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Antimicrobial Resistance of Old and Recent *Staphylococcus aureus* Isolates from Poultry: First Detection of Livestock-Associated Methicillin-Resistant Strain ST398[▽]

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The susceptibilities of 12 antimicrobial agents for two collections of *Staphylococcus aureus*, isolated in the 1970s and in 2006 from poultry, were determined. For eight antibiotics, the percentage of resistance was significantly higher in the recent isolates. Ten recent isolates were methicillin resistant and had *spa* types t011 and t567, belonging to multilocus sequence type 398. This is the first report of "livestock-associated" methicillin resistant *S. aureus* from healthy poultry.

Antimicrobial agents, including penicillin, erythromycin, and tetracyclines, are widely used for treating staphylococcal and other infections in poultry (1, 19, 23). The extensive use of antimicrobial agents in animal husbandry contributes to the selection of drug-resistant strains. Recently, the isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from animals has been reported at an increasing frequency (14, 15, 16, 18, 22, 24). This study analyzes the frequency of acquired resistance to 12 antimicrobial agents, β-lactamase activity, and the prevalence of the *mecA* gene between two groups of *S. aureus* isolates from poultry.

Ninety *S. aureus* isolates were obtained from tendon sheaths of diseased breeder chickens and from the noses and cloacae of healthy broiler breeders between 1970 and 1972 (9) (old isolates), and eighty-one *S. aureus* isolates were collected from the noses and cloacae of healthy chickens derived from 39 randomly selected industrial broiler farms in 2006 (recent isolates). The old isolates had been lyophilized and stored at −20°C until used. To collect the recent isolates, the noses and cloacae of five chickens from each flock were sampled. Samples were inoculated on Columbia agar supplemented with sheep blood, colistin, and nalidixic acid (Oxoid, Basingstoke, United Kingdom). Isolates were identified as *S. aureus* by colony morphology, standard biochemical methods, and growth on modified Baird-Parker medium (10). Multiplex PCR for the *femA* and *mecA* genes was performed to confirm the identification and methicillin resistance of *S. aureus* (17, 22). Susceptibility to oxacillin, penicillin, enrofloxacin, erythromycin, tylosin, lincomycin, gentamicin, neomycin, spectinomycin, sulfonamides, tetracycline, and trimethoprim was determined according to CLSI guidelines by using agar dilution tests (6). For interpretation of MICs, European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.escmid.org/sites/index_f.aspx?par=2.4) wild-type cutoff values were used, except for enrofloxacin and tylosin, for which we used cutoff values from our study, according to the bimodal distribution of MICs (20). β-Lactamase production was tested for the penicillin-resistant isolates by using β-lactamase diagnostic tablets (Rosco, Taastrup, Denmark) according to the manufacturer's instructions. MRSA isolates ($n = 10$) were genotyped by pulsed-field gel electrophoresis (PFGE) after SmaI macrorestriction analysis and by DNA sequence analysis of the polymorphic repeat region of protein A gene (*spa* typing) (8, 13). The *spa* types were determined with RidomStaph software, version 1.3 (Ridom GmbH, Würzburg, Germany) (http://spaserver.ridom.de/). The staphylococcal cassette chromosome *mec* (SCC*mec*) types were determined by multiplex PCR as previously described (25). Representative isolates belonging to different *spa* types were further analyzed by the Multi Locus Sequence Typing facility (http://www.mlst.net) (13).

A comparison of the antimicrobial resistance frequency in both groups for the different antimicrobial agents was performed by means of chi-square and Fisher's exact tests. A significance level of 0.05 was used.

The percentage of resistance was significantly higher for the recent isolates than for the old isolates for all antimicrobial agents tested, except for enrofloxacin, sulfonamides, and tetracycline, where no significant difference was found between results for old and recent isolates, and for penicillin, where the percentage of resistance for the old isolates was significantly higher than that for the recent isolates (Tables 1 and 2). In 91.7% of the recent isolates and 82.2% of the old isolates, acquired resistance to at least one of the antimicrobial agents that were tested was detected. All isolates resistant to penicillin were positive in the β-lactamase test. Ten recent isolates, obtained from chickens originating from five different flocks, showed resistance to oxacillin and were thus classified as MRSA. In all the MRSA isolates, the presence of the *mecA* gene was demonstrated by PCR. The *mecA* gene was absent in

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TABLE 1. Distribution of MICs of various antimicrobial agents for 90 *S. aureus* isolates obtained between 1970 and 1972 (old isolates)

Antimicrobial agent	WT[b] ≤	Number of isolates with a MIC (μg/ml) of:[a]																	%R[d]
		≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	256	512	1,024	>1,024	
Enrofloxacin[c]	2			72	18											—	—	—	—	0
Erythromycin	1			11	36	28								**1**	**14**	—	—	—	—	16.7
Gentamicin	2				32	54	2								**2**	—	—	—	—	2.2
Lincomycin	2				9	37	22	5	**3**		**1**		**1**	**2**	**10**	—	—	—	—	18.9
Neomycin	1				18	66	6									—	—	—	—	0
Oxacillin	2				4	47	35	4								—	—	—	—	0
Penicillin	0.12		31	1	**3**	**52**	**3**									—	—	—	—	64.4
Spectinomycin	128											23	66		**1**	—	—	—	—	1.1
Sulfamethoxazole	128					2		4	32	30	3	6	9	1	—	**3**				3.3
Tetracycline	1				26	17	3					**15**	**29**			—	—	—	—	48.9
Trimethoprim	4			4	37	49										—	—	—	—	0
Tylosin[c]	64					15	27	27	9					**1**	**11**	—	—	—	—	13.3

[a] The results in boldface type exceeded the breakpoint criteria for resistance. —, not applicable.
[b] MIC distribution for the wild-type (WT) organism according to EUCAST and categorized as WT ≤ the indicated number (μg/ml).
[c] Bimodal distribution of MICs (not available in EUCAST). The isolates with MICs in the higher ranges were considered to have acquired resistance.
[d] %R, percentage of isolates resistant to the respective antimicrobial agent.

all other *S. aureus* isolates. The MRSA strains were nontypeable by PFGE analysis using the SmaI restriction enzyme, showing a pattern with no attributable fragment (3). They exhibited *spa* types t011 ($n = 8$) and t567 ($n = 2$) and belonged to sequence type 398 (ST398). An analysis of SCC*mec* cassettes showed that from the strains belonging to *spa* type t011, six strains were nontypeable and two presented SCC*mec* types IVa and V, respectively. Both strains belonging to *spa* type t567 possessed SCC*mec* type III. Besides their resistance to β-lactam antimicrobials, all MRSA isolates showed resistance to erythromycin, lincomycin, tylosin, tetracycline, and trimethoprim. Seven strains were additionally resistant to gentamicin and neomycin, and five strains showed resistance to spectinomycin.

The occurrence of antimicrobial resistance among the recent and old isolates indicates that antimicrobial resistance in staphylococci of poultry origin has increased over time in Belgian industrial farms. This may be due to the frequent use of antimicrobial agents in poultry husbandry. Indeed, antimicrobial agents have been administered for many years, not only to control and prevent disease but also for growth promotion and improved feed conversion efficiency (4, 5, 11). However, since January 2006, all growth promoters in the feed have been forbidden in the European Union (2). In Belgium, the antimicrobial agents used most often for the treatment of disease in poultry are β-lactam antibiotics, fluoroquinolones, tetracyclines, macrolides, lincosamides, trimethoprim/sulfonamides, and colistin. For specific treatment of staphylococcal infections in poultry, veterinarians generally use penicillin, erythromycin, and tetracycline (19, 23). From our study, it appears that antimicrobial resistance to these agents is quite common in *S. aureus* isolates from poultry in Belgium.

In the present study, the percentage of β-lactamase-producing *S. aureus* isolates was significantly higher in the old collection than in the recent one. The reason this result is the converse of that for the other antimicrobials is not clear. Possibly, the general use of ampicillin injected into day-old grandparent chicks before transport in the 1960s and 1970s may explain this finding.

To the best of our knowledge, in this study, we detected for the first time MRSA strains in healthy poultry. These MRSA strains were nontypeable by PFGE analysis and exhibited *spa*

TABLE 2. Distribution of MICs of various antimicrobial agents for 81 *S. aureus* isolates obtained from poultry in 2006 (recent isolates)

Antimicrobial agent	WT[b] ≤	Number of isolates with a MIC (μg/ml) of:[a]																	%R[d]
		≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	256	512	1,024	>1,024	
Enrofloxacin[c]	2			29	26	11	12		**2**	**1**						—	—	—	—	3.7
Erythromycin	1			16	34	1			**1**	**1**				**1**	**27**	—	—	—	—	37
Gentamicin	2				2	65	2		**2**	1	**1**	**6**	**1**	**1**		—	—	—	—	14.8
Lincomycin	2				4	38	5			**1**		**5**	**1**		**27**	—	—	—	—	42.0
Neomycin	1				8	50	9			**8**	**3**	**1**	**2**			—	—	—	—	17.3
Oxacillin	2			6	29	25	11				**2**	**8**				—	—	—	—	12.4
Penicillin	0.12	5	39	1	**4**	**5**	**8**	**8**	**2**	**1**		**8**				—	—	—	—	44.4
Spectinomycin	128					1						15	57		**8**	—	—	—	—	9.9
Sulfamethoxazole	128					2			10	10	8	7	36	3	—	**1**		**2**	**2**	6.2
Tetracycline	1			2	1	30	2		1	2	**1**	**2**	**32**	**3**	**5**	—	—	—	—	56.8
Trimethoprim	4			4	23	35	2		2					**1**	**14**	—	—	—	—	18.5
Tylosin[c]	64				8	13	12	12	3	1		5	1		**26**	—	—	—	—	32.1

[a] The results in boldface type exceeded the breakpoint criteria for resistance. —, not applicable.
[b] MIC distribution for the wild-type (WT) organism according to EUCAST and categorized as WT ≤ the indicated number (μg/ml).
[c] Bimodal distribution of MICs (not available in EUCAST). The isolates with MICs in the higher ranges were considered to have acquired resistance.
[d] %R, percentage of isolates resistant to the respective antimicrobial agent.

types t011 and t567, which belong to "animal-associated" MRSA, multilocus ST398. The 10 MRSA isolates harbored different SCC*mec* types, suggesting the acquisition of the *mec* element on at least four occasions. This clone is emerging in livestock animal populations and in humans in contact with animals in Europe. It was described first for pigs (22) but is also highly prevalent in horses (21). Its prevalence in poultry is unknown, but our study suggests that it may be relatively high in Belgium, as it was isolated from chickens originating from 5 out of 39 sampled farms. This finding indicates that the animal reservoir of MRSA ST398 is broader than previously anticipated. This may pose a public health hazard, since it has been shown that this MRSA clone has a zoonotic potential, causing infections in people in contact with carrier animals and in relatives of those people (22).

Resistance profiles of the MRSA isolates found in this study are comparable to those described for "animal-associated" MRSA in, among other countries, The Netherlands, Belgium, and Denmark; 100% resistance to tetracycline and frequent resistance to macrolides-lincosamides and trimethoprim has been found (7, 12, 21). The treatment of poultry infected with these isolates may thus be more difficult due to their resistance to multiple antimicrobials.

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