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Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period

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Objectives: The objective of this study was to investigate the evolution of resistance to antimicrobials, corresponding mechanisms and molecular characteristics of *Staphylococcus* spp., between 1999 and 2014.

Methods: Susceptibility to 38 antimicrobials was determined for 632 clinical staphylococcal isolates obtained from companion animals (dogs, cats, horses and other animals). Twenty antimicrobial resistance genes, including *mecA* and *mecC*, were screened by PCR. Methicillin-resistant staphylococci were characterized by *spa* (*Staphylococcus aureus*), SCC*mec*, MLST and PFGE typing. Statistical analyses were performed using SAS v9.3 and differences were considered relevant if $P \leq 0.05$.

Results: The *mecA* gene was identified in 74 staphylococcal isolates (11.6%): 11 MRSA (40.7%), 40 methicillin-resistant *Staphylococcus pseudintermedius* (MRSP; 8.7%) and 23 methicillin-resistant CoNS (26.7%). Resistance to the majority of antimicrobials and the number of *mecA*-positive isolates increased significantly over time. Eighteen *spa* types were identified, including two new ones. MRSA isolates were divided into three PFGE clusters that included ST22-IV, ST105-II, ST398-V and ST5-VI. Most methicillin-resistant *Staphylococcus epidermidis* isolates were of clonal complex (CC) 5, including a new ST, and clustered in eight PFGE clusters. MRSP were grouped into five PFGE clusters and included ST45-NT, ST71-II-III, ST195-III, ST196-V, ST339-NT, ST342-IV and the new ST400-III. Methicillin-resistant *Staphylococcus haemolyticus* clustered in two PFGE clusters.

Conclusions: The significant increase in antimicrobial-resistant and *mecA*-positive isolates in recent years is worrying. Furthermore, several isolates are MDR, which complicates antimicrobial treatment and increases the risk of transfer to humans or human isolates. Several clonal lineages of MRSA and methicillin-resistant *S. epidermidis* circulating in human hospitals and the community were found, suggesting that companion animals can become infected with and contribute to the dissemination of highly successful human clones. Urgent measures, such as determination of clinical breakpoints and guidelines for antimicrobial use, are needed.

Introduction

Staphylococci are a group of bacteria with clinical, veterinary, agricultural and economic importance because of their wide range of virulence factors and ability to become resistant to antimicrobials. This feature should be considered and antimicrobial susceptibility testing is important to monitor the spread of antimicrobial-resistant staphylococci.[1] Therefore, monitoring programmes may help uncover new resistance trends and evaluate the usefulness of the available antimicrobials against staphylococci. Companion animals, in particular, are frequently treated with the same antimicrobial classes used in human medicine.[2,3]

The genus *Staphylococcus* causes a different array of infections and the most common species in companion animal practice are the coagulase-positive *Staphylococcus pseudintermedius* (formerly called *Staphylococcus intermedius*), *Staphylococcus schleiferi* and *Staphylococcus aureus*. These are mostly found in skin samples, ear samples and as the cause of urinary tract infections (UTIs).[1] CoNS, on the other hand, are usually not considered pathogenic, although they are often considered reservoirs of antimicrobial resistance genes, e.g. the *mecA* gene.[4] Presence of the *mecA* or *mecC* genes is one of the most significant features encountered in staphylococcal species. These genes mediate resistance to β-lactams, which are first-line antimicrobial choices for the treatment of infections in human and veterinary medicine and are considered by the WHO as 'critically important' antimicrobials.[5] Furthermore, knowledge on the genotype of such isolates is important to assess the risk of transfer of methicillin-resistant staphylococcal isolates between companion animals and humans.

The main objective of this study was to investigate the trends in antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period (1999–2014). Furthermore, we identified the genetic mechanisms underlying the antimicrobial resistance. Finally, we characterized the genotypes of the methicillin-resistant staphylococci to understand evolutionary steps driving the spread of these isolates in companion animals.

Materials and methods

Isolate collection

Six hundred and thirty-two staphylococcal isolates obtained from companion animals between 1999 and 2014 were included in the study. The isolates were collected at the Clinical Laboratory and the Antimicrobial and Biocide Resistance Laboratory, FMV-UL, which receive samples from the Veterinary Teaching Hospital of FMV-UL and private practices throughout the Lisbon region. The isolates were obtained from clinical infections and sent to the laboratory along with a small form including animal data, such as species, breed, age and sex, clinical description of the sample site and suspected pathology course. Each isolate was considered individually and cases where more than one isolate originated from the same animal (i.e. different staphylococci isolated at the same time from the same specimen or at different sampling times) were considered only if the staphylococcal species or genotype differed between isolates.

Staphylococcal species identification

Both the Clinical Laboratory and the Antimicrobial and Biocide Resistance Laboratory use phenotypic tests (BD™ BBL™ Crystal Gram Positive ID Kit; Becton, Dickinson and Company, MD, USA) to determine the staphylococcal species. All species were confirmed by PCR (*Staphylococcus epidermidis*-specific primers Se705-1/Se705-2, *Staphylococcus saprophyticus*-specific primers Sap1/Sap2, *Staphylococcus xylosus*-specific primers XYL F/XYL R and *Staphylococcus simulans*-specific primers SimF/SimR)[6–10] and/or sequencing of the 16S rRNA gene. The sequences were compared using the nucleotide basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/).

Antimicrobial susceptibility testing

All isolates were tested by disc diffusion according to CLSI standards and *S. aureus* ATCC 29213 was used for quality control purposes whenever a new antimicrobial batch was used.[11] A total of 38 antimicrobials (Oxoid, Hampshire, UK) was tested: amikacin (30 μg), ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefalotin (30 μg), cefotaxime (30 μg), cefovecin (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), enrofloxacin (5 μg), erythromycin (15 μg), florfenicol (30 μg), fusidic acid (10 μg), gentamicin (10 μg), kanamycin (30 μg), levofloxacin (5 μg), linezolid (30 μg), moxifloxacin (5 μg), mupirocin (5 μg), neomycin (30 μg), netilmicin (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), ofloxacin (5 μg), oxacillin (1 μg), penicillin G (10 U), quinupristin/dalfopristin (15 μg), rifampicin (5 μg), streptomycin (15 μg), sulphonamides (300 μg), teicoplanin (30 μg), tetracycline (30 μg), tobramycin (10 μg), trimethoprim (5 μg), trimethoprim/sulfamethoxazole (25 μg) and vancomycin (30 μg). Some antimicrobials were included since they are clinically relevant antimicrobial agents and others were included for antimicrobial resistance epidemiology purposes (e.g. linezolid, quinupristin/dalfopristin and teicoplanin). Results were interpreted according to CLSI VET01-S2[12] (oxacillin for *S. pseudintermedius*, enrofloxacin, gentamicin and clindamycin), CLSI M100-S24[13] (ampicillin, penicillin, cefoxitin for *S. aureus* and CoNS, teicoplanin, amikacin, kanamycin, netilmicin, tobramycin, erythromycin, tetracycline, ciprofloxacin, levofloxacin, norfloxacin, ofloxacin, moxifloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole, sulphonamides, trimethoprim, chloramphenicol, rifampicin, quinupristin/dalfopristin, linezolid), CA-SFM VET-10[14] (neomycin) and CA-SFM 10[15] (streptomycin and mupirocin). EUCAST guidelines[16] were used for fusidic acid interpretation. Breakpoints for amoxicillin/clavulanic acid, cephalosporins and vancomycin were recently removed from CLSI guidelines. However, as these antimicrobials were included in the susceptibility panel of both laboratories, we used the breakpoints given by the last CLSI document containing them (CLSI M100-S16[17] and CLSI M100-S22[18]). The breakpoints (mm) for cefovecin were retrieved from the manufacturer ($S \geq 24$, I 21–23 and $R \leq 20$). There are no breakpoints for florfenicol against staphylococci and we assessed the distribution of the zone diameters (mm) detected in our study (Figure S1, available as Supplementary data at *JAC* Online), estimating a resistance breakpoint of $R < 19$. A bacterial isolate was considered MDR when it exhibited resistance to three or more antimicrobial classes.[19] Isolates with intermediate susceptibility were regarded as susceptible.

Detection of antimicrobial resistance genes

The presence of the *mecA* and *mecC* genes was tested in all staphylococcal isolates. Other antimicrobial resistance genes were investigated only when phenotypic resistance was observed. Genes previously reported for resistance to β-lactams (*blaZ*), aminoglycosides (*aadE*, *aadD*, *aphA3* and *aacA-aphD*), macrolides/lincosamides [*erm*(A), *erm*(B), *erm*(C), *msrA* and *mph*(C)], tetracyclines [*tet*(M) and *tet*(K)], fusidic acid (*fusB* and *fusC*), chloramphenicol (*cat* pC221), florfenicol (*fexA*) and trimethoprim [*dfr*(G) and *dfr*(K)] were detected by PCR.[20,21]

Molecular characterization

All *S. aureus* isolates were subjected to *spa* typing and *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/). MLST was performed on the MRSA, methicillin-resistant *S. pseudintermedius* (MRSP; representative isolates determined by PFGE) and methicillin-resistant *S. epidermidis* (MRSE) isolates (http://www.mlst.net/databases/default.asp; http://pubmlst.org/databases/). SCC*mec* types were determined as described previously.[22] The methicillin-resistant staphylococcal isolates were compared by SmaI-PFGE using previously described protocols.[21–23] PFGE clusters were defined when the isolates had ≥80% similarity.

Statistical analyses

Statistical analyses were performed with SAS software version 9.3 (SAS Institute, Cary, NC, USA) and results were considered significant when $P \leq 0.05$. For the purpose of statistical analyses, we defined staphylococcal species as *S. aureus*, *S. pseudintermedius*, *S. schleiferi* and CoNS. The association between staphylococcal species, antimicrobial resistance and resistance genes was assessed using either the χ^2 test or Fisher's exact test (when $n \leq 5$).

The importance of animal species (dog or cat), type of infection (pyoderma, UTI or otitis) and age within animal species as possible risk factors for resistance to the different antimicrobials was analysed by logistic regression, considering each factor individually.

The evolution over time of the proportion of isolates resistant to different antimicrobials was analysed by logistic regression, using year as the independent variable. This test was also used to determine whether there was a significant increase in the proportion of *mecA*-positive isolates over time.

Results

This research study involved a total of 632 staphylococcal isolates from 614 animals, of which 537 isolates were from dogs (87.4%), 80 from cats (13.0%), 10 from horses (1.6%) and 5 from other

Table 1. Staphylococcal species distribution and frequency of the *mecA* gene

Staphylococcal species	Frequency (%)	Frequency of the *mecA* gene (%)
S. aureus	27 (4.3)	11 (40.7)
S. caprae	1 (0.2)	1 (100)
S. cohnii	2 (0.3)	0 (0.0)
S. epidermidis	20 (3.2)	11 (55.0)
S. felis	26 (4.1)	0 (0.0)
S. haemolyticus	13 (2.1)	8 (61.5)
S. hominis	1 (0.2)	1 (100)
S. kloosi	1 (0.2)	0 (0.0)
S. lentus	2 (0.3)	1 (50.0)
S. lugdunensis	1 (0.2)	0 (0.0)
S. pseudintermedius	446 (70.6)	40 (8.7)
S. saprophyticus	4 (0.6)	0 (0.0)
S. schleiferi	73 (11.6)	0 (0.0)
S. simulans	9 (1.4)	1 (11.1)
S. warneri	3 (0.5)	0 (0.0)
S. xylosus	3 (0.5)	0 (0.0)

animals (0.8%). Overall, 252 isolates were from females (41.0%), 346 from males (56.4%) and 34 were unknown (5.5%). The average age was 6.8 years for dogs, 5.5 for cats and 11.4 for horses.

Isolates were most frequently isolated from otitis (307 isolates, 48.6%), followed by 178 isolates from pyoderma (28.2%), 90 from UTI (14.2%), 10 from surgical site infections (1.6%) and 47 from other types of infection (7.4%). The frequency of each staphylococcal species is shown in Table 1, with a clear predominance of *S. pseudintermedius*, with 446 isolates (70.6%), followed by CoNS species with 86 isolates (13.6%), *S. schleiferi* with 73 isolates (11.6%) and 27 isolates of *S. aureus* (4.3%).

The frequencies of antimicrobial resistance and antimicrobial resistance genes are shown in Tables 2 and 3, respectively. All isolates were susceptible to vancomycin, teicoplanin, linezolid, netilmicin and quinupristin/dalfopristin. About 35% of the staphylococcal isolates were MDR. The *mecA* gene was identified in 74 staphylococcal isolates (11.6%): 11 *S. aureus* (40.7%), 40 *S. pseudintermedius* (8.7%) and 23 CoNS (26.7%) (Table 3). None of the isolates carried the *mecC* gene. We detected the *fexA* gene in three isolates that were resistant to florfenicol and chloramphenicol (one *S. aureus* and two *S. pseudintermedius*). In these *fexA*-positive isolates, we searched for the *cfr* gene and detected, for the first time, the *cfr* gene in an *S. pseudintermedius* isolate (confirmed by sequencing).

The CoNS and *S. aureus* isolates had higher probabilities of having the *mecA* gene ($P<0.0001$) than *S. pseudintermedius* or *S. schleiferi* (40.7% and 26.7% versus 8.7% and 0%, respectively; Table 3). Resistance to ampicillin/penicillin and the presence of the *blaZ* gene were highly associated with both *S. aureus* and *S. pseudintermedius* ($P<0.001$; Tables 2 and 3, respectively). *S. aureus* were more resistant to fluoroquinolones [enrofloxacin ($P<0.0001$), ciprofloxacin ($P<0.0001$), levofloxacin ($P<0.0001$), norfloxacin ($P<0.0001$), ofloxacin ($P<0.0001$) and moxifloxacin ($P<0.0001$)] than the other species (Table 2). *S. pseudintermedius* were more likely ($P<0.05$) to have the *erm*(B) gene and less likely ($P<0.05$) to have the *erm*(C) gene than any other species (Table 3). Streptomycin resistance was more associated with *S. pseudintermedius* ($P<0.02$; Table 2) and the *aadE* gene was only present in this species (Table 3). On the other hand, tetracycline resistance was associated with CoNS ($P<0.02$) and *S. pseudintermedius* ($P<0.0003$; Table 2), with the *tet*(K) gene more associated with CoNS ($P<0.006$) and the *tet*(M) gene with *S. pseudintermedius* ($P<0.0001$; Table 3). Resistance to fusidic acid was higher in the CoNS isolates ($P<0.02$; Table 2).

The variables gender and age were not considered risk factors ($P>0.05$) leading to more antimicrobial resistance. However, isolates from dogs were resistant to more antimicrobials (Table 4) than isolates from cats ($P<0.05$). Likewise, staphylococcal isolates from otitis were resistant to more antimicrobials than isolates from pyoderma or UTI ($P<0.05$; Table 4). No significant differences were found between isolates from pyoderma and isolates from UTI ($P>0.05$).

Using logistic regression analysis, we assessed trends over time in the resistance to the different antimicrobials. Among the 38 antimicrobials analysed, resistance increased over the period analysed ($P<0.05$) in 27 antimicrobials (Figure 1) and the number of isolates with resistance to at least one antimicrobial or with multidrug resistance also increased over time ($P<0.05$; Figure 1). The number of *mecA*-positive isolates also increased over time ($P<0.05$; Figure 2). The antimicrobials where resistance did not increase significantly ($P>0.05$) over the 16 year period under analysis were amikacin, florfenicol, fusidic acid, mupirocin, nitrofurantoin and rifampicin. The corresponding OR, CI and *P* values are given in Table S1.

The characteristics of all methicillin-resistant staphylococcal isolates are shown in Table S2. Eighteen *spa* types were identified in *S. aureus* (t002, t025, t032, t044, t084, t085, t091, t105, t108, t148, t311, t1294, t1346, t1897, t2357 and t11188), including two new *spa* types (t14112 and t14113). The MRSA isolates were divided into three PFGE clusters (Figure S2) and MLST included ST22-IV ($n=8$, including t025, t032 and t2357), ST105-II ($n=1$, t002), ST398-V ($n=1$, t108) and ST5-VI ($n=1$, t311). Isolates ST5 and ST105 belonged to clonal complex (CC) 5.

Most MRSE isolates were members of CC5: ST2-NT ($n=2$), ST5-NT ($n=2$), ST20-NT ($n=1$), ST23-IV ($n=1$), ST35-NT ($n=1$), ST57-IV ($n=1$), ST190-NT ($n=1$) and a new ST ($n=1$), which carried an SCC*mec* II. The MRSE isolates were divided into eight PFGE clusters (Figure S2), with the two MRSE ST2 isolates having <80% similarity by PFGE.

MRSP were grouped into five PFGE clusters (Figure S2) and included ST45 ($n=1$), ST71 ($n=13$), ST195 ($n=1$), ST196 ($n=1$), ST203 ($n=1$), ST339 ($n=1$), ST342 ($n=2$) and a new ST, assigned ST400 ($n=3$). ST71, ST195 and ST203 belonged to CC71, while ST342 belonged to CC261, ST45 to CC45, ST196 to CC196 and ST339 to CC84. The isolates for which MLST was not performed grouped in the same PFGE cluster and thus we assumed they belonged to CC71. The MRSP ST45 strain was non-typeable by SCC*mec* typing and SmaI-PFGE macrorestriction.

Methicillin-resistant *Staphylococcus haemolyticus* were divided into two PFGE clusters (Figure S2) and SCC*mec* V ($n=5$) was the most frequent type, followed by NT ($n=3$).

Discussion

Recent studies have evaluated antimicrobial resistance in *S. pseudintermedius*.[2,24,25] However, none of these studies combined all

Table 2. Frequency of antimicrobial resistance for the total isolates and per staphylococcal isolate

Antimicrobial	Percentage of resistance in all isolates (CI)	Percentage of resistance in CoNS	Percentage of resistance in *S. aureus*	Percentage of resistance in *S. pseudintermedius*	Percentage of resistance in *S. schleiferi*	*P*
Ampicillin	58.7 (54.7–62.6)	40.7	77.8	64.4	38.4	<0.0001
Penicillin	58.7 (54.7–62.6)	40.7	77.8	64.4	38.4	<0.0001
Amoxicillin/clavulanic acid	7.8 (5.8–10.1)	5.8	37.0	7.6	0.0	<0.0001
Cefalexin	6.0 (4.3–8.2)	2.3	22.2	6.7	0.0	0.0002
Cefovecin	10.4 (8.2–13.1)	19.8	40.7	8.5	0.0	<0.0001
Ceftriaxone	8.5 (6.5–11.0)	11.6	37.0	7.6	0.0	<0.0001
Cefotaxime	8.5 (6.5–11.0)	10.5	37.0	7.9	0.0	<0.0001
Cefoxitin[a]	28.3 (20.2–37.6)	24.4	40.7	—	—	0.1005
Oxacillin[b]	8.7 (6.3–11.8)	—	—	8.7	—	—
Enrofloxacin	12.3 (9.9–15.2)	14.0	40.7	9.2	19.2	<0.0001
Ciprofloxacin	12.3 (9.9–15.2)	14.0	40.7	9.2	19.2	<0.0001
Levofloxacin	11.4 (9.0–14.1)	14.0	40.7	9.0	12.3	<0.0001
Norfloxacin	12.7 (10.2–15.5)	16.3	40.7	9.2	19.2	<0.0001
Ofloxacin	12.8 (10.3–15.7)	16.3	40.7	9.2	20.6	<0.0001
Moxifloxacin	10.1 (7.9–12.8)	11.6	40.7	8.1	9.6	<0.0001
Tetracycline	34.8 (31.1–38.9)	23.3	3.7	44.0	4.1	<0.0001
Nitrofurantoin	0.5 (0.1–1.4)	0.0	0.0	0.7	0.0	0.7393
Chloramphenicol	4.6 (3.1–6.5)	1.2	3.7	6.1	0.0	0.0450
Florfenicol	0.5 (0.1–1.4)	0.0	3.7	0.5	0.0	0.0812
Gentamicin	7.6 (5.7–9.9)	11.6	0.0	8.3	1.4	0.0358
Neomycin	14.9 (12.2–17.9)	5.8	7.4	19.1	2.7	<0.0001
Tobramycin	7.1 (5.2–9.4)	9.3	0.0	8.1	1.4	0.0735
Amikacin	0.3 (0.0–1.1)	2.3	0.0	0.0	0.0	0.0052
Kanamycin	18.7 (15.7–21.9)	12.8	3.7	23.3	2.7	<0.0001
Streptomycin	20.6 (17.5–23.9)	8.1	7.4	26.7	2.7	<0.0001
Erythromycin	20.1 (17.0–23.4)	25.6	11.1	22.2	4.1	0.0012
Clindamycin	17.1 (14.2–20.3)	12.8	7.4	20.6	4.1	0.0014
Fusidic acid	4.1 (2.7–6.0)	24.4	3.7	0.9	0.0	<0.0001
Mupirocin	0.2 (0.0–0.9)	0.0	0.0	0.0	1.4	0.0534
Sulfamethoxazole/trimethoprim	13.3 (10.7–16.2)	8.1	0.0	16.4	5.5	0.0034
Sulphonamides	46.8 (43.0–50.7)	29.1	11.1	54.3	35.6	<0.0001
Trimethoprim	16.9 (14.1–20.1)	12.8	3.7	20.0	8.2	0.0105
Rifampicin	1.9 (1.0–3.3)	3.5	0.0	1.4	4.1	0.2272
Resistance to at least one antimicrobial	79.4 (76.1–82.5)	74.4	81.5	82.7	64.4	0.0023
Resistance to at least three antimicrobials	35.0 (31.3–38.8)	34.9	25.9	39.0	13.7	0.0003

The *P* value refers to the association between antimicrobial resistance and staphylococcal species.
All isolates were susceptible to netilmicin, vancomycin, teicoplanin, linezolid and quinupristin/dalfopristin.
[a]Used for *S. aureus* and CoNS.
[b]Used for *S. pseudintermedius*.

staphylococcal species found in clinical specimens, susceptibility for several antimicrobials, corresponding resistance mechanisms and, most importantly, molecular epidemiology of methicillin-resistant staphylococci. Moreover, we studied a long time period, 16 years, to establish trends in antimicrobial resistance and changes in genotypes.

The overall prevalence of clinical methicillin-resistant staphylococcal isolates found in this study was 11.7%, which is higher than the prevalence found in a similar study conducted in Lithuania (5.3%).[26] This difference could be due to a higher consumption of first- and second-generation cephalosporins in Portugal compared with Lithuania.[27] However, the prevalence of MRSA within *S. aureus* isolates (40.7%) and MRSP within *S. pseudintermedius* (8.7%) was similar to two studies conducted in Germany (ranging from 41.3% to 62.7% MRSA in *S. aureus* of canine, feline and equine origin; and 6.3% of MRSP within isolates belonging to the *S. intermedius* group).[28,29] In Italy, a much higher prevalence of MRSP was found in clinical samples (21% of MRSP within isolates belonging to the *S. intermedius* group).[30] Interestingly, Germany and Portugal have similar consumptions of first- and second-generation cephalosporins, but in Italy it is higher.[27] We could speculate that cephalosporins may select for methicillin-resistant isolates, but further studies are needed.

Table 3. Frequency of resistance genes for the total isolates and per staphylococcal species

Resistance gene	Percentage of the resistance gene in all isolates (CI)	Percentage of the resistance gene in CoNS	Percentage of the resistance gene in *S. aureus*	Percentage of the resistance gene in *S. pseudintermedius*	Percentage of the resistance gene in *S. schleiferi*	*P*
mecA	11.6 (9.2–14.3)	26.7	40.7	8.7	0.0	<0.0001
blaZ	59.0 (55.1–62.9)	40.7	77.8	64.6	39.7	<0.0001
erm(A)	0.8 (0.3–1.8)	4.7	3.7	0.0	0.0	<0.0001
erm(B)	18.2 (15.3–21.4)	12.8	3.7	22.4	4.1	0.0001
erm(C)	2.4 (1.3–3.9)	11.6	7.4	0.2	2.7	<0.0001
cat pC221	4.1 (2.7–6.0)	1.2	0.0	5.6	0.0	0.0335
aphA3	18.0 (15.1–21.3)	7.0	11.1	23.3	1.4	<0.0001
aacA-aphD	7.6 (5.7–9.9)	11.6	3.7	8.1	1.4	0.0803
aadD	0.5 (0.0–1.0)	3.5	0.0	0.0	0.0	0.0003
aadE	16.3 (13.5–19.4)	0.0	0.0	23.1	0.0	<0.0001
tet(K)	9.3 (7.2–11.9)	20.9	0.0	9.2	0.0	<0.0001
tet(M)	27.5 (24.1–31.2)	4.7	3.7	37.2	4.1	<0.0001
dfr(K)	0.2 (0.0–0.9)	0.0	0.0	0.2	0.0	0.9366
dfr(G)	7.0 (5.1–9.2)	3.5	3.7	9.0	0.0	0.0163
msrA	1.9 (1.0–3.3)	9.3	3.7	0.5	1.4	<0.0001
mph(C)	2.2 (1.2–3.7)	10.5	3.7	0.7	1.4	<0.0001
fusB	2.1 (1.1–3.5)	12.8	0.0	0.2	1.4	<0.0001
fusC	0.8 (0.3–1.8)	4.7	3.7	0.0	0.0	<0.0001

The *P* value refers to the association between antimicrobial resistance genes and staphylococcal species.

Most of our isolates were characterized as *S. pseudintermedius*, which was expected since most isolates were from dogs, where this species is the most frequently found.[1] Recently, two studies evaluated antimicrobial resistance in *S. pseudintermedius* over time and detected trends of increasing resistance to ampicillin/amoxicillin/penicillin, cefovecin, cefalexin, enrofloxacin, clindamycin and sulfamethoxazole/trimethoprim.[24,25] In our study, trends of increasing resistance to these antimicrobials were also observed, but we detected other trends of increasing resistance, including cefoxitin in *S. aureus* and CoNS, oxacillin in *S. pseudintermedius* and ciprofloxacin, norfloxacin, ofloxacin, moxifloxacin, tetracycline, chloramphenicol, gentamicin, neomycin, tobramycin, kanamycin, streptomycin, erythromycin, sulphonamides and trimethoprim in all staphylococcal groups analysed. Moreover, increasing trends of resistance to at least one antimicrobial and multidrug resistance were also identified, such that ∼35% of the staphylococcal isolates were MDR. The most common multidrug resistance pattern among the methicillin-susceptible isolates was ampicillin/penicillin/tetracycline/sulphonamides and in methicillin-resistant isolates it was β-lactams/fluoroquinolones/tetracycline. These resistance profiles are in accordance with the antimicrobial usage patterns in companion animal practice in Portugal.[27] In fact, penicillins+β-lactamase inhibitors, first- and second-generation cephalosporins, fluoroquinolones and tetracyclines are among the antimicrobials most used (in this order) by companion animal practitioners in this country.[27]

Isolates from otitis were more resistant to several antimicrobials and more often carried the *mecA* gene than isolates from pyoderma or UTI. This finding suggests that these antimicrobials are probably being used inappropriately for the treatment of otitis. The recommended treatment option for otitis externa is antiseptics; however, in some cases (e.g. ulceration and/or tympanic membrane rupture) there is a need for administration of systemic antimicrobial therapy and the first-line antimicrobials are β-lactams or fluoroquinolones.[31–33] However, antimicrobials that are used systemically for otitis are unlikely to achieve therapeutic concentrations within the fluid and waxy exudates of the external canals in which the infectious organisms are harboured.[26] Our study probably reflects the selective pressure imposed on staphylococci in the ear by the use of these antimicrobials, including the higher frequency of *mecA*-positive staphylococci, further supporting the urgent need for more studies on the efficacy of systemic antimicrobials for ear infections.

There are no cefoxitin or oxacillin recommended breakpoints for *S. schleiferi* subsp. *coagulans*. However, these breakpoints are important for diagnostic purposes, since this species is very common in companion animals. Although we did not find *mecA*-positive isolates, there are already descriptions of methicillin-resistant *S. schleiferi* subsp. *coagulans* isolates[27] and therefore there is an urgent need to determine appropriate breakpoints for this species. Moreover, with the increasing frequency of MDR strains, some antimicrobials are being suggested as second-line antimicrobial agents, namely florfenicol, amikacin, minocycline, doxycycline, nitrofurantoin, topical fusidic acid or mupirocin.[34–36] Even some antimicrobials that are used daily in companion animal practice (e.g. ampicillin, cefovecin or sulfamethoxazole/trimethoprim) do not have clinical breakpoints determined for these species. Thus, updated and species-specific clinical breakpoints are essential for the appropriate selection of antimicrobials.

Interestingly, the first *mecA*-positive isolate detected in our study was an MRSA isolated in 2001. ST22-IV, which represents EMRSA-15, was the most common MRSA lineage found in this study. This is in agreement with previous reports, which show that there is a shared population of this lineage infecting/colonizing

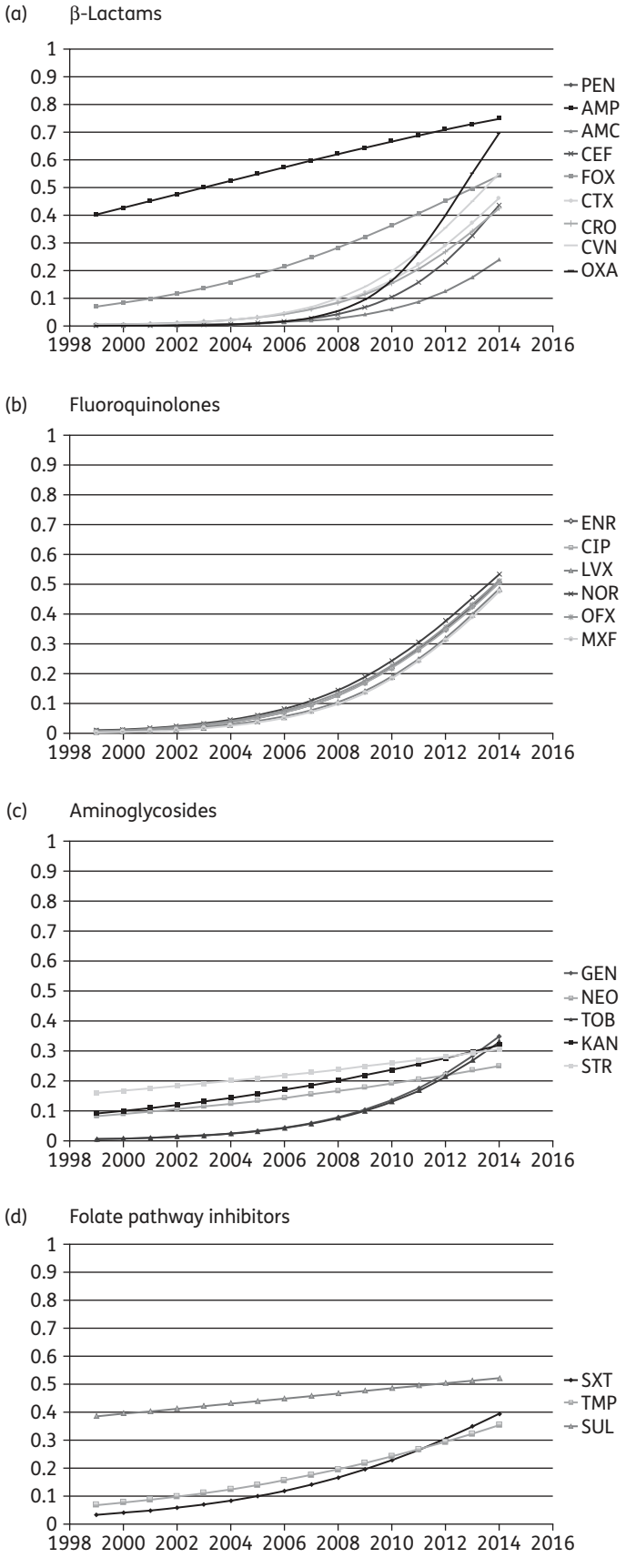
Table 4. Risk factors and corresponding OR, CI and *P* value for the occurrence of significant individual antimicrobial resistance and the *mecA* gene, from logistic regression analyses conducted by animal species and by type of infection

Risk factor	Antimicrobial/gene	OR[a]	CI	*P*
Dogs[b]	OXA	6.4	2.2–18.5	0.0005
	ENR	2.8	1.6–4.9	0.0006
	CIP	2.8	1.6–4.9	0.0006
	LVX	3.1	1.7–5.6	<0.0001
	NOR	3.1	1.8–5.5	<0.0001
	OFX	3.1	1.8–5.4	<0.0001
	MXF	3.1	1.7–5.7	0.0003
	TET	0.3	0.2–0.6	0.0005
	GEN	2.8	1.4–5.5	0.0034
	TOB	3.1	1.5–6.1	0.0016
	FUS	3.9	1.6–9.4	0.0030
	mecA	3.0	1.7–5.4	0.0002
Otitis[c]	FOX	3.7	1.2–11.1	0.0191
	OXA	5.6	2.2–14.4	0.0004
	ENR	2.4	1.3–4.4	0.0035
	CIP	2.4	1.3–4.4	0.0035
	LVX	2.7	1.4–5.0	0.0016
	NOR	2.2	1.2–3.9	0.0077
	OFX	2.1	1.2–3.7	0.0111
	MXF	3.0	1.5–6.0	0.0012
	mecA	5.1	2.6–10.1	<0.0001
Otitis[d]	ENR	2.4	1.2–4.9	0.0173
	CIP	2.4	1.2–4.9	0.0173
	LVX	2.6	1.2–5.4	0.0139
	NOR	2.2	1.1–4.4	0.0313
	OFX	2.1	1.0–4.2	0.0410
	MXF	3.0	1.3–6.7	0.0071
	FUS	4.2	1.4–12.9	0.0114
	mecA	3.1	1.4–7.3	0.0075

CIP, ciprofloxacin; ENR, enrofloxacin; FOX, cefoxitin; FUS, fusidic acid; GEN, gentamicin; LVX, levofloxacin; MXF, moxifloxacin; OXA, oxacillin; NOR, norfloxacin; OFX, ofloxacin; TET, tetracycline; TOB, tobramycin.
[a]OR for the occurrence of isolates resistant to the antimicrobial listed, considering the risk factors of animal species (dogs versus cats) and type of infection (otitis versus pyoderma and otitis versus UTI).
[b]OR relative to isolates from cats.
[c]OR relative to isolates from pyoderma.
[d]OR relative to isolates from UTI.

humans and companion animals.[37] The ST22-t032-SCC*mec* IV isolates were negative for *erm*(C), while the other ST22-IV non-t032 (t2357 and t025) isolates carried the *erm*(C) gene. It is assumed that the loss of this gene is associated with isolates coming from companion animals,[37] which suggests that the MRSA ST22 non-t032 isolates found in our study were acquired from humans very recently and have thus maintained the *erm*(C) gene.

Only one MRSA isolate was ST398-t108-V, a livestock-associated MRSA, and it was isolated from a dog. This isolate had 93% ApaI-PFGE similarity to previously isolated MRSA isolates from calves in Portugal (data not shown).[38] Surprisingly, all these isolates (from dog and calves) carried the *fexA* gene and were resistant to



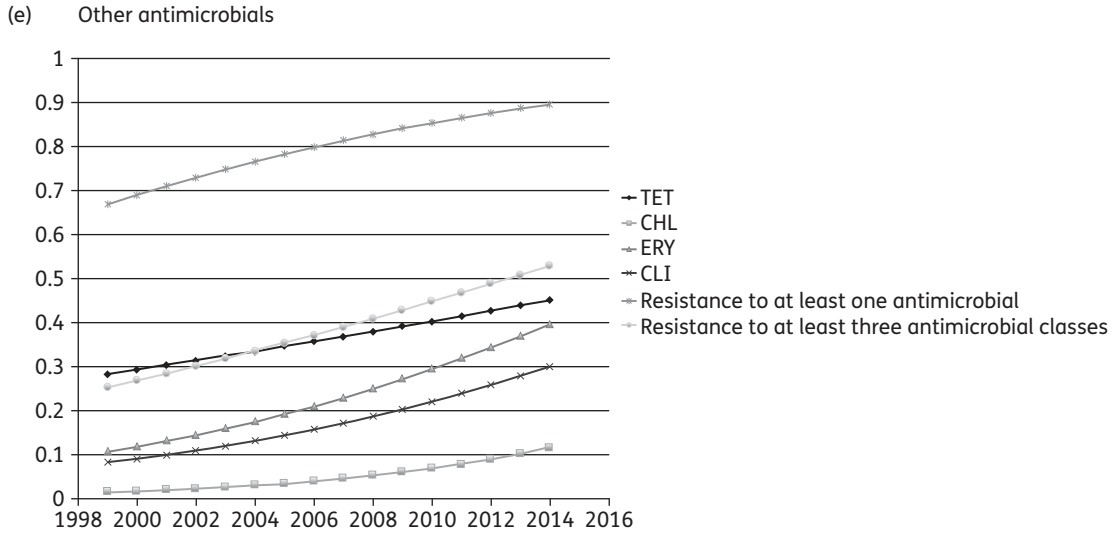


Figure 1. Evolution of antimicrobial resistance over the 16 years studied ($P < 0.05$): (a) β-lactams; (b) fluoroquinolones; (c) aminoglycosides; (d) folate pathway inhibitors; and (e) other antimicrobials. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CEF, cefalotin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CRO, ceftriaxone; CTX, cefotaxime; CVN, cefovecin; ENR, enrofloxacin; ERY, erythromycin; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; MXF, moxifloxacin; NEO, neomycin; NOR, norfloxacin; OFX, ofloxacin; OXA, oxacillin; PEN, penicillin; STR, streptomycin; SUL, sulphonamides; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin.

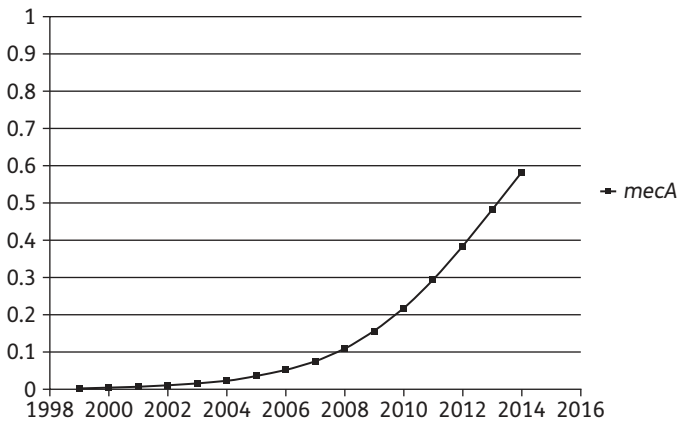


Figure 2. Proportion of *mecA*-positive isolates over the 16 years studied ($P < 0.05$).

fluoroquinolones, which suggests that they had a similar source of infection/colonization.[38] However, the dog in our study had no history of contact with farms or farm animals and therefore this remains to be elucidated. The MRSA ST5-t311-VI isolated from a cat was resistant to fusidic acid and carried the *fusC* gene, as was reported for one MRSA ST5-t062-VI isolated from a horse in a previous study.[22] Comparing the SmaI-PFGE profiles of these two isolates (data not shown) revealed they had 86% similarity. As the cat's strain was isolated in 2001, we could not determine whether there was any history of contact with horses and therefore we could not define the source of infection/colonization. However, it is interesting to notice that several MRSA lineages are disseminated in different animal species.

The first MRSP strain was identified in Portugal in 2007, but only in 2010 was an increase in the number of isolates detected. The first MRSP isolates in Europe were detected in 2005[39] and were ST71-II-III. Interestingly, the first MRSP strain in Portugal, isolated in 2007, was ST196-V. Only in 2009 did the first ST71-II-III appear in Portugal. Between 2009 and mid-2012, MRSP CC71-II-III was the only lineage detected. Yet, in 2013–14 we observed a higher genetic diversity among the MRSP isolates obtained, with other MRSP lineages appearing, including a new ST (ST400) carrying the *mecA* gene. The ST45-NT, ST339-NT and ST342-IV lineages have already been described in recent studies.[2,40] ST45 was the predominant MRSP clonal lineage in Thailand and Israel and was not typeable by SmaI-PFGE and SCC*mec* typing.[40] This lineage carried a novel pseudo-SCC*mec* element, ΨSCC*mec*$_{57395}$, that, besides *mecA*, also carried determinants of resistance to heavy metals such as arsenic, cadmium and copper.[40] It seems that this ST has also been introduced into Europe, as the MLST database reports that ST45 has been detected in England, the Netherlands and now in Portugal. The new ST, ST400, does not belong to any of the previous *mecA*-positive CCs, which suggests that SCC*mec* has been acquired by this ST. Two of the MRSP ST400 isolates were isolated from two dogs that lived in the same kennel. However, the third dog had no connection with these dogs or the kennel, which could mean that this lineage is already spreading through the dog population in Portugal.

The *fexA* gene was detected in three isolates (two *S. pseudintermedius* and one *S. aureus*). The animals (three dogs) infected with these isolates had previously been diagnosed with an infection caused by an MDR MRSA or MRSP strain and thus florfenicol (25–50 mg/kg q12h subcutaneously; Nuflor®, Merck Animal Health, USA) was being used as a last-resort antimicrobial. The use of florfenicol was very recently suggested as a second-line antimicrobial agent in dogs.[34] However, it seems that the use of this antimicrobial can lead to additional acquisition of antimicrobial resistance genes or isolates. Furthermore, one of these isolates (an *S. pseudintermedius*) also carried the *cfr* gene and, to the

best of our knowledge, this is the first description the *cfr* gene in an *S. pseudintermedius* strain isolated from a dog undergoing florfenicol treatment. Although the strain did not exhibit resistance to linezolid, this is a worrisome finding, since it shows *S. pseudintermedius* could be carriers of important resistance genes.

The MRSE STs found in this study were identical to those isolated in humans in Portugal (community- and hospital-acquired isolates).[41] This means that MRSE isolates can circulate between humans and animals, making these a reservoir of important MRSE lineages. Unfortunately, there is no MLST database for *S. haemolyticus*, which makes it impossible to compare our isolates with other animal or even human isolates. Nevertheless, it is important to notice that methicillin-resistant CoNS were more frequently isolated than might be expected from previous studies and the presence of the *mecA* gene was highly associated with these isolates. Furthermore, several methicillin-resistant CoNS exhibited a multidrug resistance pattern, suggesting that they are reservoirs of antimicrobial resistance genes.

The results reported herein might be a biased representation of the reality found in companion animals in Portugal, as they were obtained from a reference laboratory that receives samples from complicated infections observed in private practices. This suggests that our results represent only a small part of the staphylococcal isolates that in reality infect companion animals. However, since our laboratory has been collecting samples since 1999, the increased frequency of antimicrobial resistance over time reported here probably reflects what is happening in the staphylococcal population in general. Additionally, the observed time trend for the various antimicrobials reflects the development of new resistant strains, but also the spread of resistant organisms over time.

This study highlights the importance of companion animals as reservoirs of important antimicrobial-resistant pathogens. In 2005, Heuer *et al*.[42] underlined that the use of antimicrobial drugs in companion animals had received little attention and that monitoring programmes had focused solely on antimicrobial drug consumption in food animals. Ten years later, the European Surveillance of Veterinary Antimicrobial Consumption group reported information on the sales of tablets by veterinary antimicrobial class for companion animals.[27] Yet, no alterations or restrictions in antimicrobial prescription in companion animals have been imposed so far (especially considering critically important antimicrobials). International and national guidelines on antimicrobial use for companion animal practice are urgently needed. Another problem that requires immediate attention is updated and species-specific clinical breakpoints. Together, these features will hopefully improve antimicrobial stewardship and prevent the development of antimicrobial resistance.

The significant increase in antimicrobial-resistant and *mecA*-positive isolates in recent years is worrying. Furthermore, several isolates are MDR, which complicates antimicrobial treatment and raises the risk of transfer to humans or human isolates. Several clonal lineages of MRSA and MRSE circulating in human hospitals and the community were found in this study, suggesting that companion animals can become infected with and contribute to the dissemination of highly successful human clones. Thus, companion animals can act as reservoirs of important human clones, perpetuating the transmission cycle of methicillin-resistant staphylococci between humans and companion animals.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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