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Antimicrobial Resistance of Bacterial Strains Isolated from Avian Cellulitis

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ABSTRACT

Avian cellulitis is an inflammatory process in the subcutaneous tissue, mainly located in the abdomen and thighs. This problem is commonly observed in poultry at slaughter and it is considered one of the major causes of condemnation of carcasses in Brazil. The aim of this study was to perform the microbial isolation of lesions of avian cellulitis from a processing plant located in the State of Goiás in order to analyze antimicrobial resistance by antibiogram test and to detect resistance genes by polymerase chain reaction. A total of 25 samples of avian cellulitis lesions were analyzed, from which 30 bacterial strains were isolated. There were eleven (44%) strains of *Escherichia coli*, nine (36%) strains of *Staphylococcus epidermidis*, seven (28%) strains of *Proteus mirabilis* and three (12%) strains of *Manheimiahaemolytica*. The antibiogram test showed that all strains were resistant to at least one antimicrobial. The gene of antimicrobial resistance *tetB* was detected in *E. coli, S. epidermidis* and *P. mirabilis* strains, and was the most frequently observed gene. The gene of antimicrobial resistance *Sul1* was detected in all bacterial species, while *tetA* was found in *E. coli* and *S. epidermidis* strains, *SHV* in *E. coli* strains, *S. epidermidis* and *P. mirabilis,*and *cat1* in one *P. mirabilis* strain. The results suggest a potential public health hazard due to the ability of these microorganisms to transmit antimicrobial resistancegenes to other microorganisms present in the intestinal tract of humans and animals, which may affect clinical-medical usage of these drugs.

INTRODUCTION

Avian cellulitis was first described in Britain by Randall *et al*. (1984). Characterized as an acute purulent inflammation of the subcutaneous tissue, it is routinely encountered in the abdominal region and the legs of poultry (Messier *et al*. 1993, Gross 1994,). Carcasses presenting cellulitis should be partially or totally condemned, as determined by Ordinance No.210 of 10/11/1998 of the Brazilian Ministry of Agriculture (Brazil, 1998), which establishes the Technical Regulations of Technological and Sanitary-Hygienic Inspection of Poultry Meat. Some authors mention that condition as one of the main causes of carcass condemnation in broiler processing plants (Brito *et al*., 2002; Armendaris, 2006; Santana *et al*., 2008). Considering the importance of this pathology in the context of poultry meat production, this study aimed at performing microbiological isolation, testing antimicrobial resistance, and detecting antimicrobial resistance genes in microorganisms isolated from avian cellulitis lesions obtained from chicken carcasses stored in the refrigerator of a processing plant located in the state of Goiás, Brazil.

MATERIAL AND METHODS

1) Collection, isolation, and identification of avian cellulitis lesions

Samples of avian cellulitis lesions were collected from 25 carcasses that had been partially or totally condemned by officials of the Federal Inspection Service (SIF) of the Ministry of Agriculture and stored in the refrigerator of a processing plant located in the state of Goiás, Brazil. The carcasses presented irregular skin lesions, thickening, and color change, and were considered by SIF officials to be examples of avian cellulitis, according to Ordinance No. 210 of 11/10/1998 of the Ministry of Agriculture.

The methodology used for microbiological isolation was described by Konemman *et al*. (2001). Samples were duly identified and individually plated on blood agar culture medium (BioRad®) and kept at 37°C in bacteriological incubators (Quimis®) for 24 hours. Subsequently, colonies were separated and individually plated on nutrient agar (Acumedia®) for biochemical identification. The following culture media and biochemical tests were used: oxidase test, catalase by the method of analysis and GRAM KOH test 3%, TSI (Triple Sugar Iron), urea, phenylalanine, citrate, indole, methyl red, arginine, lysine, gelatin, mannitol, trehalose, sucrose, and glucose. Gram-positive colonies were cultured in Baird-Parker culture medium (HIMEDIA®). Reading and interpretation of biochemical tests were performed according to Oliveira (2000) and Baron *et al*. (1994) for species identification.

2) Antimicrobial susceptibility testing and DNA extraction of isolated strains

Each isolated and identified colony was submitted to an antibiogram test carried out by the disk diffusion method, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) 2003.The tested pharmacological principles were ampicillin (AMP) 10 mg, cefazolin (CFZ) 30 mg, gentamicin (GEN) 10 mg, spiramycin (SP) 100 mg, doxycycline (DOX) 30 mg, cephalexin (CFX) 30 mg, sulfonamide (SOUTH) 200 mg, cephalothin(CEF) 30 mg, penicillin (PEN) 6 mg, enrofloxacin (ENRO) 5 mg, tetracycline (TET) 30 mg, neomycin (NEO) 30 IU, norfloxacin (NOR) 10 mg, erythromycin (ERI) 15 mg, amoxicillin (AX) and 25 mg chloramphenicol (CLO) 30 mg. The disks were purchased fromBIO-RAD®.

Strains submitted to total bacterial DNA extraction were individually plated on nutrient agar (Acumedia ®) and incubated in a bacteriological incubator at 37°C for 24 hours. Subsequently, three colonies were selected and inoculated into 15 mL falcon tubes containing 3 mL of L-broth (1% peptone, casein, yeast extract, and 0.5% sodium chloride 1%) and were maintained in a shaker (New Brunswick Scientific Edison, NJ, USA) at 200 rpm for 12 hours at 37°C. One mL of the L-broth was used for total DNA extraction, applying the phenol-chloroform (1:1) method, according to the protocol described by Sambrook *et al*. (2001). Total DNA was read in agarose gel at 0.8% with the addition of ethidium bromide at a concentration of 5mg/mL under ultraviolet light (Majorscience®).

3) Detection of antibiotic resistance genes

The isolated bacterial colonies were individually used to perform the PCR-based protocol described by Van *et al*. (2008), with minor modifications, in which simple PCR reactions were developed to detect individual tetracycline resistance genes (*tetA, tetB and tetC*), aminoglycoside resistance gene (*aac (3 ')-I)*, and macrolide resistance gene (*ereA*). A single reaction was developed for simultaneous detection (multiplex PCR) of genes of resistance to sulfonamides (*Sul1*), beta-lactams (*VHS*), and chloramphenicol (*cat1*). The oligonucleotide probes used for each resistance gene are listed in Table 1.

The PCR for the detection of single tetracycline resistance genes *tetA, tetB* and *tetC* involved a total volume of 25 µl reaction containing 10ng of DNA extracted from each bacterial strain isolated from cellulitis samples, 10 pmol of each forward and reverse primer (RTD®), final concentration 1.5 mM MgCl2, final concentration 2.0 mMdNTPs (Invitrogen®) and 1 U Taq (Invitrogen®). The amplification conditions in the thermal cycler (BioRad®) were as follows: initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, and a final cycle of amplification at 72°C for 10 minutes. The protocol used to detect the macrolide resistance gene (*ereA*) and the aminoglycoside resistance gene (*aac (3')-I*) was the same as that described for the tetracycline resistance gene, except forMgCl2 final concentration, which was 2.5 mM, and annealing temperature, which was 59°C for the gene *ereA* and 55°C for the gene *aac*(3 ')-I. In the original protocol of Van *et al*. (2008), the PCR of these two resistance genes (*ereA* and aac (3')-I) was performed in a multiple reaction with other resistance genes.

Table 1 – Primers used for detection of genes for resistance to tetracycline, sulfonamide, chloramphenicol, aminoglycosides, beta-lactam and macrolides.

Gene	Antimicrobial resistance	Name	*Oligonucleotide* sequences5'-3'	Size of the amplified product in base pairs
Sul1	Sulfonamide	Sul-F Sul-R	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	822bp
SHV	Beta-lactam	blaSHV-F blaSHV-R	TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG	768bp
Cat1	Chloramphenicol	CAT1-F CAT1-R	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	547bp
ereA	Macrolide	Ere(A)-F Ere(A)-R	GCCGGTGCTCATGAACRRGAG CGACTCTATTCGATCAGG1GC	419bp
aac(3)-I	Aminoglycoside	aac(3)-I-F aac(3)-I-R	ACCTACTCCCAACATCAGCC ATATAGATCTCACTACGCGC	157bp
tetA	Tetracycline	tet(A)-F tet(A)-R	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG	887bp
tetB	Tetracycline	tet(B)-F tet(B)-R	CCTTATCATGCCAGTCTTTTGC ACTGCCGTTTTTTCGCC	773bp
tetC	Tetracycline	tet(C)-F tet (C)-R	ACTTGGAGCCACTATCGAC CTACAATCCATGCCAACCC	880bp

Source: Van *et al*. (2008).

The PCR for the simultaneous detection (multiplex PCR) of the sulfonamide, chloramphenicol and beta-lactams resistance genes (*sul1, cat1* and *SHV*) was performed in a total volume of 25 µl reaction containing 10ng of DNA extracted from each isolated strain, with final concentration of 3.0 mM MgCl2. This concentration was modified, as in the original protocol proposed by Van *et al*. (2008), the concentration was 4.0 mM. A final concentration of 2.0 mMdNTP was used, with 10 pmol of each reverse and forward primer and 1 U of Taq polymerase (Invitrogen®). Amplification conditions were denaturation at 94°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds – the annealing temperature was 58°C in the original protocol described by Van *et al*. (2008) – and extension at 72°C for 1 minute and one final cycle of amplification at 72°C for 10 minutes. PCR products were visualized and photo documented (Majorscience®).

RESULTS

1) Microbiological isolation from samples of avian cellulitis

Out of the 25 analyzed samples of avian cellulitis, 11 (37%) strains of *Escherichia coli*, 9 (30%) of *Staphylococcus epidermidis*, seven (23%) of *Proteus mirabilis* and 3 (10%) of *Manheimia haemolytica* were isolated. A total of 30 bacterial strains, and two bacterial genera were isolated from some samples of cellulitis. The results of bacterial isolation in this study revealed the presence of more than one type of microorganism present in the lesions of avian cellulitis obtained from the processing plant.

2) Detection of resistance genes in isolates from avian cellulitis lesions

The results of antimicrobial resistance genes are shown in Table 2 and Figure 1. One strain of *E. coli* and

Table 2 – Detection of resistance genes in 11strains of *E.coli*, 9 of *Staphylocccus epidermidis*, 7 of *Proteus mirabilis* and 3 of *Manheimia haemolytica* isolated fromavian cellulitis lesions in broilers carcasses stored in a refrigerator located in the state of Goiás.

Genes	Antimicrobialagent	*E.coli*	*Staphyloccusepidermidis*	*Proteusmirabilis*	*Manheimiahaemolytica*
tet(A)	Tetracycline	3/11	1/9	0	0
tet(B)	Tetracycline	5/11	2/9	3/7	0
tet(C)	Tetracycline	0	0	0	1/3
Sul1	Sulfonamide	2/11	3/9	2/7	1/3
Cat1	cloranphenicol	0	0	1/7	0
Aac(3')-1	aminoglycosides	0	0	0	0
ereA	macrolides	0	0	0	0
SHV	Beta-lactam	1/11	1/9	1/7	0

one of *P. mirabilis* that showed the gene for beta-lactam resistance (*SHV*) were also phenotypically resistant to this class of antimicrobial agents (amoxicillin, ampicillin and penicillin). One strain of *E. coli* and two strains of *S. epidermidis* were positive for the gene *Sul1*, and these were phenotypically sensitive to the drug. Two of the seven *P. mirabilis* strains were resistant to sulfonamide. The *P. mirabilis* strain that was phenotypically resistant to chloramphenicol presented the resistance gene to this antimicrobial agent (*cat1*). Macrolide-resistant genes (*ereA*) and those resistant to aminoglycosides (*aac (3) -1*) were not detected in any of the isolated bacterial species.

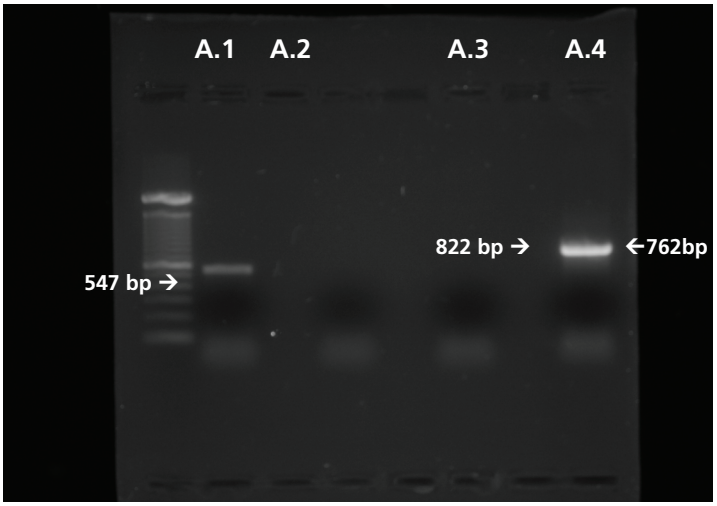

Figure 1.PCR used for the identification of the antimicrobial resistance genes SHV, cat1, Sul1. A.1)100bp DNA Ladder marker (Invitrogen®); A.2)547 bp fragment of the cat1 gene in a of Proteus mirabilis strain; A. 3) Sample of E. coli genes tested negative for the evaluated genes; A.4) gene; 822 bp fragment of the Sul1 gene in a Proteus mirabilis strain; A.4)762 bp fragment of the SHV gene in E. coli strain.

DISCUSSION

The results of bacterial isolation in this study revealed the presence of more than one type of microorganism present in the samples of avian cellulitis lesions collected from carcasses stored in a refrigerator of a processing plant located in the state of Goiás. This is in contrast with the study by Vieira *et al*. (2005), who reported the isolation of only *Escherichia coli* in all 20 (100%) samples of avian cellulitis lesions collected from broiler carcasses stored in a refrigerator of a processing plant in the state of Rio de Janeiro. Andrade (2005) also found *Escherichia coli* in cellulitis lesions of broiler carcasses in the state of São Paulo. In a study conducted in England with broilers, Randall *et al*. (1984) were the first to report an association between *Escherichia coli* and *Pasteurella multocida* in avian cellulitis lesions. Messier *et al*. (1993) also isolated *Escherichia coli* and *Streptococcus dysgalactiae* in cellulitis samples of chicken carcasses in Canada. In a study on cellulitis in chicken carcasses in Iran, Derakhshanfar *et al*. (2002) isolated *Escherichia coli* in 91.8% of the carcasses, and reported that *E. coli* strains were associated or not with *Staphylococcus aureus*. *Actinomyces pyogenes* and *Staphylococcus aureus* were also isolated as the sole agent in some samples. In general, research on avian cellulitis shows that *Escherichia coli* is the most frequently encountered bacterium in this type of lesion.

The results of the present study are consistent with the findings of other authors relative to the phenotypic resistance to antibiotics. Working with *E. Coli* strains isolated from airsacculitis, pericarditis and tracheitis lesions sampled from broilers in the state of Rio de Janeiro, Gonçalves *et al*. (2005) found 100% resistance to penicillin, erythromycin, and other antimicrobial agents. In his review of studies on the antimicrobial resistance profile of *E. coli* strains in chickens conducted in the United States, Canada, France, Australia and Iceland, Gyles (2008) mentioned that Avian Pathogenic *E. coli* (APEC) strains were often found to be highly resistant to tetracycline, sulfonamide, and streptomycin. Xin-Sheng *et al*. (2007), in a study of 70 strains of *E. coli* isolated from the livers of chickens reared on different farms in China, reported high resistance to ampicillin (83%). Zanatta *et al*. (2004) found similar results in a study with 120 *E. coli* samples obtained in the necropsy of commercial broilers in Descalvado, SP, Brazil, out of which 54.6% were resistant to cephalexin and cephalothin.

Resistance to chloramphenicol was detected in 18.19% of the isolated *E. coli* strains, which was lower compared with the other tested antimicrobial agents tested in this study. This may explained by the fact that the use of chloramphenicol in animal production is forbidden since 2003 in Brazil(Brazil, 2003).Brito *et al*. (2000), in a study of 10 *E. coli* strains isolated from cellulitis lesions in quails reared in commercial farms in northern region of the state of Paraná, observed that 90% of the isolated strains were resistant to chloramphenicol.

To the best of our knowledge, there are no reports of microbiological isolation of *Proteus mirabilis*, *Staphylococcus epidermidis* and *Manheimia haemolytica* from avian cellulitis lesions. However, some authors have reported the occurrence of antimicrobial resistance of these organisms isolated from other sources. Shin-hee *et al*. (2005), in a study with ground beef, turkey, chicken and pork, isolated 64 multi-resistant strains of *Proteus mirabilis* from a commercial establishment located in Stillwater, Oklahoma, United

States, but only from samples of homogenized turkey, chicken and pork meat. The isolated strains were resistant to at least four antimicrobial agents, especially to ampicillin, tetracycline, gentamicin and kanamycin. Qiongfen *et al*. (2011), in a study of stool samples from pigs reared on a farm located in eastern China, found a single *P. mirabilis* strain that was resistant to chloramphenicol, trimethoprim/sulfamethoxazole and tetracycline. In a study conducted in France, Even *et al*. (2010) isolated 33 strains of *Staphylococcus epidermidis* from cheese and dry fermented sausages samples, and clinical samples from hospitalized patients, and reported that 73% were resistant to at least one of the antimicrobials: 9.18% to tetracycline, 36% to erythromycin, 39% to penicillin, and 18% to norfloxacin, among other antimicrobial agents. Onni *et al*. (2011), in a study of 131 strains of *S. epidermidis* isolated from mastitis in goats reared in different geographical areas of the region of Sardinia, Italy, found that 38% of the strains were resistant to penicillin, 7.6% were resistant to tetracycline, and 2.3% resistant to both penicillin and tetracycline.

Some authors isolated *Manheimia haemolytica* strains from sources other than avian cellulitis. Klima *et al*. (2011), in a study with bovine nasopharyngeal samples collected at the beginning and end of feedlot in two farms in southern Alberta, Canada, isolated of *M. haemolytica* strains and found that all isolates were resistant to sulfametazole/trimethoprim, ceftiofur, enrofloxacin, florfenicol and gentamicin. In that study, the authors demonstrated that out of the 409 *Manheimia haemolytica* isolates, 39 (9.54%) were resistant to only one type of antimicrobial agent, and five (1.2%) were resistant to more than one antimicrobial agent. Hendriscken *et al*. (2008) conducted a survey of data on the antimicrobial susceptibility of pathogenic bacteria, including *M. haemolytica* isolated from samples of cattle reared in different European countries between 2002 and 2004.

Further studies should be conducted to determine true origin of the antimicrobial resistance observed in present study. The resistance of bacteria isolated from this type of lesion may pose a considerable public health hazard, because these lesions may be a source of cross-contamination of chicken carcasses during processing, disseminating antimicrobial-resistant bacteria, when the carcasses are only partially used.

Relative to the results obtained in the detection of antimicrobial resistance genes, in a study of *E. coli* isolated strains from broiler chickens in Vietnam, Van *et al*. (2008) observed the concomitant presence of *tetA* and *tetC*, and *tetB* and *tetC* genes. This is consistent with the detection of two concomitant resistance genes in the same strain of *E. coli* in the present study. Xin-Sheng *et al*. (2007) detected the presence of the *cat1* gene in 11 of 70 *E. coli* strains isolated from livers of sick chickens reared in China. Abdullah *et al*. (2010), in a study with samples of raw chicken meat sold in supermarkets of the city of Taif, in Saudi Arabia, detected the presence of the *Sul1* gene in all isolated *E. coli* strains. Soufi *et al.* (2009) also detected sulfonamide (*Sul1*) and cetyltransferase (CAT) resistance genes in of *E. coli* strains isolated from chicken and turkey meat sampled in a processing plant in Tunisia. Simeoni *et al*. (2008), in a study with samples obtained at various processing stages in two pig processing plants of northern Italy, isolated *S. epidermidis*, which presented tetracycline (*tetM*, *tetO,* and *tetK*), beta-lactams (blaZ), aminoglycosides (aac (6 ') aph2), methicillin (mecA), and macrolide-lincosamide-streptogramins (*ermA* , *ermB* and *ermC*) resistance genes. The presence of multidrug-resistant bacteria was also observed in the present study.

There was general consistency between the results obtained in the analysis of phenotypic antimicrobial resistance and the detection of resistance genes. The presence of the chloramphenicol resistance gene may suggest that this gene is still present in bacterial strains, although its use is forbidden in Brazil since 2003. According to Even *et al*. (2010), *S. epidermidis* in foods may be a significant reservoir of antimicrobial resistance genes, and may be involved in resistance transfer among microorganisms present in foodstuffs. Further studies should be conducted to establish the origin and the possibility resistance transfer by microorganisms in foodstuffs.

CONCLUSION

The present study identified *Escherichia coli*, *Staphylococcus epidermidis*, *Proteus mirabilis,* and *Manheimiahaemolyica* strains in samples of avian cellulitis lesions. The presence of genes for resistance to antimicrobial agents was detected by PCR in those four bacterial species. This is the first study that isolated and detected resistance genes to antimicrobial agents in this type of lesion in Brazil. The presence of antimicrobial resistance in bacteria isolated from a feedstuff intended for human consumption was demonstrated. Further studies are needed to verify the origin of this resistance and to analyze the probable risk carcass contamination at the time of inspection when only part of the carcass is used.

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