	-	-	0.12	2
Cefazolin						25	58	61	31	14								-	-	-	-	-	-	0.5	1
Cephalexin				10	4		7	13	18	73	42	16	6					-	-	-	-	-	-	2	8
Cephalonium				72	35	76	6											-	-	-	-	-	-	0.06	0.12
Cephapirin				34	43	57	40	12	2	1								-	-	-	-	-	-	0.12	0.25
Cefquinome			1	1	1	6	53	75	40	9	3							-	-	-	-	-	-	0.5	1
Cloxacillin					3	5	38	68	51	15	9							-	-	-	-	-	-	0. 5	2
Oxacillin*			1		6	38	61	53	19	6	4			1				106	56.1	-	-	83	43.9	0.25	1
Penicillin G*	2	1	14	47	25	45	17	5	7	8	2	9	7					134	70.9	-	-	55	29.1	0.12	2
Danofloxacin			1	1	6	71	70	37	1		2							-	-	-	-	-	-	0.25	0.5
Enrofloxacin		1	2	3	30	73	71	7		1	1							-	-	-	-	-	-	0.12	0.25
Marbofloxacin					1	14	79	76	17	1	1							-	-	-	-	-	-	0.5	1
Erythromycin*					7	83	62	5	1	2	2	2	8	2		15		157	83.1	5	2.7	27	14.3	0.25	16
Tylosin						1	2	21	62	72	18	2				11		-	-	-	-	-	-	2	4
Kanamycin						21	59	68	27	5			3	5			1	-	-	-	-	-	-	0.5	1
Kanamycin/cephalexin						22	81	50	27				4	4	1			-	-	-	-	-	-	0.25	1
Neomycin						165	17	4	1	1			1					-	-	-	-	-	-	≤0.12	0.25
Lincomycin						2	10	45	33	11	17	27	10	8	5	6	15	-	-	-	-	-	-	2	128
Lincomycin/spectinomycin						1		12	43	35	11	18	27	20	17	2	3	-	-	-	-	-	-	2	64
Pirlimycin					2	19	60	59	18	11	7	2				11		-	-	-	-	-	-	0.5	4
Penicillin/framycetin		6	50	82	22	19	7	1	2									-	-	-	-	-	-	0.03	0.12
Penicillin/streptomycin		1	6	49	15	52	27	17	14	4	2			2				-	-	-	-	-	-	0.12	1
Rifaximin				4	6	16	90	60	8	3		1		1				-	-	-	-	-	-	0.25	0.5
Rifaximin/cefacetrile				6	27	85	62	7	2									-	-	-	-	-	-	0.12	0.25
Tetracycline*					1	8	91	40	6	5		4	1	14	8	11		151	79.9	4	2.1	34	18.0	0.25	64

Amoxicillin/clavulanic acid (2:1), concentration for amoxicillin is given; kanamycin/cephalexin (10:1), concentration for kanamycin is given; lincomycin/spectinomycin (1:2), concentration for lincomycin is given; penicillin/framycetin (2:1) and penicillin/streptomycin (1:2), concentration for penicillin is given; rifaximin/cefacetrile (1:2), concentration for rifaximin is given. The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. Breakpoints are employed according to VET08. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines, respectively. For antibiotics without intermediate zone, a single green line is indicated. A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available. *indicates that the breakpoint is based on human interpretive data included in VET08. Countries included (number of isolates in parentheses) are Belgium (30), France (25), Germany (26), Italy (31), the Netherlands (29), Switzerland (32), United Kingdom (16).

[a] Coagulase-negative *Staphylococcus* species comprise (number of isolates in parentheses) *S. xylosus* (45), *S. chromogenes* (38), *S. epidermidis* (31), *S. haemolyticus* (22), *S. equorum* (8), *S. sciuri* (8), *S. simulans* (8), *S. cohnii* (7), *S. hyicus* (5), *S. warneri* (5), *S. hominis* (3), *S. saprophyticus* (2), *S. succinus* (2), *S. kloosii* (1), *S. pasteuri* (1), *S. lentus* (1).

3.4. Coagulase-negative Staphylococcus spp. (Table 4)

In the case of CoNS, breakpoints have been set for four of the tested compounds. Similar to *S. aureus*, a high percentage of resistance was recorded for penicillin G (29.1 %), as observed in the preceding VetPath study. Oxacillin resistance amounted to 43.9 % (n = 83). Thirteen of the oxacillin-resistant CoNS isolates (15.7 %) harboured *mecA*: twelve *S. epidermidis* isolates and one *S. sciuri* isolate, and originated from four countries. The remaining *mecA*-negative, oxacillin-resistant isolates (n = 70; 84.3 %) did not contain the *mecC* gene. Most other compounds were characterized by a mono- or multimodal MIC distributions. Prevalence rates of resistance to erythromycin and tetracycline were moderate. The MIC distribution of tylosin was similar to that of erythromycin, with MIC_{50} and MIC_{90} values of 2 and 4 µg/mL. $MIC_{50/90}$ of kanamycin and kanamycin/cephalexin were identical at 0.25 – 0.5 and 1 µg/mL, respectively. $MIC_{50/90}$ of both lincomycin and lincomycin/spectinomycin amounted to 2 and 64 – 128 µg/mL, respectively. MIC values of the other combination products were very low (0.03–1 µg/mL).

3.5. S. uberis (Table 5)

Most of the β-lactam antibiotics showed a bimodal distribution. A large majority of the isolates were susceptible to ampicillin and penicillin G. Around 24 % of the isolates were resistant to erythromycin and 37.5 % were resistant to tetracycline. While 0.5 % was additionally intermediate to tetracycline, no isolate was in this category for erythromycin. For tylosin, 38 isolates (18 %) showed high MICs (≥ 32 µg/mL). Whilst kanamycin shows MIC_{50} and MIC_{90} values of 64 and > 128 µg/mL, the combination kanamycin/cephalexin had much higher *in vitro* activity with $MIC_{50/90}$ of 2 and 4 µg/mL. High MIC_{50} and MIC_{90}, i.e. > 64 µg/mL, were calculated for neomycin. MIC_{50} and MIC_{90} values of lincomycin and lincomycin/spectinomycin did not differ. Pirlimycin resistance was 15.9 %. MIC_{50} and MIC_{90} of penicillin/framycetin, penicillin/streptomycin, rifaximin and rifaximin/cefacetrile were low at 0.12 – 0.25 and 0.12 – 0.5 µg/mL, respectively.

3.6. S. dysgalactiae (Table 6)

Of all mastitis pathogens tested in this study, MICs for *S. dysgalactiae*

Table 5
MIC distribution for *Streptococcus uberis* (n = 208) from acute mastitis in dairy cows (For interpretation of the references to color in this table note, the reader is referred to the web version of this article.).

Antimicrobial Agent	MIC (μg/ml)																	Susceptible		Intermediate		Resistant		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	[n]	[%]	[n]	[%]	[n]	[%]		
Amoxicillin			3	47	30	11	95	21	1									-	-	-	-	-	-	0.25	0.5
Amoxicillin/clavulanate				56	25	10	101	15	1									-	-	-	-	-	-	0.25	0.25
Ampicillin*			1	12	65	18	102	9	1									198	94.7	-	-	-	-	0.25	0.25
Cefazolin					2	72	13	109	10	1				1				-	-	-	-	-	-	0.5	0.5
Cephalexin						3	127	67	8	1			1		1			-	-	-	-	-	-	0.25	0.5
Cephalonium				103	94	1	3			1	1		1		4			-	-	-	-	-	-	0.06	0.06
Cephapirin				71	10	11	104	10				1	1					-	-	-	-	-	-	0.25	0.25
Cefquinome		1	48	31	1	49	71	5		1		1						-	-	-	-	-	-	0.12	0.25
Cloxacillin					3	3	39	36	7	43	74	1	2					-	-	-	-	-	-	2	4
Penicillin G*		2	28	50	25	77	24	1		1								182	87.1	-	-	-	-	0.06	0.25
Danofloxacin							3	53	130	24								-	-	-	-	-	-	1	2
Enrofloxacin							9	127	72									-	-	-	-	-	-	0.5	1
Marbofloxacin								1	135	74								-	-	-	-	-	-	1	2
Erythromycin*				11	137	10	1		4	1	4	1				39		159	76.4	0	0.0	49	23.6	0.06	> 64
Tylosin							2	80	73	15				2		36		-	-	-	-	-	-	1	> 64
Kanamycin											2	9	13	33	88	41	22	-	-	-	-	-	-	64	> 128
Kanamycin/cephalexin									1	118	84	4	1					-	-	-	-	-	-	2	4
Neomycin							1	1			1	4	10	20	46	125		-	-	-	-	-	-	> 64	> 64
Lincomycin					1	71	22	6	2	12	33	13	4	2	6	28	8	-	-	-	-	-	-	2	128
Lincomycin/ spectinomycin					1	5	77	14	8		14	39	12	6		10	22	-	-	-	-	-	-	1	> 128
Pirlimycin					69	49	21	7	11	18	7	8	3	1	9	5		175	84.1	-	-	33	15.9	0.12	8
Penicillin/framycetin		2	26	51	16	85	26		1	1								-	-	-	-	-	-	0.12	0.25
Penicillin/streptomycin		3	19	33	28	12	88	22	2		1							-	-	-	-	-	-	0.25	0.5
Rifaximin				1	9	108	68	5	3	1				2	4	7		-	-	-	-	-	-	0.12	0.5
Rifaximin/cefacetrile				2	83	105	16			1	1							-	-	-	-	-	-	0.12	0.12
Tetracycline*						8	104	16	1		1	1		29	26	22		129	62.0	1	0.5	78	37.5	0.25	> 64

Amoxicillin/clavulanic acid (2:1), concentration for amoxicillin is given; kanamycin/cephalexin (10:1), concentration for kanamycin is given; lincomycin/spectinomycin (1:2), concentration for lincomycin is given; penicillin/framycetin (2:1) and penicillin/streptomycin (1:2), concentration for penicillin is given; rifaximin/cefacetrile (1:2), concentration for rifaximin is given.
The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. Breakpoints are employed according to VET08. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines, respectively. For antibiotics without intermediate zone, a single green line is indicated. A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available. *indicates that the breakpoint is based on human interpretive data included in VET08. Countries included (number of isolates in parentheses) are Belgium (32), Czech Republic (15), France (29), Germany (30), Italy (3), the Netherlands (31), Switzerland (28), United Kingdom (40).

were the lowest. For all β-lactam antibiotics, MICs were numerically lower than for *S. uberis*, as is apparent from the MIC_{50} and MIC_{90} values. For both ampicillin and penicillin G 100 % susceptibility was recorded. Neomycin and kanamycin displayed 32 and 64 as MIC_{50} and MIC_{90} values, respectively; the combination kanamycin/cephalexin, however, showed $MIC_{50/90}$ values of 2 and 4 μg/mL. Resistance to erythromycin was moderate (10.6 %); MIC values of tylosin were in the same range. Resistance to tetracycline was 43.2 %. While none of the isolates were additionally intermediate to erythromycin, 35.6 % were in this category for tetracycline. Resistance to pirlimycin amounted to 7.6 %. MIC_{90} for penicillin/framycetin, penicillin/streptomycin, rifaximin and rifaximin/cefacetrile were very low at 0.008 – 0.12 μg/mL.

3.7. *S. agalactiae, T. pyogenes and Corynebacterium spp.* (Table 7)

With respect to *S. agalactiae*, the majority of the β-lactam antibiotics MICs is ≤0.25 μg/mL; penicillin MICs were 0.015 – 0.06 μg/mL. A specification of the resistance rate was only possible for erythromycin and pirlimycin (both at 27.3 %). The results for *T. pyogenes* confirm low MIC values for various β-lactam antimicrobials including penicillin (MIC range of ≤0.004 – 0.06 μg/mL). In addition, for tylosin, recommended for therapy of *T. pyogenes* infections, the majority of the isolates were characterized by low MICs. Whereas one isolate had a deviating high MIC (16 μg/mL) to tylosin, 70.2 % had high MICs (≥4 μg/mL) to tetracycline. For *Corynebacterium* spp. MIC_{50} values were for all antimicrobials ≤1 μg/mL (except cephapirin and cloxacillin), and frequently ≤0.25 μg/mL. Similarly, MIC_{90} values were very low with exception of rifaximin. Resistance to penicillin G was absent, whereas resistance to erythromycin was low at 5.7 %.

3.8. *Time period comparisons* (Table 8)

One objective of each monitoring study is to determine whether the percentage resistance of different time periods has changed. Therefore, the prevalence of resistance for five antibiotics (having CLSI clinical breakpoints) of VetPath 2002–2006 and 2009–2012 were compared with the current VetPath study (2015–2016) for the six major pathogens common in the surveys. Resistance of *E. coli* and *Klebsiella* spp. to AMC remained stable between the time periods. In contrast, resistance to kanamycin and tetracycline has significantly increased ($P \leq 0.05$) for *E. coli* isolates whereas for both compounds slight numerical decreases of resistance were observed for *Klebsiella* spp. Erythromycin resistance in *S. aureus* and streptococci was relatively constant, but for CoNS a marked increase ($P < 0.01$) was observed. Penicillin resistance of *S. uberis* and CoNS were mostly constant, but for *S. aureus* a significant decrease ($P < 0.01$) was noted for 2015–2016 *versus* 2002–2006. Among the Gram-positive pathogens, a significant increase of tetracycline resistance ($P < 0.01$) was observed for CoNS. Comparison of MIC_{50} values of the 2009–2012 and 2015–2016 surveys didn't reveal any significant changes (data not shown).

4. Discussion

The present survey is an ongoing antimicrobial susceptibility monitoring programme for udder pathogens from dairy cows in Europe applying a uniform, standardized methodology for the collection of the isolates and centralized, standardized quantitative susceptibility testing by using the CLSI broth microdilution method. In this new study presented here three additional pathogens were collected, and eight

Table 6
MIC distribution for *Streptococcus dysgalactiae* (n = 132) from acute mastitis in dairy cows (For interpretation of the references to color in this table note, the reader is referred to the web version of this article.).

Antimicrobial Agent	MIC (μg/ml)																	Susceptible		Intermediate		Resistant		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	[n]	[%]	[n]	[%]	[n]	[%]		
Amoxicillin		18	109	2	1	2												-	-	-	-	-	-	0.015	0.015
Amoxicillin/clavulanate				129	1	2												-	-	-	-	-	-	≤0.03	≤0.03
Ampicillin*		5	116	8	1	2												132	100.0	-	-	-	-	0.015	0.015
Cefazolin					56	75	1											-	-	-	-	-	-	0.12	0.12
Cephalexin						4	103	22	1	1		1						-	-	-	-	-	-	0.25	0.5
Cephalonium				125	2		2	1						1	1			-	-	-	-	-	-	≤0.03	≤0.03
Cephapirin				128	1	2	1											-	-	-	-	-	-	≤0.03	≤0.03
Cefquinome		109	21		1		1											-	-	-	-	-	-	≤0.008	0.015
Cloxacillin				4	107	17	2			1	1							-	-	-	-	-	-	0.06	0.12
Penicillin G*	21	106		1	2	2												132	100.0	-	-	-	-	0.008	0.008
Danofloxacin							1	52	78	1								-	-	-	-	-	-	1	1
Enrofloxacin						1	7	102	22									-	-	-	-	-	-	0.5	1
Marbofloxacin								10	120	2								-	-	-	-	-	-	1	1
Erythromycin*				24	90	2	2		2		1	2				9		118	89.4	0	0.0	14	10.6	0.06	1
Tylosin						2	91	30	2							7		-	-	-	-	-	-	0.25	0.5
Kanamycin												19	5	90	5	1	12	-	-	-	-	-	-	32	64
Kanamycin/cephalexin									1	83	46	2						-	-	-	-	-	-	2	4
Neomycin											1		8	79	32	12		-	-	-	-	-	-	32	64
Lincomycin					1	17	91	2	2	1		4	3			3	8	-	-	-	-	-	-	0.25	16
Lincomycin/spectinomycin						2	14	96	2	1	2	1	5	1	1		7	-	-	-	-	-	-	0.5	16
Pirlimycin					89	25	5	2		1	3	1	2	2	1	1		122	92.4	-	-	10	7.6	≤0.06	0.25
Penicillin/framycetin		124	2	2	2	1	1											-	-	-	-	-	-	≤0.008	≤0.008
Penicillin/streptomycin	81	26	20	3		2												-	-	-	-	-	-	≤0.004	0.015
Rifaximin				14	73	41	1				1	1				1		-	-	-	-	-	-	0.06	0.12
Rifaximin/cefacetrile				28	102	2												-	-	-	-	-	-	0.06	0.06
Tetracycline*							1		3	24	47	7		10	40			28	21.2	47	35.6	57	43.2	4	64

Amoxicillin/clavulanic acid (2:1), concentration for amoxicillin is given; kanamycin/cephalexin (10:1), concentration for kanamycin is given; lincomycin/spectinomycin (1:2), concentration for lincomycin is given; penicillin/framycetin (2:1) and penicillin/streptomycin (1:2), concentration for penicillin is given; rifaximin/cefacetrile (1:2), concentration for rifaximin is given.
The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. Breakpoints are employed according to VET08. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines, respectively. For antibiotics without intermediate zone, a single green line is indicated. A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available. *indicates that the breakpoint is based on human interpretive data included in VET08. Countries included (number of isolates in parentheses) are Belgium (20), Czech Republic (10), France (31), Germany (17), Italy (1), the Netherlands (18), Switzerland (20), United Kingdom (15).

antibiotics/antibiotic combinations have additionally been included, and this study was therefore more extensive than the previous study (de Jong et al., 2018). This study showed that various levels of resistance to classes of antimicrobial agents commonly used in mastitis treatment are present among mastitis pathogens. Nevertheless, compounds of several classes represent valuable therapeutic options.

Among Gram-negative mastitis pathogens, the highest number of isolates was found for *E. coli*. Our results suggest a low to high incidence of resistance among isolates. *E. coli* resistance profiles from the national surveys in Sweden and Germany are in agreement with the results presented herein, *e.g.,* 24 % resistance to ampicillin (SVARM, 2019) and 1 − 4% resistance to AMC (GERM-Vet, 2018). The MIC distributions of enrofloxacin and neomycin in the SVARM studies were apparently similar to our results. Fourth generation cephalosporin (cefquinome) and FQs exhibited low MIC_{90} values (0.06 − 0.12 µg/mL) in our study. Tetracycline resistance was 8–16 % in the Swedish surveys (SVARM, 2019) and 10–16 % in the German surveys (GERM-Vet, 2018), *i.e.* slightly lower than in our study (23.6 %). Low resistance to tetracycline was observed in Swedish isolates with 4.9 % originating from clinical mastitis (Bengtsson et al., 2009) and 5.9 % from subclinical mastitis (Persson et al., 2011). Similar MIC ranges and $MIC_{50/90}$ values have been reported for several β-lactam antibiotics and neomycin in France as compared to our study (Guérin-Faublée et al., 2003).

Klebsiella spp. is another important Gram-negative species associated with mastitis, but the occurrence is clearly lower than for *E. coli* (Guérin-Faublée et al., 2003; Bengtsson et al., 2009). As with *E. coli*, two national surveys in Europe frequently provided quantitative susceptibility data for *Klebsiella* spp. (GERM-Vet, 2018; n = 395 over 6 years; SVARM, 2019; n = 163 over 4 years); the numbers of isolates in other studies were too low to enable meaningful conclusions or comparisons within a given time period or region. Our data is similar to those of GERM-Vet (2018) and SVARM (2019), except for tetracycline (14.3 %), which was lower in the SVARM study (6–12 %) and displayed large annual fluctuations in the GERM-Vet study in the period 2011–2016 (5–39 %). The high rate of resistance to ampicillin is expected because of the inherently low susceptibility of this genus to this antimicrobial agent. Low MIC_{90} values were found for cefquinome (0.06 − 0.12 µg/mL) and FQs (0.12 µg/mL) (GERM-Vet, 2018), and is compatible with the results of our work. First/second generation cephalosporins also displayed low MIC values in the current study.

With respect to Gram-negative bacteria, milk can be a reservoir of ESBL/AmpC producing pathogens that could be transferred to humans (Geser et al., 2012; Dahmen et al., 2013). In this study, the occurrence of ESBL/AmpC producers amounted to 4.0 % for *E. coli* and 2.9 % for *Klebsiella* spp. For the preceding VetPath study (2009–2012) these figures amounted to 1.9 % (CTX-M-1, CTX-M-2) and 0.0 %, respectively, were found (unpublished data; de Jong et al., 2018). In addition to resistance to β-lactam antibiotics including extended-spectrum cephalosporins, ESBL/AmpC producing isolates frequently carry resistance determinants that confer resistance to classes of antibiotics like FQs, aminoglycosides and trimethoprim/sulfamethoxazole (Coque et al., 2008; Freitag et al., 2017). In several European countries ESBL/AmpC

Table 7
MIC_{50} /MIC_{90} values, MIC ranges and percentage of resistance for *Streptococcus agalactiae, Trueperella pyogenes* and *Corynebacterium* spp.

	Streptococcus agalactiae $n = 44$				*Trueperella pyogenes* $n = 94$				*Corynebacterium* spp.[a] $n = 35$			
	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Range (μg/mL)	% resistance	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Range (μg/mL)	% resistance	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Range (μg/mL)	% resistance
Amoxicillin	0.06	0.06	0.03 – 0.12	–	0.06	0.06	0.015 – 0.12	–	0.25	0.25	0.06 – 0.25	–
Amoxicillin/ clavulanate	0.06	0.06	≤0.03 – 0.12	–	≤0.03	0.12	≤0.03 – 0.12	–	0.12	0.25	0.06 – 0.25	–
Ampicillin	0.12	0.12	0.06 – 0.25	–	0.03	0.06	0.015 – 0.12	–	0.25	0.25	0.06 – 0.5	–
Cefazolin	0.12	0.12	0.12 – 0.25	–	0.25	0.5	≤0.06 – 2	–	1	1	0.25 – 1	–
Cephalexin	2	4	2 – 4	–	2	4	1 – 8	–	1	1	0.12 – 2	–
Cephalonium	≤0.03	≤0.03	≤0.03 – 0.06	–	0.12	0.25	0.12 – 2	–	0.06	0.12	≤0.03 – 0.12	–
Cephapirin	0.12	0.25	0.12 – 0.25	–	0.12	0.12	≤0.03 – 0.5	–	0.12	0.12	0.06 – 0.25	–
Cefquinome	0.06	0.06	0.03 – 0.06	–	0.25	0.5	0.03 – 0.5	–	0.12	0.25	0.03 – 0.5	–
Cloxacillin	1	2	0.5 – 2	–	0.25	0.5	0.06 – 1	–	2	4	0.5 – 8	–
Penicillin G	0.03	0.06	0.015 – 0.06	–	0.008	0.015	≤0.004 – 0.06	–	0.12	0.25	0.03 – 0.25	0.0
Danofloxacin	1	2	0.5 – 4	–	2	2	1 – 4	–	0.12	0.25	0.06 – 1	–
Enrofloxacin	1	1	0.5 – 2	–	0.5	1	0.5 – 4	–	0.12	0.25	0.06 – 0.5	–
Marbofloxacin	2	2	1 – 2	–	1	1	0.25 – 4	–	0.5	0.5	0.03 – 1	–
Erythromycin*	0.06	> 64	≤0.03- > 64	27.3	≤0.03	1	≤0.03- > 64	–	≤0.03	≤0.03	≤0.03- > 64	5.7
Tylosin	1	> 64	0.5- > 64	–	≤0.03	0.12	≤0.03 – 16	–	0.5	0.5	0.25- > 64	–
Kanamycin	128	> 128	8- > 128	–	2	2	1 – 8	–	0.25	0.25	≤0.12- > 128	–
Kanamycin/ cephalexin	32	32	16 – 32	–	1	2	1 – 8	–	0.25	0.25	≤0.12 – 4	–
Neomycin	> 64	> 64	16- > 64	–	8	16	4 – 32	–	≤0.12	0.25	≤0.12 – 16	–
Lincomycin	0.25	> 128	≤0.06- > 128	–	0.12	0.25	≤0.06- > 128	–	0.5	1	0.25- > 128	–
Lincomycin/ spectinomycin	0.5	64	0.12 – 128	–	0.25	0.5	≤0.06 – 4	–	1	1	0.5 – 8	–
Pirlimycin	0.12	> 64	≤0.06- > 128	27.3	≤0.06	0.25	≤0.06- > 64	–	0.12	0.25	≤0.06- > 64	–
Penicillin/ framycetin	0.03	0.06	0.015 – 0.12	–	≤0.008	0.015	≤0.008 – 0.06	–	0.06	0.12	0.03 – 0.12	–
Penicillin/ streptomycin	0.06	0.12	0.03 – 0.12	–	0.015	0.06	≤0.004 – 0.12	–	0.25	0.25	0.12 – 0.5	–
Rifaximin	0.25	0.5	0.06- > 64	–	≤0.03	0.06	≤0.03- > 64	–	≤0.03	> 64	≤0.03- > 64	–
Rifaximin/ cefacetrile	0.12	0.12	0.06 – 0.12	–	≤0.03	≤0.03	≤0.03 – 0.12	–	≤0.03	≤0.03	≤0.03 – 0.06	–
Tetracycline*	32	64	0.12 – 64	68.2	8	32	≤0.06 – 32	–	0.25	0.5	0.12 – 8	0.0

Countries included (number of isolates in parentheses) for *Streptococcus agalactiae* are Belgium (34), Czech Republic (15), France (17), Germany (28), Italy (39), the Netherlands (29), Switzerland (30), United Kingdom (33).
Countries included (number of isolates in parentheses) for *T. pyogenes* are Belgium (20), France (5), Germany (27), the Netherlands (17), Switzerland (17), United Kingdom (8).
Countries included (number of isolates in parentheses) for *Corynebacterium* spp. are France (21), the Netherlands (12), Switzerland (30), United Kingdom (33).
[a] *Corynebacterium* species comprise (number of isolates in parentheses) *Corynebacterium bovis* ($n = 34$) and *Corynebacterium amycolatum* ($n = 1$). A dash indicates that no figure could be calculated because no CLSI interpretive criteria are available.

producers were found in low numbers of milk samples in dairy herds. In an extensive French survey 0.4 % of the genetically unrelated Enterobacteriaceae isolates carried an ESBL gene (Dahmen et al., 2013). In a Swiss study none of 100 bulk tank milk samples and only one of 67 mastitis milk samples contained an ESBL-producing (CTX-M-14) isolate (Geser et al., 2012). In a German study (Freitag et al., 2017), the most prevalent ESBL genes of *E. coli* (1.4 %; 12/878) belonged to $bla_{CTX\text{-}M\text{-}1}$ (42 %), followed by $bla_{CTX\text{-}M\text{-}15}$ (33. %), $bla_{CTX\text{-}M\text{-}2}$ (17 %) and $bla_{CTX\text{-}M\text{-}14}$ (8%). In contrast, the $bla_{CTX\text{-}M\text{-}14}$ gene was most prevalent in the French study (Dahmen et al., 2013), whereas the $bla_{CTX\text{-}M\text{-}15}$ gene has been detected in 17 samples from one farm in the United Kingdom (Timofte et al., 2014). A similar incidence (4.0 %) as in our study was observed for *E. coli* from mastitic milk in South Korea, carrying ESBL/AmpC genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CMY\text{-}2}$) (Tark et al., 2017). In another study from the United Kingdom a low prevalence of ESBL/AmpC was found (Snow et al., 2012); herds using extended-spectrum cephalosporins were almost four times more likely to have ESBL/AmpCs. Hordijk et al. (2019) have examined the faecal shedding of ESBL/AmpC producing *E. coli* by individual dairy cows on selected Dutch dairy farms and concluded that animals carrying ESBL/AmpC *E. coli* were either absent or at a low prevalence. It was also reported that the presence of ESBL/AmpC could only be partly explained by antimicrobial drug use and no link was shown with humans or the environment. In The Netherlands, ESBL/AmpC bacteria in the milk are generally considered as contaminants from the environment, rather than being an intramammary pathogen.

In udder pathogens, antibiotic resistance is usually most prominent in Gram-positive bacteria. For *S. aureus* five clinical breakpoints are available, but are based mainly on data from antibiotics used in humans. Based on human breakpoints, our study suggests a marked resistance to penicillin (25.5 %) whereas low resistance was observed for erythromycin, pirlimycin and tetracycline. Similar results were observed in the national survey of Germany (Kaspar et al., 2017). In Swedish *ad hoc* data, penicillin resistance was 3.6 % for isolates from subclinical cases (Persson et al., 2011) and 7.1 % from acute clinical cases (Bengtsson et al., 2009); resistance to all other compounds tested was lower in both studies than in our study. In contrast, resistance prevalence of *S. aureus* to penicillin was 44.5 % in France (Guérin-Faublée et al., 2003) and 26 % in isolates from French and Swiss farms (Sakwinska et al., 2009). In France, MIC_{90} for cloxacillin, cephalexin, cephapirin, cefquinome and neomycin were found to be identical or at most one dilution step different compared to our data (Guérin-Faublée et al., 2003). Similar to our study, erythromycin showed very low levels of resistance in the national surveys (SVARM, 2016; Kaspar et al., 2017). Of all antimicrobial agents tested, pirlimycin is the only one with a defined veterinary-specific breakpoint for staphylococci isolated from cattle mastitis. Thereby, 3.2 % of the isolates were resistant to pirlimycin. In the GE*RM*-Vet survey pirlimycin resistance of 1–10 %

Table 8
Comparison of antimicrobial resistance (%) in bacteria isolated from cases of mastitis in three time periods (2002–2006; 2009–2012; 2015–2016).[a]

	E. coli			*Klebsiella* spp.		*S. aureus*			Coagulase-negative *Staphylococcus* spp.		*S. uberis*			*S. dysgalactiae*	
	2002 – 06 (n = 280)	2009 – 12 (n = 207)	2015 – 16 (n = 225)	2009 – 12 (n = 87)	2015 – 16 (n = 70)	2002 – 06 (n = 250)	2009 – 12 (n = 192)	2015 – 16 (n = 247)	2009 – 12 (n = 246)	2015 – 16 (n = 189)	2002 – 06 (n = 282)	2009 – 12 (n = 188)	2015 – 16 (n = 208)	2009 – 12 (n = 95)	2015 – 16 (n = 135)
Amoxicillin/clavulanate	1.1	3.9	2.7	4.6	1.4	–	–	–	–	–	–	–	–	–	–
Kanamycin	–	7.7	14.2[b]	4.6	0.0	–	–	–	–	–	–	–	–	–	–
Erythromycin	–	–	–	–	–	0.8	1.0	3.6	5.5	14.3[b]	18.8	20.2	23.6	13.7	10.7
Penicillin G	–	–	–	–	–	36.0	25.0	25.5[c]	29.1	29.1	0.0	0.0	–	–	–
Tetracycline	14.3	14.5	23.6[b,c]	19.5	14.3	5.2	5.2	7.3	7.3	18.0[b]	28.7	36.7	37.5	56.8	43.2

Superscript b indicates a significant difference of the 2015 – 16 resistance % compared to the corresponding value of 2009 – 12; superscript c indicates a significant difference of the 2015 – 16 value compared to the corresponding value of 2002 – 06. All figures refer to the resistance breakpoints defined in CLSI VET08 which were the same in the three time periods.

[a] A dash indicates no breakpoints are available to calculate the percentage resistance.

was noted (Kaspar et al., 2017). Resistance to tetracycline varied from 4 to 14 % in German isolates (Kaspar et al., 2017). Kanamycin MICs in our work were in line with those reported by Overesch et al. (2013). These researchers, however, have observed lower $MIC_{50/90}$ values of rifampicin (≤ 0.016 µg/mL), a derivative of rifaximin, than in our survey for rifaximin. Note that based on human breakpoints not reported in VET08, Overesch et al. (2013) reported an absence of rifampicin resistance. Low MIC values for several combination products were determined and seem valuable, although the rate of resistance cannot be assessed owing to the lack of breakpoints.

CoNS is another predominant group of mastitis pathogens (Pitkälä et al., 2004; Tenhagen et al., 2006). The proportion of penicillin-resistant CoNS isolates (29.1 %) was similar to that for *S. aureus* (25.1 %) in our present work. Overall, resistance to antimicrobials other than penicillin and oxacillin (43.9 %) was moderate (14.3–18.0 %) and similar to or higher than those reported in other studies (Pitkälä et al., 2004; Persson et al., 2011; Frey et al., 2013). It is well known that antimicrobial resistance profiles may differ significantly among CoNS species. In our study 16 CoNS species have been identified (Table 4). The most common species in our study were *S. xylosus* and *S. chromogenes*, as has been found in various other studies (Lüthje and Schwarz, 2006; Frey et al., 2013). Other frequent species were *S. epidermidis, S. haemolytica, S. equorum, S. sciuri* and *S. simulans* (Table 4). The prevalence of other CoNS species was relatively low. The numbers of isolates per species were too low for meaningful comparisons of the antimicrobial susceptibility.

Methicillin resistance, encoded by the *mecA* gene, which confers resistance to β-lactam antimicrobials including various broad-spectrum cephalosporins, has been detected in *S. aureus* and CoNS. Oxacillin resistance, which is one indicator of *mec* gene-mediated methicillin resistance, was the most frequent resistance phenotype in this study (43.9 %) and the preceding VetPath study (56.4 %). Oxacillin resistance was attributed to the *mecA* gene in 15 methicillin-resistant strains; 2 *S. aureus* and 13 CoNS. This corresponds to 0.8 and 6.9 %, respectively, of the respective staphylococci collections. All *mecA*-negative, oxacillin-resistant isolates (84.3 %; $n = 70$) exhibited an oxacillin MIC of 0.5 or 1 µg/mL (mode 0.5 µg/mL), which is just above the clinical resistance breakpoint. In contrast, the *mecA*-positive isolates had oxacillin MICs of 1- > 16 with a mode value of 2 µg/mL. Reports on methicillin resistance in *S. aureus* (MRSA) and CoNS associated with mastitis are limited (Guérin-Faublée et al., 2003; Tavakol et al., 2012; Frey et al., 2013). Among CoNS species, *mecA* has been frequently demonstrated in *S. epidermidis* and *S. sciuri* (Guérin-Faublée et al., 2003; Bengtsson et al., 2009; Sampimon et al., 2011; Frey et al., 2013), which is in line with our previous (de Jong et al., 2018) and our present results. Prevalence of MRSA in dairy milk is generally considered to be very low as compared to other animal species, and pigs in particular, though geographic differences can exist (Vanderhaeghen et al., 2010). Similar findings on the very low incidence of MRSA have been reported in Canada (Saini et al., 2012). Although the prevalence of MRSA in mastitis samples is very low, these findings underscore the importance of antibiogram performance prior to therapy, and the need of resistance monitoring programmes.

As for the *S. uberis* results presented here, other studies also describe high levels of susceptibility to penicillin (Guérin-Faublée et al., 2003; Bengtsson et al., 2009; Persson et al., 2011; Minst et al., 2012; Overesch et al., 2013; Käppeli et al., 2019). In our previous work we reported of 35.6 % intermediate susceptible to penicillin which was compatible with the results of Haenni et al. (2010), who reported a subpopulation of *S. uberis* isolates exhibiting high penicillin MIC values, despite no clinical resistance was present. However, CLSI breakpoints have since then been revised, which results in the reporting of percentage susceptibility only. For erythromycin, resistance rates found in our study (23.6 %) were apparently similar to those in the preceding VetPath study (20.2 %) (de Jong et al., 2018) and in the German national data of 5–27 % (GE*RM*-Vet, 2018) and 22.9 % (Minst et al., 2012), whereas in

Switzerland percentages of 10.6 and 15.7 % were observed (Overesch et al., 2013; Käppeli et al., 2019). In contrast, no erythromycin resistance was found in Sweden (Bengtsson et al., 2009; Persson et al., 2011). Pirlimycin, the only compound with a veterinary-specific breakpoint for *Streptococcus* spp., displayed 15.9 % resistance. In other studies 3–32 % and 11.8 % were noted (GERM-Vet, 2018; Käppeli et al., 2019). As with the tetracycline resistance results presented here (37.5 %) and in our previous work (36.7 %), high levels of resistance to tetracycline were found in Switzerland with 28.4 % (Overesch et al., 2013), in the Czech Republic with 63.2 % (Zouharová and Nedbalcová, 2019) and in Germany with over 40 %, (GERM-Vet, 2018) and 42.3 % (Minst et al., 2012), whereas resistance to tetracycline was 1.8–4.0 % in Sweden (Bengtsson et al., 2009; Persson et al., 2011). The combination kanamycin/cephalexin, frequently first or second drug of choice for treatment of mastitis, displayed low MICs as compared to kanamycin and similar MICs as cephalexin. Similar results may have been observed by Käppeli et al. (2019). However, a comparison with this agent and other agents wasn't feasible due to the limited MIC concentration range at the lower MIC end in the Käppeli et al. (2019) study. This also refers to the combination kanamycin/cephalexin ($\leq 4/0.4$ µg/mL). Rifaximin and rifaximin/cefacetrile exhibited low MICs in our survey, which has been confirmed for rifampicin in other studies (Zouharová and Nedbalcová, 2019; Käppeli et al., 2019).

In all studies reported, *S. dysgalactiae* isolates had very low MICs to penicillin resulting in 100 % susceptibility (*e.g.,* Guérin-Faublée et al., 2003; Overesch et al., 2013). Generally the lower MICs have been observed for *S. dysgalactiae* as compared to other mastitis pathogens (Tenhagen et al., 2006). Whilst in our study and in Germany erythromycin resistance was around 10 % (GERM-Vet, 2018; Minst et al., 2012), for Sweden an absence of resistance was reported (Bengtsson et al., 2009; Persson et al., 2011); for Swiss isolates 6.5 % resistance was reported (Overesch et al., 2013). Similarly, whereas tetracycline resistance was high in this and the preceding survey (43.2 and 56.8 %) as well as in German and Swiss work (19–72 % and 65.2 %, respectively) (de Jong et al., 2018; Minst et al., 2012; GERM-Vet, 2018), it was much lower (12 %) in Sweden (Bengtsson et al., 2009; Persson et al., 2011). Hence, marked differences seem to exist for at least part of the antimicrobial compounds among EU countries. Additional European *S. dysgalactiae* data is limited, but in a North American monitoring survey on mastitis pathogens a resistance percentage of 4.2 % was recorded for erythromycin; pirlimycin resistance amounted to 10.6 %, whereas tetracycline was not included in the study (Lindeman et al., 2013).

MIC data are rather limited as to *S. agalactiae*, *T. pyogenes* and *Corynebacterium* spp. Penicillins are recommended for treatment of intra-mammary infections caused by *S. agalactiae,* but an accurate classification into susceptible or resistant strains is not feasible due to the lack of interpretive criteria for this class of antimicrobials. German data as to *S. agalactiae* revealed – as for *S. dysgalactiae* – a very high resistance to tetracycline (48–72 %); for erythromycin and pirlimycin, two other agents recommended for medication of *S. agalactiae* infections, resistance was around 10 % and varied from 8 to 18 %, respectively (GERM-Vet, 2018). This corresponds rather well with our results. In another study the occurrence of resistance to erythromycin (16.7 %), to penicillin (0%) and to tetracycline (33.3 %) was similar as compared to our study, but the limited number of isolates precludes any conclusions (Bengtsson et al., 2009). A very limited number of studies reporting MIC values is available for *T. pyogenes* because this organism is assumed to be fully susceptible to penicillin, the drug of first choice for *T. pyogenes* intramammary infections. It has been reported that *T. pyogenes* from several indications has not been found resistant to penicillins (Werckenthin et al., 2007). This is compatible with our results showing very low MICs to penicillin and other β-lactam antibiotics (Table 7). In contrast, for neomycin and tetracycline elevated MICs were observed ($MIC_{50/90}$ 8–16 and 8 – 32 µg/mL, respectively), which agrees to the results of Werckenthin et al. (2007). The genetic basis for tetracycline resistance has been analyzed in detail by Billington et al. (2002). For *Corynebacterium* spp. in addition to low MIC values to β-lactam antibiotics, neomycin and tetracycline MICs were contrary to those for *T. pyogenes* also $\leq$ 0.5 µg/mL. In Germany the prevalence of *Corynebacterium bovis* was the most predominant mastitis pathogen, but the antimicrobial susceptibility has not been reported (Tenhagen et al., 2006).

The present study demonstrates that for a few compounds the resistance rates have significantly increased when compared to 2009–2012; for most drug/microorganism combinations resistance rates remained stationary whereas for one compound a significant decrease was noted compared to 2002–2006. For all 26 antimicrobial agents marked shifts of MIC_{50} were absent. The large German and French national surveys also suggest that overall the susceptibility of the major udder pathogens has not essentially changed over the past decade (GERM-Vet, 2018; RESAPATH, 2019). Recently Boireau et al. (2018) have investigated the changes of levels of resistance over time by analyzing the RESAPATH susceptibility data of the three major mastitis pathogens (*E. coli,* coagulase-positive staphylococci, *S. uberis*) for several commonly used antimicrobials in France. This extensive analysis was based on 27,888 antibiograms over the time period 2006 – 2016. Although the analysis is based on disks results and based on national breakpoints, the resistance trends are meaningful because the methodology remained similar over the whole period. With few exceptions the trends were stationary for *E. coli*, for *S. uberis* some significant non-linear variations without a common pattern were observed whereas for coagulase-positive staphylococci trends were stationary or decreasing. Similarly, in North American studies trends are in general stationary (Barlow, 2011; Lindeman et al., 2013; Awosile et al., 2018).

As applies for any monitoring programme, there are some limitations to the study design of the VetPath project. For instance, the number of countries and isolates per country differed slightly from the preceding VetPath surveys hampering comparison between the time periods. The current study comprises a total of 1244 clinical isolates and, while this is a substantial number, it remains a small representative sample of the total mastitis pathogen population in the EU and is too small to draw definitive conclusions. The sample size per country precludes to perform meaningful country comparisons. A small portion of isolates were from animals with unknown clinical history. However, the major pitfall is the lack of clinical breakpoints. In our study only pirlimycin has veterinary-specific mastitis breakpoints against Gram-positive mastitis pathogens. To improve the interpretation of our data, we have applied breakpoints defined for infections in humans not related to intra-mammary infections, but included in the CLSI document VET08. This has resulted in additionally up to five compounds (depending on the pathogen) which MIC distribution could be categorized in terms of susceptibility and resistance. Thus, categorization of susceptibility and resistance still relies on clinical breakpoints developed for humans, the validity of which has not been established in veterinary medicine. As a result, the reported frequencies of *in vitro* antibiotic resistance reported herein, or in other studies, must be interpreted cautiously in view of clinical efficacy. Despite these shortfalls, inherent in all surveillance studies, we are unaware of any European collection of mastitis isolates that is as representative of the European population of dairy cattle, both in size and geographic diversity and conducted over a long period of time.

5. Conclusions

The present VetPath study aligns with published literature in that there is little resistance to most antimicrobials commonly used to treat bovine mastitis. This study shows that in Europe mastitis pathogens were susceptible to most antimicrobials with exceptions of staphylococci against penicillin and streptococci against erythromycin or tetracycline. In the present study for only one antimicrobial the breakpoint was mastitis-specific, some others still rely on breakpoints based

on data specific for humans whereas for the majority no breakpoint was available. The lack of clinical breakpoints emphasizes the need for establishing additional mastitis-specific clinical breakpoints to ensure a correct interpretation of the results for their use in the field.

Declaration of Competing Interest

Some authors are connected with pharmaceutical companies, however the testing, interpretation of results and preparation of the manuscript have been carried out independently.

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