Research Note

# Antimicrobial Susceptibility of Environmental *Staphylococcus aureus* Strains Isolated from a Pigeon Slaughterhouse in Italy

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**ABSTRACT** No information is available concerning the antimicrobial susceptibility of *Staphylococcus aureus* isolated from pigeon slaughterhouses. In the present study, 59 staphylococcal strains isolated from a pigeon slaughterhouse in central Italy were compared according to their antibiotic resistance. On the basis of cultural and biochemical properties, all isolates could be identified as *S. aureus*. The strains were checked for the productions of enterotoxins A, B, C, D by reversed passive latex agglutination. Resistance to 26 antibiotics was also determined paying particular attention to resistance to those antimicrobial agents frequently used in human medicine and in poultry breeding. Only one strain was positive for the production of enterotoxins type C and D. It was isolated from the evisceration tube after slaughtering. Enterotoxin B was produced by 2 strains isolated from the eyebrows and conjunctivas of the worker operating the crop rinsing tube. As to the susceptibility to antibiotics, all strains were sensitive to amoxicillin/clavulanic acid, bacitracin, cephalothin, fusidic acid, gentamicin, kanamycin, linezolid, oxacillin, quinupristin/dalfopristin, rifampicin, tobramycin, trimethoprim-sulfamethoxazole, vancomycin. Some (15.2%) of the strains were resistant to ampicillin and to penicillin G; 6.8% were resistant to chloramphenicol, 20.3% to enrofloxacin, 16.9% to erythromycin and to ciprofloxacin, 8.5% to clindamycin, and 11.9% to lincomycin. The highest percentages of strains were resistant to tetracycline and oleandomicin (37.3 and 25.4% respectively). Methicillin-resistant staphylococci were also found (3.4%). Only one strain had a multiple antibiotic resistance index >0.30. The results were statistically analyzed and clustered in 6 groups. This work provides the antibiotic resistance pattern of *S. aureus* strains isolated from a pigeon slaughtering plant and represents a study on a quite unknown field in meat production.

(*Key words*: *Staphylococcus aureus,* pigeon, antimicrobial susceptibility, slaughterhouse)

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## INTRODUCTION

*Staphylococcus aureus* is a common aetiological agent of foodborne intoxication and is a significant marker of food quality and surface cleanliness (Ròaska and Wojto, 1998; Losito et al., 2004; Normanno et al., 2005). Poultry meat is a common vehicle of foodborne illness, with *S. aureus* usually being one of the causes of outbreaks involving large numbers of people (Georgnaras and von Holy, 2001). The presence of this microrganism in poultry emphasizes the need for laboratory surveillance for this bacterial pathogen even in relation to the demonstrated antimicrobial resistance (antimicrobial agents are, in fact, widely used in the treatment and control of staphylococcal infection in poultry; Moller et al., 2000). However, no information is available concerning the antimicrobial susceptibility of *S. aureus* strains isolated from pigeon slaughterhouses. The aim of this study was to characterize the antibiotic resistance of 59 *S. aureus* strains isolated from a pigeon slaughterhouse in central Italy.

## MATERIALS AND METHODS

### Bacterial Strains

During 4 different samplings, environmental samples (Table 1) were collected in a pigeon slaughterhouse with a daily production of 1,500 birds and a slaughtering speed of 500 pigeons/h. During each sampling, 18 surfaces were tested by means of wet swabbing method (9 after cleaning and 9 after the process) by using a Swab Rinse Kit.[2] The sampling area covered 100 $cm^2$ per sampling site. Eyebrows and conjunctivas of 10 workers were also sampled by wet swabbing during each sampling (Giaccone et al., 2000). The samples were brought under refrigeration to the laboratory and analyzed within the following 3 h. Isolation was carried out on Baird Parker + rabbit plasma


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**Abbreviation Key:** MAR = multiple antibiotic resistance; MRSA = methicillin-resistant *Staphylococcus aureus*.

**Table 1.** Tested surfaces contaminated by *S. aureus* in a pigeon slaughterhouse

| | Sampling I | | Sampling II | | Sampling III | | Sampling IV | |
|---|---|---|---|---|---|---|---|---|
| Tested surfaces | B[1] | A[2] | B[1] | A[2] | B[1] | A[2] | B[1] | A[2] |
| Crop rinsing tube | 1 | 2 | ND | ND | ND | ND | ND | 4 |
| Defeathering equipment | ND[3] | 2 | 1 | 5 | ND | 2 | ND | 2 |
| Leg defeathering equipment (I finger) | ND | 2 | ND | ND | 1 | 1 | ND | ND |
| Leg defeathering equipment (V finger) | 2 | 2 | ND | ND | ND | ND | ND | ND |
| Gutting equipment | ND | 1 | ND | ND | ND | 1 | ND | ND |
| Tunnel entrance | ND | 2 | ND | ND | ND | 1 | ND | 1 |
| Tunnel exit | ND | 2 | ND | ND | ND | 1 | ND | 3 |
| Workers' hands | 2 | 2 | ND | ND | ND | ND | ND | 1 |
| Workers' overalls | ND | 2 | ND | 3 | ND | 1 | ND | 4 |

[1]B = number of analyzed strains per each surface tested after cleaning.
[2]A = number of analyzed strains per each surface tested after process.
[3]ND = not detected.

**Table 2.** Tests contained in Gram-Positive Identification Cards[1] for the Vitek system

| | | |
|---|---|---|
| Peptone-glucose | Bacitracin | Ethylhydrocupreine hydrochloride |
| Hemicellulase | Sodium chloride | 10% bile |
| 40% bile | Esculin-ferric ammonium citrate | Decarboxylase base control |
| Arginine monohydrochloride | Urea | 2,3,5-triphenyl tetrazolium chloride |
| Novobiocin sodium salt | Dextrose | Lactose |
| Mannitol | Raffinose | Salicin |
| Sorbitol | Sucrose | Trehalose |
| Arabinose | Pyruvic acid | Pullulan |
| Inulin | Melibiose | Melezitose |
| Cellobiose | Ribose | Xylose |

[1]BioMérieux Vitek Systems, Inc., Hazelwood, MO.

fibrinogen agar.[3] The strains of coagulase-positive isolates were maintained on tryptic soy agar[4] slants and stored at 4°C.

## Cultural and Biochemical Tests

***Gram's Staining and Catalase Test.*** Colonies isolated colonies from tryptic soy agar were tested for Gram staining and evolution of gas bubbles with 30 vol of hydrogen peroxide.

***β-Haemolysin Production.*** The production of hemolysins was determined by cultivation of the isolates on Columbia agar with 5% sheep blood plates[4] incubated aerobically at 37°C for 24 h (Rosec et al., 1997). Strains producing broad discolored zones with edges sharply demarcated as if drawn by a pencil, clearing at 4°C, were recorded as positives.

***Vitek Identification Test.*** Isolates were subcultured twice on Columbia agar with 5% sheep blood[4] and grown overnight at 35°C. Suspensions were prepared by emulsifying bacterial isolates in 0.45% saline to the equivalent of 0.5 McFarland turbidity standard.[3] The Gram-Positive Identification Cards[5] contained 30 tests (Table 2). A card was automatically filled by a vacuum device, sealed, inserted into the VITEK reader-incubator module[5] (incubation temperature of 35.5°C), and measured every 60 min. The results were interpreted by a database, and final results were obtained automatically (Ligozzi et al., 2002).

***Detection of Enterotoxin Production.*** Enterotoxins were detected by a reversed passive latex agglutination test (Adesiyun et al., 1998; Klotz et al., 2003) for staphylococcal enterotoxins A (SEA), B (SEB), C (SEC), and D (SED) using a test kit.[6]

**Table 3.** Tests and antimicrobial concentration ranges of Gram-Positive Susceptibility 108 Cards[1] for the Vitek system

| Antimicrobial agent | MIC range[2] (μg/mL) |
|---|---|
| Amoxicillin-clavulanic acid | 2–8 |
| Ampicillin | 0.12–16 |
| Ceftiofur | 1–8 |
| Cephalothin | 2–32 |
| Chloramphenicol | 1–32 |
| Clindamycin | 0.5–8 |
| Enrofloxacin | 0.25–2 |
| Erythromycin | 0.5–8 |
| Gentamicin | 2–16 |
| Oxacillin | 0.25–8 |
| Penicillin G | 0.03–16 |
| Tetracycline | 1–16 |
| Trimethoprim-sulfamethoxazole | 10–320 |
| Vancomycin | 0.5–32 |

[1]BioMérieux Vitek Systems, Inc., Hazelwood, MO.
[2]Minimum inhibitory concentration.

[3]BioMérieux, Marcy-l'Etoile, France.
[4]Oxoid, Hampshire, England.
[5]BioMérieux Vitek Systems, Inc., Hazelwood, MO.
[6]Oxoid, Hampshire, England.

**Table 4.** Minimum inhibitory concentrations (MIC) and respective *Staphylococcus aureus* strain percentages obtained with a Vitek instrument[1]

| Antimicrobial agent | MIC[2] (strain percentage) | | | | |
|---|---|---|---|---|---|
| Amoxicillin-clavulanic acid | ≤2 (100%) | | | | |
| Ampicillin | ≤0.12 (84,7%) | ≥16 (15.3%) | | | |
| Ceftiofur | ≤1 (90%) | 2 (5%) | 4 (5%) | | |
| Cephalothin | ≤2 (100%) | | | | |
| Chloramphenicol | ≤1 (1.8%) | 2 (6.9%) | 4 (32.4%) | 8 (52%) | ≥32 (6.9%) |
| Clindamycin | ≤0.5 (91.5%) | ≥8 (8.5%) | | | |
| Enrofloxacin | ≤0.5 (67.8%) | 0.5 (6.8%) | 1 (5%) | ≥2 (20.4%) | |
| Erythromycin | ≤0.5 (71.2%) | 1 (10%) | 4 (1.8%) | ≥8 (17%) | |
| Gentamicin | ≤2 (100%) | | | | |
| Oxacillin | ≤0.25 (40.6%) | 0.5 (57.7%) | 2 (1.7%) | | |
| Penicillin G | ≤0.03 (84.8%) | ≥16 (15.2%) | | | |
| Tetracycline | ≤1 (62.7%) | ≥16 (37.3%) | | | |
| Trimethoprim-sulfamethoxazole | ≤10 (98.3%) | 20 (1.7%) | | | |
| Vancomycin | ≤0.5 (71.2%) | 1 (1.7%) | 2 (27.1%) | | |

[1]BioMérieux Vitek Systems, Inc., Hazelwood, MO.

[2]The MIC values are expressed in micrograms per milliliter.

## Antibiotic Resistance

***Antibiotic Susceptibility Testing by VITEK.*** The 0.5 McFarland bacterial suspension was diluted to $1.5 \times 10^7$ cfu/mL in 0.45% saline. Cards were automatically filled, sealed, and loaded into the VITEK instrument[5] for incubation and reading. The Gram-Positive Susceptibility 108 Cards[5] used for staphylococci contained 14 different antibiotics (Table 3). Final results were obtained automatically as previously reported. The results allowed us to classify the strains as susceptible, intermediate or resistant.

***Antibiotic Susceptibility Testing by Disk Diffusion Method.*** The disk diffusion method of Bauer et al. (1966) was used to determine the antibiograms of *S. aureus* strains. The following antimicrobial agents[6] were used: kanamycin (30 μg), streptomycin (10 μg), rifampicin (5 μg), ciprofloxacin (5 μg), bacitracin (10 IU), fusidic acid (10 μg), lincomycin (2 μg), linezolid (30 μg), oleandomycin (15 μg), quinupristin/dalfopristin (15 μg), and tobramycin (10 μg). Resistance to antimicrobial agents was determined based on zone size as recommended by disk manufacturer.

***Methicillin Resistance.*** The assessment of the susceptibility to the methicillin was carried out by selection for PBP2′ with a Slidex methicillin-resistant *S. aureus* (MRSA) detection kit.[3]

## Multiple Antibiotic Resistance Index

The multiple antibiotic resistance (MAR) index is defined as *a/b* where *a* represents the number of antibiotics to which the strain was resistant, and *b* represents the number of antibiotics to which the strain was exposed (Krumperman, 1983; Moschetti et al., 1997). Strains with intermediate resistance to an antibiotic were classified as resistant.

[7]SPAD software, 2000, Version 4, DECISIA/SPAD, Paris, France.

All tests described above were repeated twice for each bacterial strain.

## Statistical Analyses

Data on antibiotic resistance were first analyzed by multiple correspondence analyses (CORMU procedure of the SPAD software[7]). Based on the results of multiple correspondence analyses, it was possible to carry out a cluster analysis (RECIP procedure) to cluster the bacterial strains showing similar characteristics (Escofier and Pagès, 1988; Bolasco, 1999). In particular, in cluster analysis, we considered the first 5 factors drawn out from the multiple correspondence analyses, which, altogether, explained approximately 75% of the total variance of the phenomenon.

# RESULTS AND DISCUSSION

Table 1 shows tested surfaces contaminated by *S. aureus*. It is important to emphasize the presence of *S. aureus* on a worker's hand before slaughtering; humans are in fact considered an important potential reservoir of this microorganism (Adesiyun et al., 1998; Capita et al., 2002). *S. aureus* is frequently found in living animals, but the incidence of enterotoxigenic *S. aureus* in animals is lower than in processed animal products because of the potential for cross-contamination during processing and because of the contamination coming from workers. The defeathering equipment and workers' overalls showed constant contamination after slaughter. During the second sampling, *S. aureus* was present on the defeathering machine even after cleaning. This equipment is an important cross-contamination point because of the structure of the rubber fingers (Lindsay et al., 1996).

Only one strain was positive for the production of enterotoxin types C and D, which were isolated from the evisceration tube after slaughter. During the fourth sampling, the eyebrows and conjunctivas of the worker op-

erating the crop rinsing tube tested positive for the production of enterotoxin B.

As to the susceptibility to antibiotics, there was no significant variability between test repetitions. All strains were susceptible to amoxicillin/clavulanic acid, cephalothin, gentamicin, oxacillin, trimethoprim-sulfamethoxazole, vancomycin, kanamycin, tobramycin, linezolid, bacitracin, fusidic acid, quinupristin/dalfopristin, and rifampicin. Only 11.9% of the strains were positive for $\beta$-lactamase. All *S. aureus* strains were resistant (11.9%) or had intermediate susceptibility to lincomycin (88.1%). Some (15.2%) of the strains were resistant to ampicillin and to penicillin G; 6.8% were resistant to chloramphenicol, 8.5% to clindamycin, 20.3% to enrofloxacin, 16.9% to erythromycin and ciprofloxacin, and 25.4% to oleandomycin. The highest percentage (37.3%) of strains was resistant to tetracycline. Some strains showed intermediate susceptibility to ceftiofur (10.2%), ciprofloxacin (8.5%), streptomycin (1.7%), enrofloxacin, and erythromycin (11.9%). The data for minimum inhibitory concentration obtained with a VITEK instrument are shown in Table 4. Our results are quite similar to the data of Moller et al. (2000) and White et al. (2003), who conducted studies on the susceptibility to antimicrobial agents among clinical poultry staphylococci isolates. In fact, *S. aureus* is one of the most common causes of infections in birds, and antimicrobial agents are widely used in the treatment and the control of staphylococcal infections.

Two MRSA strains were isolated from the defeathering equipment and the hand of the worker responsible for wings trimming and decapitation. Although the environment contributes to the transmission of MRSA, transmission through food products has not been thoroughly investigated. There are no former data about the presence of MRSA strains in pigeons, and surveys about poultry (Lee, 2003) are very limited.

As to the MAR index of the staphylococcal strains, only a single strain isolated from the defeathering machine during the last sampling showed resistance to 9 antibiotics exhibiting a MAR index of 0.35. This strain was also resistant to methicillin. Two strains had MAR of 0.23 and 0.27 because they were resistant to 6 and 7 antibiotics, respectively. Most (36 strains) had a MAR index between 0.12 and 0.19. The other strains analyzed showed lower values on the MAR index; 18 strains were monoresistant.

For an evaluation of the results obtained by antibiotic resistance tests, the similarities among strains are represented as a dendrogram (Figure 1). The results were statistically clustered in 6 groups and are indicated by different roman numerals (Table 5). Groups I, IV, and VI included 15.2% of the strains, group II included 13.6%, and group V included 10.2%. Group III was represented by the most of the isolated strains (30.6%) and was characterized by susceptibility to erythromycin, tetracycline, enrofloxacin, ciprofloxacin, oleandomycin. Amoxicillin/clavulanic acid, cephalothin, gentamicin, oxacillin, trimethoprim-sulfamethoxazole, vancomycin, kanamycin, tobramycin, linezolid, bacitracin, fusidic acid, quinupristin/dalfopristin, and rifampicin were not considered for the final clus-

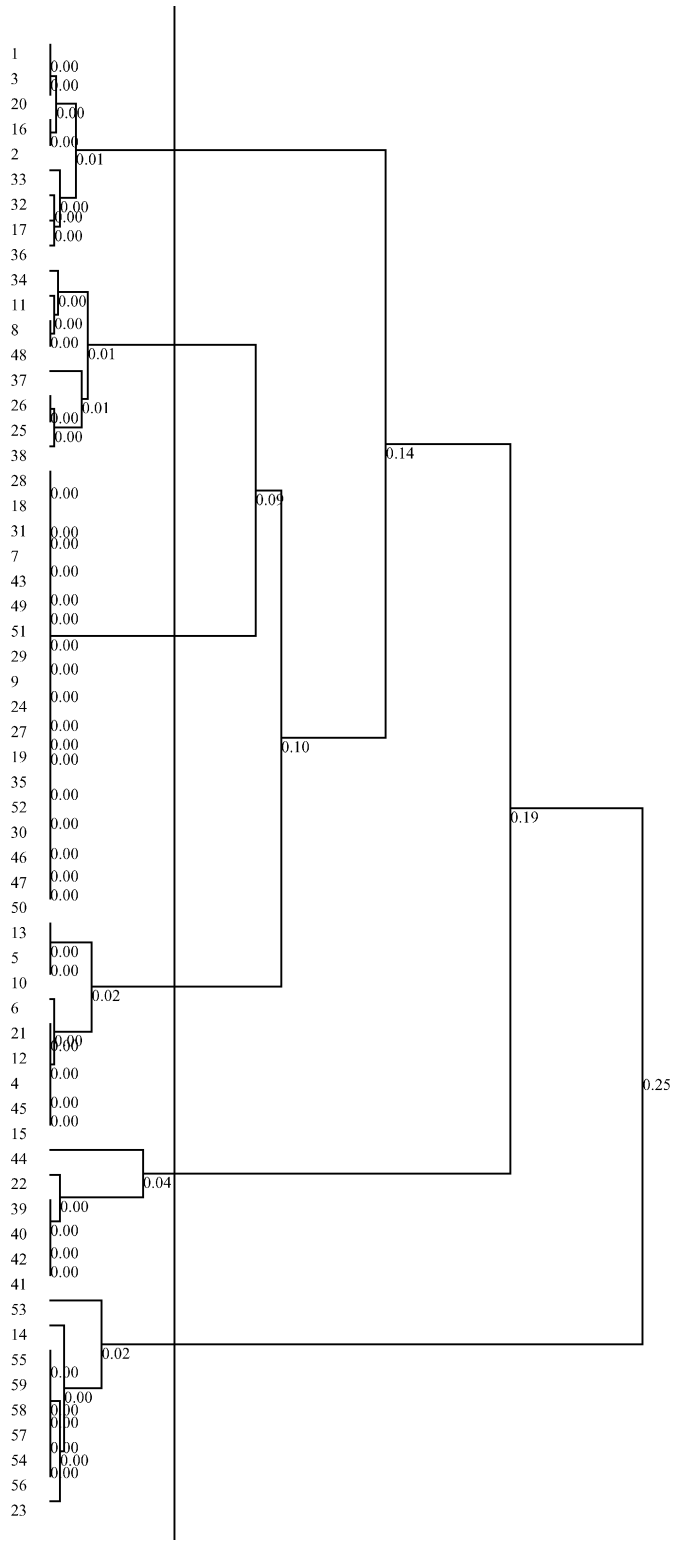


**Figure 1.** Dendrogram showing the degree of similarity of antibiotic resistance patterns of *Staphylococcus aureus* strains isolated from a pigeon slaughterhouse. It is possible to distinguish 6 groups. The smallest cluster is represented by 6 strains, and the biggest by 18 strains. Clusters were generated using RECIP method of the SPAD software (Version 4, DECISIA/SPAD, Paris, France). Tested strains are indicated by numbers in ascending order from 1 to 59.

**Table 5.** Group analysis: antibiotic resistance characteristics of the single groups of *Staphylococcus aureus* strains

| Group I[1] | Group II[2] | Group III[3] |
|---|---|---|
| Ciprofloxacin: resistant<br>Enrofloxacin: resistant | Ciprofloxacin: intermediate<br>Enrofloxacin: intermediate | Erythromycin: susceptible<br>Tetracycline: susceptible<br>Enrofloxacin: susceptible<br>Ciprofloxacin: susceptible<br>Oleandomycin: susceptible |
| **Group IV[4]** | **Group V[5]** | **Group VI[6]** |
| Erythromycin: intermediate<br>Tetracycline: resistant<br>Oleandomycin: resistant | Erythromycin: resistant<br>Clindamycin: resistant<br>Tetracycline: resistant<br>Oleandomycin: resistant<br>Lincomycin: resistant | Tetracycline: susceptible<br>Ampicillin: resistant<br>Penicillin G: resistant<br>$\beta$-Lactamase: positive |

[1]Group I included 9 strains deriving from the first (strains 1, 2, 3, 16, 17, and 20) and the third samplings (strains 32, 33, and 36).

[2]Group II included 8 strains. The first and the second samplings provided 2 strains per each (strains 8, 11 and 25, 26), the third sampling provided 3 strains (strains 34, 37, and 38), and the fourth sampling provided only 1 strain (strain 48).

[3]Group III included 18 strains derived mostly from the second (strains 24, 27, 28, 29, 30, and 31) and fourth (strains 43, 46, 47, 49, 50, 51, and 52) samplings; 4 strains were derived from the first sampling (strains 7, 9, 18, and 19), and only 1 strain was derived from the third sampling (strain 35).

[4]Group IV included 9 strains isolated during the first (strains 4, 5, 6, 10, 12, 13, 15, and 21) and the fourth (strain 45) samplings.

[5]Group V included only 6 strains isolated during the first (strain 22) and last (strains 39, 40, 41, 42, 44) samplings.

[6]Group VI included 9 strains were derived from the first (strain 14), the second (strain 23) and the fourth (strains 53, 54, 55, 56, 57, 58, and 59) samplings. All the strains producing enterotoxins were from this group.

tering because they were not discriminant for the characterization of the strains. The statistical analysis was useful for quantifying similarity on the basis of antibiotic resistance characteristics and providing an objective basis for grouping bacterial strains into homogenous clusters. On the basis of the dendrogram (Figure 1) it was possible to discriminate 6 different antibiotypes, which showed the extreme heterogeneity among the strains of *S. aureus* that could be harbored by a pigeon slaughterhouse.

Pigeon processing plants can provide a favorable environment for the survival and transmission of *S. aureus*. Infections due to antibiotic-resistant strains of *S. aureus* are an increasingly serious problem clinically, and, because antibiotic exposure in food-animal species may lead to antibiotic-resistant bacteria, it is possible that processed poultry may constitute a reservoir for distributing antibiotic resistance into the community (Bertolatti et al., 2003). Further studies are necessary to investigate the antibiotic resistance of *S. aureus* present in pigeon breeding and slaughterhouses in wider geographic locations.

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