Investigation of Antimicrobial Resistance Status of Aerobic Bacteria Causing Arthritis in Broilers

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ABSTRACT

It is recognized that bacterial arthritis, which causes significant losses in the poultry industry, occurs frequently after septicemia or localized infection to the joints and is associated with many bacterial agents. This study was aimed to identify aerobic bacterial agents and to investigate the antibiotic resistance of the isolates which cause arthritis in commercial broilers in mid-west Turkey. A total of 152 joint fluid samples were collected from the one commercial broiler farm from broilers with arthritis. Joint fluid samples were inoculated in mannitol salt agar, enterococcosel agar, eosine methylene blue agar and 7% ovine blood agar after pre-enrichment and then incubated for 48 hours at 37˚C. Identification of bacteria and detection of antibiotic resistance was performed with VITEK II automated system (bioMérieux, France). After the culture period, no growth was observed in 53 (34.9%) joint fluid samples; a total of 112 aerobic bacteria (52 Gram negative and 60 Gram positive) were isolated from 99 (65.1%) broilers. While pure agent was isolated from eighty-six broilers, two microorganisms were isolated from 13 broilers. The data revealed that *Staphylococcus* spp. (56/112, 50.0%), *Escherichia* spp. (44/112, 39.3%) *Salmonella* spp. (7/112, 6.2%), *Enterococcus* spp. (4/112, 3.6%), *Enterobacter* spp. (1/112, 0.9%) were the significant pathogens isolated from joint fluid samples from broilers with arthritis. Fifty-two Gram negative bacterial isolates were the most resistant to cephalexin (90.0%) followed by cephalotine (78.8%), doxycycline (71.2%), tetracycline and chloramphenicol (69.2%). Sixty Gram positive bacterial isolates were the most resistant to marbofloxacin (78.3%) followed by amoxicillin/clavulanic acid (75.0%), benzylpenicillin (68.3%), clindamycin, tetracycline and doxycycline (66.6%). To the best knowledge of the authors this is the first study that evaluated simultaneously the prevalence of broad spectrum beta lactamase (ESBL) producing *Escherichia coli* and methicillin-resistant coagulase negative staphylococci (CNS) isolated from broiler with arthritis in Turkey. Twelve (12/46, 26.1%) CNS isolates were resistant to cefoxitin and eight (8/44, 18.2%) *E. coli* isolates had ESBL activity, indicating that these isolates could serve as a problem for public health. Further infection control measures need to be investigated and implemented in order to reduce the spread of these resistant bacteria.

Keywords: Aerobic bacteria; Antibiogram; Arthritis; Broiler Chickens; Cefoxin resistance; ESBL.

INTRODUCTION

Septic arthritis is an inflammation of the synovial membrane and synovial fluid in the joints caused by bacterial, viral or fungal agents. The synovial membrane is a highly vascular structure. Since it does not have a protective membrane, microorganisms that are blood borne can easily settle there (1). Bacteria can enter joint synovia from various foci of infection, such as local trauma or osteomyelitis (2).

One of the most common conditions in poultry farming is leg problems (arthritis, synovitis, tenosynovitis, osteomyelitis, etc.). Purulent arthritis is assumed to constitute a significant proportion of cases of leg weakness (3). Purulent

arthritis in broilers is mostly seen at 35 days of age, but is usually reported in birds of ages ranging from 14 to 70 days (4). The most common causative aerobic bacterial agent is *Staphylococcus aureus* (4, 5). Gram negative bacteria such as *Escherichia coli* (6), *Salmonella* spp., *Pasteurella multocida*, *Pseudomonas aeroginosa* (5) and Gram positive bacteria such as *Streptococcus* spp., *Enterococcus* spp., *Listeria monocytogenes* (5, 7) are among the other important bacteria isolated from joints with arthritis. *Erysipelothrix rhusiopathiae* (8), *Ornithobacterium rhinotracheale* (5) and *Mycoplasma synoviae,* are among the microaerophilic/facultative anaerobic agents which have been reported to be isolated from arthrititic conditions in broilers (5).

Today, antibiotic resistance has become an important problem for animal health as well as for humans. Due to antibiotic resistance, the treatment of infectious diseases is becoming increasingly difficult (9). Methicillin-resistant staphylococci and broad-spectrum beta-lactamase (ESBL) enzymes producing *E. coli*, especially those with multiple antibiotic resistance, are an important public health issue (10,11). Food-producing animals, especially poultry, have been suggested to be a potential source of direct transmission of these bacteria to humans (12).

Death due to inability to reach food and water due to leg problems are seen in poultry houses. This problem, which is observed whole world, causes economic losses in the poultry industry due to poor performance. While there is no data on the extent of the economic losses in Turkey it is reported that the annual economic loss due to commercial skeletal problems in the US is between 80-120 million USD in broilers (13). There is little information in the literature about the isolation of aerobic bacteria in broilers with arthritis. In this study, it was designed to identify the aerobic bacteria causing arthritis in commercial broilers and to investigate the antibiotic resistance status of isolates by using the Vitek II automated system.

MATERIAL AND METHOD

Material

In this study, synovial fluid samples taken from 152 broilers, Ross 308, aged between 16-41 years of age, with arthritis from a single commercial farm between February and April 2019 were used as material. Sample broilers were not treated with antibiotics during the last two weeks before sampling.

Broilers that had arthritis was taken to separate rooms from other broilers. When they were about 40 days old, they were cut in the slaughterhouse. If these broilers die before slaughter, necropsy was done for diagnostic purposes and samples were taken. Samples were taken from the outer surface of the femorotibial joint of all the birds, which was cleaned with 70%, alcohol, dried, followed by opening the joint. Synovial fluid samples were collected using a sterile cotton swabs. The tip of the swap was touched only on the site of suspected infection. The samples were stored in Stuart medium (Oxoid, UK) at room temperature until transported to within 2 h to the laboratory.

Isolation and identification

Pre-enrichment was performed for enterococci (BBL™ Enterococcosel™ Broth, BD, USA), staphylococci (Tryptone Soya Broth with 7.5% sodium chloride, TSB, Merck 1.00525) and salmonella (Buffered Peptone Water, Oxoid, CM0509) species. All broths were incubated at 37°C for 18-24 h. Thereafter, a loopful of broth was streaked on BBL™ Enterococcosel Agar (EA, Becton Dickinson, Germany) for enterococci, BD mannitol salt agar for staphylococci (MSA, Germany) for staphylococci, xylose lysine deoxycholate (XLD, Merck 1.05287) agar for *Salmonella* spp., eosin methylene blue agar (EMB, Merck 1.01347, Germany) for other Gram negative bacteria and 7% sheep blood agar (BA, Oxoid, UK CM0271) for all aerobic bacteria. The plates were incubated at 37°C for 72 h under aerobic conditions. When bacteriological growth occurred, the bacterial colonies were collected and purified. The classical characteristics (colony morphology, hemolysis, Gram stain, catalase, coagulase, potassium hydroxide 3% and oxidase test) were examined for the isolated microorganisms (14).

The confirmation of the identifications was carried out with the Vitek II system (Biomerieux, France). All salmonella suspicious colonies were tested serologically using specific *Salmonella* polyvalent O, monovalent O:9, and type-specific H antisera (Bio-Rad Laboratories, Inc. Richmond, CA, USA) (15). The isolates were stored in Brain Heart Infusion Broth (BHIB, Oxoid CM 1135, UK) containing glycerol 20% at -20°C.

Antimicrobial susceptibility

Gram negative bacteria: The activities of 17 antibiotics (gentamicin, neomycin, amikacin, amoxicillin-clavulanic acid,

ampicillin, cephalothin, cephalexin, ceftiofur, cefpodoxime, chloramphenicol, doxycycline, tetracycline, enrofloxacine, marbofloxacine, nitrofurantoin, trimethoprim-sulfamethoxazole, imipenem) and determination of the presence of extended spectrum beta lactamase (ESBL) were carried out with the Vitek II compact system using the Vitek II AST-GN97. The *E. coli* ATCC 25922 strain was used as quality control.

Gram positive bacteria: The activities of 16 antibiotics (gentamicin, neomycin, kanamycin, amoxicillin-clavulanic acid, benzylpenicillin, cefoxitin, oxacillin, ceftiofur, chloramphenicol, doxycycline, tetracycline, enrofloxacine, marbofloxacine, trimethoprim-sulfamethoxazole, erythromycin, clindamycin) and determination of the inducible clindamycin resistance (ICR) were carried out with the Vitek II compact system using the Vitek II AST- GP80. The *S. aureus* ATCC 29213 strain was used as quality control.

Antibiotic susceptibility results were evaluated according to the CLSI criteria (16).

RESULTS

Clinically

Unilateral or bilateral swelling of the femoro-tibial joints, edema, bleeding, purulent inflammation, swelling of the tendons, and abscess formation were present. Foot problems such as arthritis, tenosynovitis, femoral head lesions, osteomyelitis and reluctance to move were seen starting from 2 weeks of age in broilers. Later, due to lack of access to food or water, slimming and death were observed.

Isolation and identification

From these 152 synovial fluid samples, 53 (53/152, 34.9%) had no bacterial growth and from remaining 99 samples (99/152, 65.1%) a total of 112 aerobic bacteria (52/112,

Table 1: Identification results of all isolates

Bacteria	Number of Isolates (N=112)	Isolation Ratio (%)
E. coli	44	39.3
S. xylosus	19	16.9
S. aureus	10	8.9
S. saprophyticus	7	6.2
Salmonella spp.	7	6.2
S. lentus	4	3.6
S. hominis ssp. hominis	4	3.6
S. cohnii ssp. urealyticus	4	3.6
S. capitis	2	1.8
E. faecium	2	1.8
E. faecalis	2	1.8
S. sciuri	1	0.9
S. epidermidis	1	0.9
S. auricularis	1	0.9
S. lugdunensis	1	0.9
S. kloosii	1	0.9
S. warneri	1	0.9
E. aerogenes	1	0.9
TOTAL	112	100

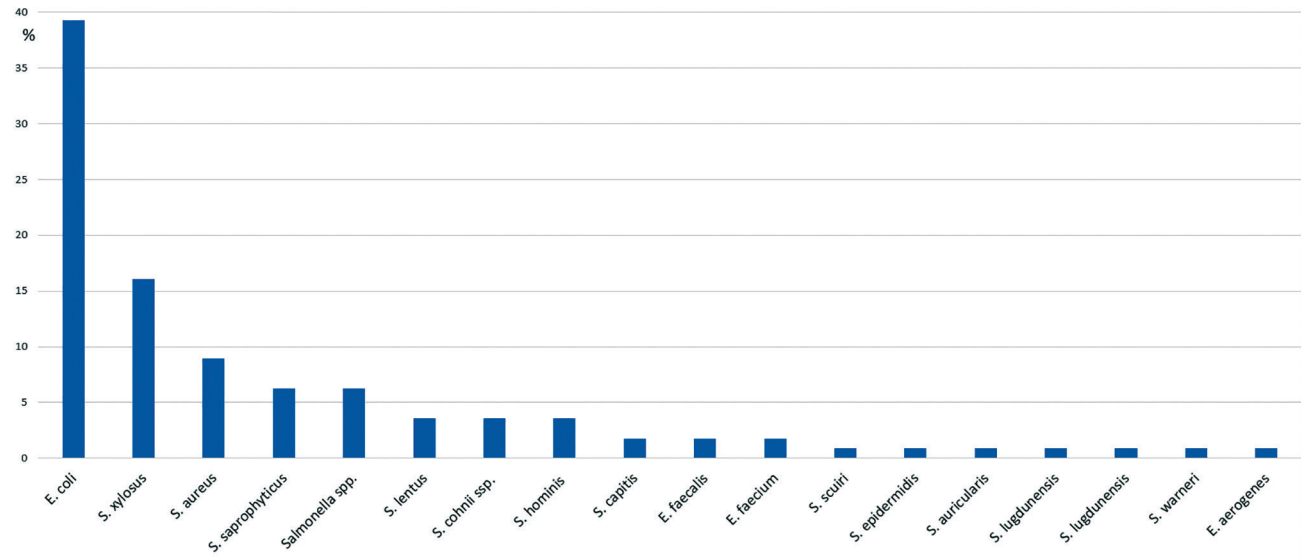

Figure 1: Proportion of bacteria isolated from samples

46.4% Gram negative and 60/112, 53.6% Gram positive) were isolated. Pure agent was isolated from 86 samples; two microorganisms were grown together from 13 samples. A total of 5 aerobic bacteria species were identified by Vitek II identification system: *Staphylococcus* spp. (56/112, 50.0%) followed by *Escherichia* spp. (44/112, 39.3%), *Salmonella* spp. (7/112, 6.2%), *Enterococcus* spp. (4%112, 3.6%), *Enterobacter* spp. (1/112, 0.9%). The identification results of culture positive 112 samples are presented in Table 1 and Figure 1.

Among the Gram negative bacteria, the most common species were *E. coli* (44/112, 39.3%) followed by *Salmonella* spp. (7/112, 6.2%) and *Enterobacter aerogenes* (1/112, 0.9%).

Among the Gram positive bacteria, the most common species were *Staphylococcus xylosus* (19/112, 16.9%) followed by *S. aureus* (10/112, 8.9%), *Staphylococcus saprophyticus* (7/112, 6.2%), *Staphylococcus lentus* (4/112, 3.6%), *Staphylococcus cohnii* spp. *urealyticus* (4/112, 3.6%), *Staphylococcus hominis* ssp. *hominis* (4/112, 3.6%), *Staphylococcus capitis* (2/112, 1.8%), *Enterococcus faecalis* (2/112, 1.6%), *Enterococcus faecium* (2/112, 1.6%), *Staphylococcus sciuri* (1/112, 0.9%), *Staphylococcus epidermidis* (1/112, 0.9%), *Staphylococcus auricularis* (1/112, 0.9%), *Staphylococcus lugdunensis* (1/112, 0.9%), *Staphylococcus kloosii* (1/112, 0.9%), *Staphylococcus warneri* (1/112, 0.9%).

Antibiotic resistance

In antibiotic susceptibility tests for Gram negative bacteria, 17 antibiotics belonging to 9 antimicrobial families (aminoglycoside, penicillin, cephalosporin, phenicol, tetracycline, quinolone, nitrofurantoin, folate pathway inhibitor, carbapenem) which are most commonly used in the treatment of poultry diseases were utilized. The resistance rates of the 52 Gram negative isolates to antibiotics were as the following: 90.4% to cephalexin, 78.8% to cephalothin, 71.2% to doxycycline, 69.2% to tetracycline and chloramphenicol, 65.4% to ampicillin, 61.5% to nitrofurantoin, 59.6% to cefpodoxime, 46.2% to enrofloxacin and marbofloxacine, 44.2% to amikacin, 36.5% to gentamicin, 36.4% to trimethoprim/sulfamethoxazole, 32.7% neomycin, 26.9% to amoxicillin-clavulanic acid, 11.5% to ceftiofur and 3.9% to imipenem.

Eight (8/44, 18.2%) *E. coli* isolates had ESBL activity (Table 2, Figure 2). Antibiotic resistance of Gram negative isolates was found to be the most resistant to cephalosporin antibiotics, followed by tetracycline, phenicol, penicillin and folate pathway inhibitors.

In antibiotic susceptibility tests for Gram negative bacteria, 16 antibiotics belonging to 9 antimicrobial families (aminoglycoside, penicillin, cephalosporin, phenicol, tetracycline, quinolone, folate pathway inhibitor, erythromycin, linkosamide) which are most commonly used in the treatment of poultry diseases were utilized. The resistance rates of the 60 Gram positive isolates to antibiotics were 78.3% to marbofloxacine, 75.0% to amoxicillin-clavulanic acid, 68.3% to benzylpenicillin, 66.6% to clindamycin, tetracycline and doxycycline, 63.3% to chloramphenicol, 60.0% to enrofloxacin and erythromycin, 45.0% to oxacillin, 25.0% to trimethoprim/sulfamethoxazole, 15.0% to ceftiofur, 13.3% to neomycin, 10.0% to gentamicin and kanamycin. Seven (7/60, 11.6) isolates had inducible clindamycin resistance and 12 (12/46, 26.1%) CNS isolates had cefoxitin resistance (Table 3, Figure 3). Antimicrobial resistance of Gram-positive isolates was found to be the most resistant to quinolone group antibiotics, followed by penicillin, linkozamide, tetracycline and phenicol group antibiotics.

DISCUSSION

In broilers, arthritis may have an infectious or noninfectious etiology (17). In the world (18,19) as well as in our country (20) there are very few studies examining aerobic bacteria causing arthritis in broilers. Therefore, the aim of this study was to identify the aerobic bacteria causing arthritis in broilers and to investigate the antibiotic resistance status of the isolates.

In this study, 65.1% (99/152) aerobic bacteria were isolated from arthritic broiler joint fluids. There are two possible reasons why aerobic bacterial agents cannot be isolated from 34.9% (53/152) of these samples. However, other agents that are microaerophilic/facultative anaerobic (*O. rhinotracheale, E. rhusiopathiae, M. synoviae*) or viral (*Reovirus, Adenovirus, Retrovirus, Herpesvirus*) may also cause arthritis.

Isolation rates of bacterial species isolated from arthritis broiler joints may vary depending on the method of breeding, age, hygiene methods applied in poultry farms. *S. aureus* has been reported to be the most common bacterial aerobic arthritis agent in broilers in previous studies (21). *E. coli* (6), *Salmonella* spp., *P. multocida* and *P. aeroginosa* (5), *Streptococcus* spp., *Enterococcus* spp. (20), *L. monocytogenes* (5,7) bacteria have been isolated from arthritis. Tawfik *et al.* (19) isolated *E. coli* (70%), *S aureus* (25%), *S. enterica* (5%), and *M. synoviae*

Table 2: Antibiotic resistance of Gram negative bacteria

Antibiyotikler	*E. coli* (n=44) (%)	*Salmonella* spp. (n=7) (%)	*E. aerogenes* (n=1) (%)	Resistant Gram (-) bacteria (n=52) (%)
Gentamicin	14 (31.8)	5 (71.4)	0	19 (36.5)
Neomycin	12 (27.2)	4 (57.1)	1 (100.0)	17 (32.7)
Amikacin	17 (31.8)	6 (85.7)	0	23 (44.2)
Amoxicillin-clavulanic acid	13 (29.5)	0	1 (100.0)	14 (26.9)
Ampicilin	33 (75.0)	0	1 (100.0)	34 (65.4)
Cephalothin	33 (75.0)	7 (100.0)	1 (100.0)	41 (78.8)
Cephalexin	39 (88.6)	7 (100.0)	1(100.0)	47 (90.4)
Ceftiofur	6 (13.6)	0	0	6 (11.5)
Cefpodoxime	29 (65.9)	2 (28.6)	0	31 (59.6)
Chloramphenicol	32 (72.7)	2 (28.6)	0	36 (69.2)
Doxycycline	29 (65.9)	7 (100.0)	1 (100.0)	37 (71.2)
Tetracycyline	29 (65.9)	6 (85.7)	1 (100.0)	36 (69.2)
Enrofloxacine	24 (54.5)	0	0	24 (46.2)
Marbofloxacine	24 (54.5)	0	0	24 (46.2)
Nitrofurantoin	24 (54.5)	7 (100.0)	1(100.0)	32 (61.5)
Trimethoprim-sulfamethoxazole	18 (40.9)	0	0	18 (36.4)
Imipenem	2 (4.6)	0	0	2 (3.9)
ESBL	8 (18.2)	0	0	8 (15.4)

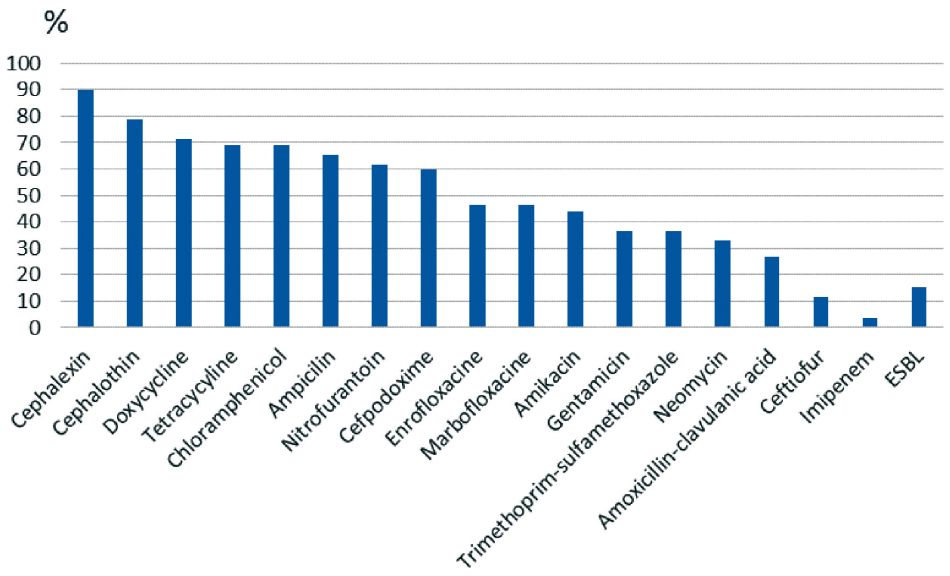

Figure 2: Antibiotic resistance rates of Gram negative isolates

(10%) from the broiler with arthritis. In this study, a total of five species of aerobic bacteria (*Staphylococcus* spp., *Escherichia* spp., *Salmonella* spp., *Enterococcus* spp., *Enterobacter* spp.) were identified.

In many parts of the world, antimicrobial resistance is becoming a major threat. Empirical treatment, inappropriate antibiotic prescriptions and widespread use of over-the-counter antibiotics have led to the emergence of antibiotic resistance (22). The most important risks of acquiring ESBL producing and methicillin resistance in bacteria are misuse of antibiotics and transmission of resistance genes from the community, animals and the environment (23,24). However, this is the first study to evaluate concurrent prevalence of ESBL producing *E. coli* and methicillin-resistant CNS iso-

Table 3: Antibiotic resistance of Gram positive bacteria

Antibiyotikler	CNS (n=46)	*S. aureus* (n=10)	*E. faecalis* (n=2)	*E. faecium* (n=2)	Resistant Gram (+) bacteria (n=60) (%)
Gentamicin	1 (2.2)	1 (10.0)	2 (100.0)	2 (100.0)	6 (10.0)
Neomycin	4 (8.8)	0	2 (100.0)	2 (100.0)	8 (13.3)
Kanamycin	2 (4.4)	0	2 (100.0)	2 (100.0)	6 (10.0)
Amoxicillin-clavulanic acid	34 (73.9)	7 (70.0)	2 (100.0)	2 (100.0)	45 (75.0)
Benzylpenicillin	34 (73.9	7 (70.0)	0	0	41 (68.3)
Cefoxitin	12 (26.1)	0	0	0	12 (20.0)
Oxacillin	21 (45.6)	3 (30.0)	2 (100.0)	1 (50.0)	27 (45.0)
Ceftiofur	4 (8.8)	1 (10.0)	2 (100.0)	2 (100.0)	9 (15.0)
Chloramphenicol	29 (63.0)	6 (60.0)	2 (100.0)	1 (50.0)	38 (63.3)
Doxycycline	29 (63.0)	7 (70.0)	2 (100.0)	2 (100.0)	40 (66.6)
Tetracycyline	31 (67.3)	5 (50.0)	2 (100.0)	2 (100.0)	40 (66.6)
Enrofloxacine	28 (60.8)	5 (50.0)	1 (50.0)	2 (100.0)	36 (60.0)
Marbofloxacin	37 (80.4)	6 (60.0)	2 (100.0)	2 (100.0)	47 (78.3)
Trimethoprim-sulfamethoxazole	10 (22.0)	1 (10.0)	2 (100.0)	2 (100.0)	15 (25.0)
Erythromycin	27 (58.6)	6 (60.0)	1 (50.0)	2 (100.0)	36 (60.0)
Clindamycin	29 (63.0)	8 (80.0)	2 (100.0)	1 (50.0)	40 (66.6)
ICR	2 (4.4)	1 (10.0)	2 (100.0)	2 (100.0)	7 (11.6)

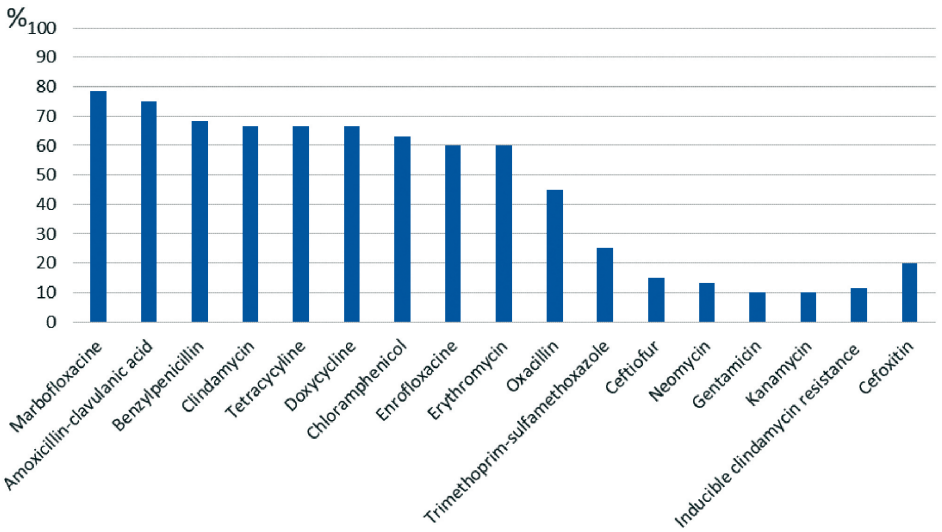

Figure 3: Antibiotic resistance rates of Gram positive isolates

lated from broiler with arthritis in Turkey. It is important to identify the prevalence of these microorganisms in the specific geographical areas and to establish an appropriate screening policy.

Coagulase negative staphylococci (CNS) are the most common microorganisms found in human and animal skin and isolated in clinical microbiology laboratories. The clinical importance of CNS has increased due to the fact that they are true pathogens that cause cardiovascular, joint and hematic infections (25). Most of the studies of methicillin resistance in staphylococci in poultry are related to *Staphylococcus aureus* (24). Methicillin-resistant CNS was first isolated from the respiratory tract and skin of healthy chickens at 1-8 weeks of age in 1996 (26). In this study, 45 methicillin resistant CNS isolates were identified as 37 *Staphylococcus sciurii*, 5 *S. epidermidis*, and 3 *S. saprophyticus*. In our study, the methicillin resistance rate in CNS was found to be 26.2%. Recently, the increase of CNS poses a threat to public health. While

methicillin resistant CNS types cause important infections; carriage between animals and humans has also become an important risk. These organisms must be considered a potential threat to broilers and veterinarians who care for them. These isolates were resistant to many beta-lactam antibiotics, and some isolates were also resistant to macrolide and aminoglycoside antibiotics.

Over the past few years, resistance to antimicrobial agents has increased among *E. coli* from both human and animal sources (27). Initially, resistance has been described to specific agents such as ampicillin, trimethoprim or tetracycline's (28). Recently, resistance has expanded with the emergence of broad resistance to large families of antimicrobial agents between human and veterinary isolates. *E. coli* isolates have been reported in ESBL producing poultry in Turkey as well as in various parts of the world. However, most studies have focused on *E. coli* isolated from feces (29,30) or meat of chickens (12,31,32). A study on ESBL producing *E. coli* isolated from arthritic joints is not available to the best of our knowledge. In our study, it was found that 18.2% (8/44) of *E. coli* isolates produced ESBL. This finding is lower than that of previous studies reported for ESBL *E. coli* in chicken meat (31,32). Carbapenems have been reported to be the most effective antibiotics for the treatment of infections caused by extended-spectrum beta-lactamase-producing bacteria (16). Two of the isolates obtained in this study were resistant to imipenem.

Clindamycin is a beneficial option in the treatment of skin and soft tissue infections caused by macrolide-resistant (e.g. erythromycin) staphylococci (33). The resistance to clindamycin is structural or inducible. Inducible clindamycin resistance can be determined by the antagonist effect of a macrolide on the effect of clindamycin (16). In this study, 60.0% (36/60) of total Gram positive bacteria isolates were erythromycin resistant. In 19.5% (7/36) of erythromycin resistant isolates, inducible clindamycin resistance was also present. These findings are similar to the rates reported by some researchers (34).

In this study, *E. coli* was the most common (39.3%) isolated bacterial species. This suggests that hygienic conditions in the poultry farms may not be good enough and that *E. coli* contributes to systemic infections in broilers. Similarly, CNS has been shown to be an important group of bacteria contributing to systemic infection. Staphylococci have a high affinity for collagen-rich surfaces such as the articular surface of joints and synovial sheaths placed around joints and tendons. This organism also tends to be localized in the growth plate of actively growing bones (1,2,5). It has been shown that in this study, joint lesions of broiler chickens can be caused by many different types of CNS. Therefore, it was concluded that CNS should be taken into consideration in cases of broiler arthritis.

According to the results of this study in ESBL resistance in *E. coli* and methicillin resistance in CNS has started to develop in broilers in Midwest of Turkey. Both forms of resistance are a public health problem. Furthermore, the possibility of ESBL and methicillin resistance should be considered in infections with treatment failure. It is thought that the molecular epidemiology and antimicrobial resistance mechanisms of these isolates should be examined together in future studies.

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