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Phenotypic characterisation of *Enterococcus* spp. from femoral head necrosis lesions of chickens

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Abstract: Two types of *Enterococcus* species were isolated from femoral head necrosis lesions of chickens. A total of 150 femoral articular tissues, of which 121 were from commercial broilers, 18 from broiler breeders, and 11 from layer breeders, were sampled. Out of 48 *Enterococcus* isolates of these clinical samples, 37 and 11 isolates were identified as *Enterococcus faecalis* and *Enterococcus faecium*, respectively. Additionally, 42 (28%) *Escherichia coli* and 33 (22%) *Staphylococcus aureus* isolates were obtained either alone or together with 1 of the 2 *Enterococcus* species from the same clinical samples, while no bacteria were detected from the remaining 56 samples. None of the *Enterococcus* isolates showed gelatinase activity, while only 1 *Enterococcus faecalis* isolate was found to be positive for both aggregation substance and cytolysin production. Resistance rates to tetracycline, erythromycin, ciprofloxacin, chloramphenicol, and ampicillin in *Enterococcus faecalis* were 94.6%, 43.2%, 37.8%, 17.8%, and 2.8%, respectively, while they were 81.8%, 45.5%, 18.2%, 9.8%, and 9.8% for the *Enterococcus faecium* isolates. All of the *Enterococcus* isolates were susceptible to both vancomycin and gentamicin. Resistance rate to 3 or more antibiotics was 18.9% and 18.1% for *Enterococcus faecalis* and *Enterococcus faecium* isolates, respectively.

Key words: *Enterococcus,* femoral head necrosis, chicken

Femur başı nekrozu lezyonlarından *Enterococcus* türlerinin fenotipik karakterizasyonu

Özet: Bu çalışmada 121 ticari broyler, 18 broyler damızlık, 11 yumurtacı damızlıktan elde edilen toplam 150 femur başı nekrozu incelendi ve iki farklı *Enterococcus* türü izole edildi. Elde edilen 48 *Enterococcus* izolatından 37'si *Enterococcus faecalis*, 11'i *Enterococcus faecium* olarak identifiye edildi. Ayrıca aynı örneklerden iki *Enterococcus* türünden biri ile birlikte ya da ayrı olarak 42 (% 28) *Escherichia coli* ve 33 (% 22) *Staphylococcus aureus* izole edilirken, 56 örnekten hiçbir bakteri saptanmadı. *Enterococcus* izolatlarından hiçbiri jelatinaz aktivitesi göstermezken, bir *Enterococcus faecalis* izolatı agregasyon maddesi ve sitolizin üretimi yönünden pozitif bulundu. *Enterococcus faecalis* ve *Enterococcus faecium*'un tetrasiklin, eritromisin, siprofloksasin, kloramfenikol ve ampisilin direnç yüzdeleri sırasıyla % 94,6, % 43,2, % 37,8, % 17,8, % 2,8, ve % 81,8, % 45,5, % 18,2, % 9,8, % 9,8 olarak bulundu. Sonuç olarak; tüm *Enterococcus* izolatlarının vankomisin ve gentamisine duyarlı olduğu, *Enterococcus faecalis* ve *Enterococcus faecium* izolatlarında 3 ya da daha fazla antibiyotiğe çoklu dirençlilik oranının sırasıyla % 18,9 ve % 18,1 olduğu belirlendi.

Anahtar sözcükler: *Enterococcus,* femur başı nekrozu, piliç

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Introduction

Enterococcus spp. can be encountered in different kinds of disease lesions in poultry, and can cause septicaemia and amyloid arthropathy (AA) in poultry. There are only a few field and/or experimental studies related to *Enterococcus* species and arthropathy/arthritis in chickens and/or in poultry (1-4). Abe et al. (5) observed encephalomalacia caused by *Enterococcus durans* (*E. durans*) in chicks. Wood et al. (6) isolated *Enterococcus fecorum* from bone lesions of broiler chicks. Chadfield et al. (7), in their 2 studies, showed the aetiological role of enterococci and *Enterococcus hirae* (*E. hirae*), respectively, in septicaemia cases in broilers. Petersen et al. (8) reported the isolation of normal and small colony variants of *Enterococcus faecalis* (*E. faecalis*) from field cases of AA in chickens. Steentjes et al. (9) found that *E. faecalis* was responsible for AA in broiler breeders and was isolated in 77% of amyloid arthritic joints. In an experimental study by Çiftci (3), chickens inoculated with a gelatinase-positive *E. faecalis* strain by intra-articular and intravenous injections produced AA lesions at the rates of 65.2% and 75.0%, respectively, and gelatinase, which is one of the virulence factors of *E. faecalis*, was reported as an important factor for developing AA in chickens. Another experimental study, by Sevimli et al. (4), demonstrated that excessive consumption of vitamin A increased morbidity and severity of AA. Beside these relations between enterococci and bone problems in chickens, there is only one study reporting the isolation of an *Enterococcus* species, *Enterococcus cecorum*, from femoral head necrosis lesions of broiler chickens (6).

Poultry production is regarded as the most heavily medicated sector among animal husbandry (10), where antibiotic resistant bacteria, including enterococci, can gradually emerge. Then, through this source, they can find ways to transmit to humans directly or indirectly through the consumption of meat, fish, and vegetables (11). Furthermore, these resistant enterococci can persist longer in the environment than faecal coliforms (12), which significantly increases their chance to re-infect animal or human hosts (13).

In this study we aimed to both screen for the presence of *Enterococcus* strains in femoral head necrosis (FHN) lesions in commercial broilers, broiler, and layer breeder chickens, and to examine the antibiotic resistance profiles of the isolates.

Materials and methods

Samples**:** A total of 150 femoral articular tissue samples from the necrosis lesions of 121 commercial broiler chickens from a 30,000 capacity Ross breed commercial broiler flock, of 18 broiler breeders from a Ross breed commercial broiler flock with 4000 capacity, and from 11 layer breeders of a 4000 capacity Isa Brown breed commercial layers with arthritis symptoms were obtained in sterile conditions for bacteriological analysis.

Bacteria: A gelatinase positive, cytolysin, and aggregation substance (AS) negative *E. faecalis* control strain was provided from Dr. Alper Çiftci, Department of Microbiology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey, and was used for control in experiments for growth, gelatinase, and cytolysin production, and for the presence of AS. *E. faecalis* OG1X and isogenic variants (Table 1) were kindly provided by Dr. Serap Savaşan, Department of Microbiology, Faculty of

Table 1. Properties of *Enterococcus faecalis* OG1X and isogenic variants used in this study.

Enterococcus fecalis isogenic variant	Presence of aggregation substance	Cytolysin production	Gelatinase production
OG1X	negative	negative	negative
OG1X (pAM 714)	positive	positive	negative
OG1X (pAM 944)	negative	positive	negative
OG1X (pAM 9058)	positive	negative	negative
OG1RF	negative	negative	positive

Veterinary Medicine, Adnan Menderes University, Aydın, Turkey. *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*) ATCC 35283, and *Staphylococcus aureus* (*S. aureus*) strains were provided from the bacterial culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Uludağ University, Bursa, Turkey. Reference strain of *E. faecalis* ATCC 29212 was used as the control strain in antibiotic susceptibility tests.

Isolation procedure**:** Articular tissue samples were homogenised using a sterile mortar and pestle containing 10 mL of Tryptic Soy Broth (TSB, Merck 1.05459). The homogenate was centrifuged at 1500 × g for 15 min. Ten microlitres of supernatant was streaked onto MacConkey Agar (Merck, 1.05465) and Mannitol Salt Phenol Red Agar (Merck, 1.05404) plates for *E. coli* and *Staphylococcus* spp., respectively. In addition, 1 mL from the supernatant was inoculated into 10 mL of Enterococcus Presumptive Broth (EPB) prepared as described by Çiftci (3) and incubated at 37 °C for 48 h. Ten µL from EPB broth showing yellow coloration after incubation were streaked onto Enterococcosel Agar (BD BBL 212205, Becton Dickinson, USA) plates and were incubated at 37 °C for 48 h. *Enterococcus* suspect colonies were tested for catalase activity, and catalase negative colonies were examined by API 20 strep test kit (Biomerieux, Marcy l'Etoile, France) for identification. Gram and catalase positive cocci were subjected to coagulase test by Staphylase test kit (Oxoid, DR0595A) for the identification of staphylococci. Isolated gram-negative rod shaped bacteria were identified by carbohydrate fermentation tests.

Antibiotic susceptibility testing: Antimicrobial resistance patterns of the enterococci were determined by disc diffusion method as described in the National Committee for Clinical Laboratory Standards (NCCLS) Guidelines (14). Antibiotic discs (Oxoid) used were ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), erythromycin (30 µg), gentamicin (120 µg), chloramphenicol (30 µg), and ciprofloxacin (5 µg). Interpretation of the inhibition zone diameter was performed according to the recommendations of NCCLS (14). Isolates considered intermediate by this method were recorded as sensitive.

Virulence factor determination in *Enterococcus* isolates: Isolated enterococci were examined for AS, cytolysin and gelatinase production as described by Çiftci and by Elsner et al. (3,15).

Statistical analysis: Data obtained were evaluated statistically by chi-square test (Minitab Release 13.20, 2001).

Results

Bacteriology results: A total of 48 (32%) *Enterococcus* isolates were obtained from 150 articular tissue samples of all chicken types. Twenty-two (18.2%), 1 (5.6%), and 3 (27.3%) of the 121 commercial broiler, 18 broiler breeder, and 11 layer breeder chickens had enterococci, respectively (Table 2).

Table 2. Comparison of single isolation and coisolation rates of *Enterococcus* spp., *E. coli*, and *S. aureus* according to the breeding type of chickens.

Breeding Type	n	E (%)	S (%)	Ec (%)	E + S (%)	E + Ec (%)	S + Ec (%)	E + S + Ec (%)	No isolation (%)
Commercial Broiler	121	22 (18.2)	10 (8.3)	31 (25.6)	12 (9.9)	4 (3.3)	2 (1.7)	5 (4.1)	35 (28.9)
Broiler Breeder	18	1 (5.6)	3 (16.7)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	13 (72.2)
Layer Breeder	11	3 (27.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (72.7)
Total	150	26 (17.3)	13 (8.7)	31 (20.7)	13 (8.7)	4 (2.7)	2 (1.3)	5 (3.3)	56 (37.3)

n, number of samples; E, number (%) of only *Enterococcus* isolation; S, number (%) of only *S. aureus* isolation; Ec, number (%) of only *E. coli* isolation; E+S, number (%) of both *Enterococcus* and *S. aureus* isolation; E+Ec, number (%) of both *Enterococcus* and *E. coli* isolation; S+Ec, number (%) of both *S. aureus* and *E. coli* isolation; E+S+Ec, number (%) of *Enterococcus*, *S. aureus*, and *E. coli* isolation.

Thirty-seven and 11 of the *Enterococcus* isolates were identified as *E. faecalis* and *E. faecium*, respectively. All *E. faecalis* isolates were from commercial broilers, whereas *E. faecium* isolates belonged to all chicken types as follows: 6 (54.5%) isolates from commercial broilers, 2 (18.1%) isolates from broiler breeders, and 3 (27.2%) isolates from layer breeders.

Twelve out of 121 (9.9%) commercial broilers were found to harbour both *Enterococcus* spp. and *S. aureus*, whereas only 1 (5.6%) broiler breeder out of 18 was found to have both of these bacteria. No coinfection with *Enterococcus* spp. and *S. aureus* was observed in the layer breeders (Table 2).

In addition, *E. coli* was isolated from 4 (3.3%) commercial broiler chickens together with *Enterococcus*, while there was no *E. coli* isolation either from broiler or from layer breeders (Table 2).

E. coli isolation numbers alone and as coinfection with either *Enterococcus* spp. or *S. aureus*, or with both *Enterococcus* and *S. aureus* from 121 commercial broiler articular samples were as 31 (25.6%), 4 (3.3%) or 2 (1.7%), and 5 (4.1%), respectively. No *E. coli* was isolated either alone or with other bacteria from broiler or layer breeders (Table 2).

No *S. aureus* was isolated from any of the layer breeder samples, while 10 (8.3%) and 3 (16.7%) of the commercial broilers and broiler breeders were found to harbor *S. aureus*, respectively (Table 2).

Isolations of *Enterococcus* with or without *S. aureus* and/or *E. coli* from FHN lesions were compared. Sole *Enterococcus* spp. isolation rate (17.3%) was significantly higher than coinfection with *Enterococcus* spp., *S. aureus* and *E. coli* (3.3%) ($P < 0.05$). There was an increase in *E. coli* and/or *S. aureus* isolations (30.6%) ($P < 0.05$) when no enterococci was isolated from the samples (Table 3).

Some virulence factors of *Enterococcus* isolates**:** All *Enterococcus* isolates were gelatinase negative. Only 1 isolate (*E. fecalis* isolate no. 12) from commercial broilers was positive for both aggregation substance and cytolysin production.

Antibiotic resistance of *Enterococcus* isolates**:** Numbers and percentages of enterococci resistance profiles tested against 7 different antibiotics are summarised in Table 4. Resistance rates to tetracycline, erythromycin, ciprofloxacin, chloramphenicol, and ampicillin in *E. faecalis* were 94.6%, 43.2%, 37.8%, 17.8%, and 2.8%, respectively, while they were 81.8%, 45.5%, 18.2%, 9.8%, and 9.8%, respectively, for the *E. faecium* isolates. No resistance was observed to vancomycin and gentamicin in either species' isolates. Antibiotic resistance patterns of all *E. faecalis* and *E. faecium* isolates are given in detail in Table 5. Resistance to the 3 antibiotics tested was determined in 7 (18.8%) and 2 (18.2%) of the *E. faecalis* and *E. faecium* isolates, respectively (Table 5).

Table 3. Statistical analyses of *Enterococcus* spp. isolation with or without *S. aureus* and/or *E. coli*.

	Enteroccoccus			
	Positive		Negative	
S. aureus and/or *E. coli*	n	%	n	%
Positive	5^{A}	3.3	46^{B}	30.6
Negative	26^{C}	17.3	56^{D}	37.3

$^{A-D}$ Different superscripts in the same line indicate statistical difference ($P < 0.05$) between groups.

Table 4. Antibiotic resistance profiles of *E. faecalis* and *E. faecium* isolates.

	Antibiotic resistance to						
Enterococcus (n)	Amp (%)	Van (%)	Tet (%)	Ery (%)	Gen (%)	Chl (%)	Cip (%)
faecalis (37)	1 (2.8)	0.0 (0.0)	35 (94.6)	16 (43.2)	0.0 (0.0)	4 (17.8)	14 (37.8)
faecium (11)	1 (9.8)	0.0 (0.0)	9 (81.8)	5 (45.5)	0.0 (0.0)	1 (9.8)	2 (18.2)

Table 5. Antibiotic resistance profiles of *Enterococcus* isolates from chickens with FHN.

E. faecalis Isolate No.	Resistance to	*E. faecalis* Isolate No.	Resistance to	*E. faecalis* Isolate No.	Resistance to
1	Tet, Ery	20	Tet, Cip	1	Amp, Ery
2	Tet, Cip	21	Tet, Ery	2	Tet
3	Tet, Cip	22	Tet, Chl, Cip	3	Cip
4	Ery, Cip	23	Tet, Cip	4	Tet, Ery
5	Tet, Ery, Amp	24	Tet, Cip	5	Tet, Ery, Cip
6	Tet, Ery, Chl	25	Tet	6	Tet
7	Tet, Ery	26	Tet	7	Tet
8	Tet	27	Tet, Ery	8	Tet
9	Tet, Chl	28	Tet	9	Tet
10	Tet	29	Tet, Ery, Cip	10	Tet, Ery, Chl
11	Tet, Ery, Cip	30	Tet, Ery	11	Tet, Ery
12	Tet, Cip	31	Tet, Cip		
13	Ery	32	Tet, Cip		
14	Tet, Ery	33	Tet, Ery		
15	Tet	34	Tet		
16	Tet, Ery	35	Tet, Ery, Cip		
17	Tet, Ery	36	Tet		
18	Tet	37	Tet, Chl, Cip		
19	Tet, Ery				

Discussion

In this study, we isolated 2 *Enterococcus* species at different rates from 3 breeding types of chickens with FHN. Thirty-seven *Enterococcus* isolates were identified as *E. faecalis* and 11 isolates as *E. faecium*. All *E. faecalis* isolates were only from commercial broiler chickens, while *E. faecium* was isolated from 6 (54.5%) commercial broilers, 2 (18.1%) broiler breeders, and 3 layer breeders (27.2%). Similar to our study, these 2 *Enterococcus* species have previously been reported as the most dominantly isolated enterococci from chickens (16), while some other *Enterococcus* species such as *E. durans*, *E. gallinarum*, and *E. hirae* can also be isolated (10,17,18). As mentioned in the literature, major predisposing factors for the development of FHN are immunosuppressive viruses such as Infectious Bursal Disease Virus and Chicken Infectious Anaemia Virus. Bacteria such as *Mycoplasma synoviae* and *S. aureus* (19) can accompany the viral pathologies as secondary infection agents, which increase both the mortality rate and the severity of FHN lesions in the flocks. On the other hand, sometimes bacteria such

as enteroccoci, staphylococci, and streptococci can find ways to cause primary bone infections in chickens, as well (20). In light of the findings in this study, and regardless of the predisposing factor(s), the presence of *Enterococcus* in FHN lesions should always be taken into consideration as an important cause of primary or secondary infection in chickens. These types of cases also require immediate action with appropriate antibiotic treatment.

Our results revealed that enterococci could be found at different rates, and together with or without *S. aureus* and/or *E. coli*. These bacteria had been reported as other frequently isolated bacteria from articular problems in chickens (19,21), depending on the chicken breeding type. We observed a significant increase in *E. coli* and *S. aureus* isolations from the samples without enterococci (Table 3). Here, we should indicate that there was no particular relation between these 3 types of bacteria causing a dual or triple infection in femur head and the breeding type of chicken, but our findings indicate that they can coexist in FHN infections. Thus, it is important to examine the clinical sample at least for these 3 bacteria to determine the appropriate antibiotic regimen during treatment.

None of the enteroccoci isolates gelatinase except one in our study was AS and cytolysin positive. This suggests that these virulence factors may not have a significant effect on the persistence/presence of enterococci in FHN cases. A study by Çiftci (3), where he had experimentally developed arthritis by an AS, gelatinase, and cytolysin negative *E. faecalis* isolate at the rate of 100% in chickens, supports our suggestion.

Resistance to tetracycline among *Enterococcus* spp. is very common, particularly among those of poultry origin in the United States (22) and in other countries (16,23). Tetracycline resistance has also been previously demonstrated to be linked closely to poultry production environments (10), with observations of similar distributions of MICs (24). However, surveys on *E. faecalis* and *E. faecium* isolates from poultry flocks (25,26), poultry products (27), and from other animals (28) in the United States reported that these isolates were not resistant to vancomycin. We also found a high level of resistance to tetracycline, but no resistance to vancomycin in enterococci isolated from FHN lesions of chickens in this study.

Additionally, we observed a high frequency of resistance to erythromycin, tetracycline, and ciprofloxacin in both of the *Enterococcus* species isolated in this study. This fact had previously been related to the extensive use of quinolones, tetracyclines, and erythromycin in animal husbandry (29).

Our finding for resistance to chloramphenicol in 5 enterococci was surprising, since this antibiotic has been banned for veterinary use since 2002 in Turkey (30).

Contrary to similar studies from Japan (23), Denmark (31), Portugal (32) and USA (17,28), no gentamicin resistance was detected among either *E. faecalis* or in *E. faecium* isolates in this study. This is another interesting finding, particularly for this country, because currently gentamicin is the widely used antibiotic for treating bacterial infections in chickens in Turkey.

Ampicillin resistance in 1 *E. faecalis* and 1 *E. faecium* isolate in our study is partially different from that previously reported (23). In that study, layer and broiler chickens' *E. faecium* isolates were 22.2% and 64.1% resistant to ampicillin, respectively, whereas none of the *E. faecalis* isolates from either breeder type was resistant. Quednau et al. (33) however, had similarly found low resistance to ampicillin in enterococci isolated from chickens in their study.

Our results in this study indicate that enterococci are important causative agents in FHN lesions of chickens in Turkey. Antibiotic resistance profiles of these FHN-related enterococci, to the best of our knowledge, were also examined and reported for the first time.

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