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Antibiotic resistance profile of *Enterococcus faecium* and *Enterococcus faecalis* isolated from broiler cloacal samples

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Abstract: The present study was performed to isolate and identify *Enterococcus* spp. from broiler cloacal samples to species level, to determine their resistance patterns to various antibiotics, and to detect vancomycin resistance genes. Cloacal samples of broilers collected from slaughterhouses were inoculated in Slanetz and Bartley agars with and without vancomycin (6 µg/mL). Antibiotic resistance/susceptibility testing of the isolated and identified enterococci was performed by using the disk diffusion test. Multiplex PCR was used to identify the species and to detect vancomycin resistance genes. The majority of the isolated enterococci was *Enterococcus faecium* (60.43%, n = 142) and *Enterococcus faecalis* (33.62%, n = 79). *E. casseliflavus* and *E. gallinarum* were identified from 8 (3.42%) and 6 (2.56%) isolates, respectively. It was found that 88.9% of the enterococci were resistant to tetracycline and 83.4% of them were resistant to erythromycin. As a result, none of the strains isolated from cloacal samples of broilers carried the *van*A and *van*B genes. It was observed that 54.9% of *E. faecium* isolates and 78.4% of *E. faecalis* isolates were multidrug resistant (resistant to 3 or more antibiotic groups). The lack of vancomycin-resistant *Enterococcus* among the enterococci isolates was important for public health.

Key words: Antibiotic resistance, broiler, *Enterococcus faecalis*, *Enterococcus faecium*, *van*A

1. Introduction

Enterococci are commonly found in soil, water, and plants in nature. In addition, they are a part of the normal gastrointestinal flora of humans and animals (1). *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly isolated *Enterococcus* species from the gastrointestinal system of humans and animals (2). Although enterococci are not an important pathogen of animals (3), *E. faecalis* and *E. faecium* are the most frequent causes of nosocomial infections in humans in the world (4). Enterococci are used as indicators of fecal contamination and for monitoring of antimicrobial resistance of bacteria (5).

Enterococci have either intrinsic or acquired resistance to most of the antibiotics used in humans. Enterococcal infections, particularly nosocomial infections, may be life threatening in humans, as antibiotic treatment of these infections is difficult (6). In the veterinary medicine field, antibiotics are commonly used for the control and treatment of diseases, and antibiotic usage results in a selection of resistant enterococci in the intestinal flora of animals. Antibiotic resistant isolates can pass to humans either by food products or direct contact, and antibiotic resistance genes on mobile genetic elements may be transferred to human bacteria (6).

Detection of vancomycin resistant *Enterococcus* (VRE) isolates in chicken products may result in prohibition of the exportation of these products (7). Eight types of acquired vancomycin resistance genes (*van*A, *van*B, *van*D, *van*E, *van*G, *van*L, *van*M, and *van*N) in enterococci have been identified, with the most common being the *van*A gene. This vancomycin resistance gene is associated with mobile genetic elements and may be transferred to clinical enterococci and other pathogens (4).

The present study was aimed to isolate and identify enterococci from broiler cloacal samples to species level, to determine their resistance patterns to different antibiotics, and to identify vancomycin resistance genes.

2. Materials and methods

2.1. Identification and isolation of enterococci

Two hundred and forty cloacal swab samples, which were collected in Cary-Blair transport medium from the slaughterhouses of three different integrated broiler companies in 2011 and 2012, were inoculated onto Slanetz and Bartley agar plates supplemented with vancomycin

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(6 µg/mL) and without vancomycin. All *Enterococcus* suspected colonies were subcultured on 5% sheep blood agar. Pure cultures of catalase negative *Enterococcus* isolates that grow in bile esculin agar and 6.5% NaCl broth (8) were identified to species level using BBL Crystal Gram-Positive Identification System kits.

2.2. Antimicrobial susceptibility test

Antimicrobial susceptibility testing of enterococci to 11 different antibiotics, namely ampicillin (10 µg), vancomycin (30 µg), teicoplanin (30 µg), quinupristin/dalfopristin (15 µg), tetracycline (30 µg), rifampicin (5 µg), erythromycin (30 µg), gentamicin (120 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), and ciprofloxacin (5 µg), was performed by disc diffusion method using Mueller–Hinton agar and the test results were interpreted according to the Clinical and Laboratory Standards Institute recommendations (9).

2.3. DNA Isolation

For DNA extraction, the enterococci incubated in Mueller–Hinton broth for one night were centrifuged at 5000 rpm for 10 min to collect the bacteria and 1 mL of TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA) was added to the pellet. The solution was centrifuged at 14,000 rpm for 10 min; then the pellet was washed twice with TE buffer. The supernatant was discarded and 50 µL of lysostaphin (100 µg/mL) was added to the pellet. The solution was left for incubation for 10 min at 37 °C. After adding 50 µL of proteinase K (100 µg/mL), the solution was again incubated for 10 min at 37 °C and the DNAs that were extracted from samples incubated at 100 °C for 10 min for the inactivation of proteinase K. The extracted DNAs were stored at −20 °C until analyses (10).

2.4. Multiplex PCR method

The PCR mixture for amplification was prepared with 5 pmol *van*A primers and 2.5 pmol of each other primer (*van*B, *van*C1, *van*C2/C3, *rrs*, *E. faecalis*-specific and *E. faecium*-specific), so that the total volume of the final mixture would be 25 µL. The mixture was prepared such that it contained 1X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, and 0.5 U of Hot Start *Taq* DNA polymerase in the total volume. The amplified product was subjected to agarose gel electrophoresis, DNA bands were visualized by imaging system, and the results were evaluated.

Vancomycin resistance genes (*van*A and *van*B) in enterococci and *E. faecium*, *E. faecalis*, *E. gallinarum* (*van*C1), and *E. casseliflavus* (*van*C2/C3) species-specific genes were identified using multiplex PCR. Multiplex PCR reaction mixtures were prepared and target genes were amplified as described by Getachew et al. (11), and optimization was done by positive strains in the laboratory. The primers used in the present study are shown in Table 1.

2.5. Reference strains

E. faecalis ATCC 29212, *E. faecalis* WHO3 (*van*A), *E. faecalis* WHO14 (*van*B), *E. gallinarum*, and *E. casseliflavus* strains were used for the identification of enterococci, analyses of antibiotic resistance profiles, and optimization of multiplex PCR in the laboratory studies.

Table 1. Primers used for the identification of *Enterococcus* species and vancomycin resistance genes (11).

Primer specificity	Primer	Sequence of primer pairs	(bp)
*van*A gene	*van*A	5'ATGAATAGAATAAAAGTTGCAATA-3' 5'CCCCTTTAACGCTAATACGATCAA-3'	1030
*van*B gene	*van*B	5'-AAG CTA TGC AAG AAG CCA TG-3' 5'-CCG ACA ATC AAA TCA TCC TC-3'	536
E. gallinarum	*van*C1	5'-GGTATCAAGGAAACCTC-3' 5'-CTTCCGCCATCATAGCT-3'	822
E. casseliflavus	*van*C2/C3	5'-CGGGGAAGATGGCAGTAT-3' 5'-CGCAGGGACGGTGATTTT-3'	484
E. faecalis	ddl*E. faecalis*	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941
E. faecium	ddl*E. faecium*	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658
PCR internal control	*rrs* (16S rRNA)	5'-GGATTAGATACCCTGGTAGTCC-3' 5'-TCGTTGCGGGACTTAACCCAAC-3'	320

3. Results

In this research, 235 *Enterococcus* species were isolated using Slanetz and Bartley agars with or without vancomycin. Among the enterococci, 142 (60.43%) *E. faecium*, 79 (33.62%) *E. faecalis*, 8 (3.4%) *E. casseliflavus*, and 6 (2.55%) *E. gallinarum* (Table 2) were identified by using BBL Crystal Gram-Positive Identification System kits. Only one *E. faecium* and four *E. faecalis* isolates from all *Enterococcus* isolates grew on Slanetz and Bartley agars containing 6 µg/mL vancomycin. However, none of these isolates showed bands for *van*A and *van*B genes in PCR.

Antibiotic resistance rates of *E. faecium* (n = 142) isolates to tetracycline, erythromycin, rifampin, ciprofloxacin, chloramphenicol, quinupristin/dalfopristin, gentamicin, ampicillin, teicoplanin, nitrofurantoin, and vancomycin were 88.7%, 82.3%, 40.1%, 26.0%, 21.8%, 20.4%, 8.4%, 2.1%, 1.4%, 1.4%, and 0.7%, respectively (Table 2). Antibiotic resistance rates of *Enterococcus faecalis* (n = 79) isolates to tetracycline, erythromycin, quinupristin/dalfopristin, chloramphenicol, ciprofloxacin, gentamicin, rifampin, vancomycin, and teicoplanin were 88.6%, 82.2%, 82.2%, 49.3%, 36.7%, 27.8%, 20.2%, 5.0%, and 1.2%, respectively. No resistance to ampicillin and nitrofurantoin was detected (Table 2).

Multiplex PCR positive strains and field isolates are shown in the Figure.

Considering all the enterococci, tetracycline, erythromycin, quinupristin/dalfopristin, chloramphenicol, rifampin, ciprofloxacin, gentamicin, vancomycin, ampicillin, teicoplanin, and nitrofurantoin resistance rates were found as 88.9%, 83.4%, 42.9%, 33.1%, 32.7%, 31.0%, 17.8%, 2.1%, 1.2%, 1.2%, and 0.8%, respectively (Table 2).

In this research, it was observed that 54.9% of *E. faecium* isolates and 78.4% of *E. faecalis* isolates were multidrug resistant (resistant to 3 or more antibiotic groups).

4. Discussion

In the present study, the most commonly isolated *Enterococcus* species from broiler cloacal samples was *E. faecium* (142/235, 60.4%), followed by *E. faecalis* (79/235, 43.7%). This result was consistent with the results of previous studies, which reported that *E. faecium* was the most commonly isolated *Enterococcus* species from poultry cloacal swabs (12) and poultry neck skin samples in Turkey (13), poultry fecal samples in Southeast Asian countries (14), and meat from poultry and other animals in Greece (15). However, it was reported in Germany (16) that the most commonly isolated strain from the samples of various poultry showing clinical symptoms was *E. faecalis* (88%). This discrepancy might have resulted from the differences in geographical region, sampling time, taking samples from animals with clinical symptoms, and the methods used.

Table 2. Antibiotic resistance rates of *Enterococcus* species with disk diffusion test.

Antibiotic	Isolates and their antibiotic resistance status n (%)				
	E. faecium (142)	*E. faecalis* (79)	*E. casseliflavus* (8)	*E. gallinarum* (6)	Total (235)
AM	3 (2.1)	-	-	-	3 (1.2)
VA	1 (0.7)	4 (5.0)	-	-	5 (2.1)
TEC	2 (1.4)	1 (1.2)	-	-	3 (1.2)
QD	29 (20.4)	65 (82.2)	6 (75.0)	1(16.6)	101 (42.9)
TE	126 (88.7)	70 (88.6)	7 (87.5)	6 (100)	209 (88.9)
E	117 (82.3)	65 (82.2)	8(100)	6(100)	196 (83.4)
CN	12 (8.4)	22 (27.8)	5 (62.5)	3 (50.0)	42 (17.8)
C	31 (21.8)	39 (49.3)	3 (37.5)	5 (83.3)	78 (33.1)
F/M	2 (1.4)	-	-	-	2 (0.8)
CIP	37 (26.0)	29 (36.7)	1 (12.5)	6 (100)	73 (31.0)
RA	57 (40.1)	16 (20.2)	3 (37.5)	1 (16.6)	77 (32.7)

AM: Ampicillin (10 µg), VA: Vancomycin (30 µg), TEC: Teicoplanin (30 µg), QD: Quinupristin/dalfopristin (15 µg), TE: Tetracycline (30 µg), E: Erythromycin (30 µg), CN: Gentamycin (120 µg/mL), C: Chloramphenicol (30 µg), F/M: Nitrofurantoin (300 µg), CIP: ciprofloxacin (5 µg), RA: Rifampin (5 µg).

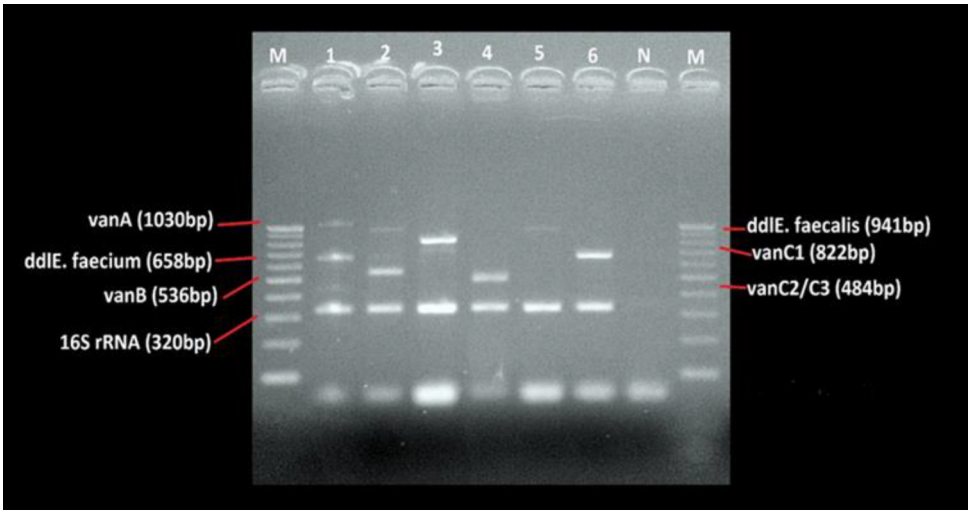

Figure. Multiplex PCR positive strains and field isolates.
M: Marker 100–1000 bp, 1: *Enterococcus faecium van*A positive strain (*van*A, ddlE. faecium and 16S rRNA genes) 2: *Enterococcus faecalis van*B positive strain (ddlE. faecalis, *van*B and 16S rRNA genes) 3: *van*C1 positive *E. gallinarum* strain (*van*C1 and 16S rRNA genes) 4: *van*C2/C3 positive *E. casseliflavus* strain *(van*C2/C3 and16S rRNA genes) 5: *Enterococcus faecalis* strain (ddlE. faecalis and 16S rRNA genes) 6*: E. faecium* strain (ddlE. *faecium* and 16S rRNA genes) N: Negative control.

This study evaluated antimicrobial resistance/susceptibility of enterococci to several antibiotics. Among the antibiotics tested in the present study, the highest resistance rate was to tetracycline (88.9%) and erythromycin (83.4%). Tetracycline and erythromycin resistance rates ranged from 55% to 100% and from 45% to 100%, respectively, in the previous studies performed in Turkey (12,13,17). The research in the other countries also demonstrated high rates of resistance to tetracycline and erythromycin (14,16,18,19). Usui et al. (14) found that 92% of *E. faecium* isolates from poultry feces showed resistance to oxytetracycline, 82.8% to enrofloxacin and 79.4% to erythromycin, while 70.9% of *E. faecalis* isolates showed resistance to erythromycin, 69.2% to oxytetracycline and 17.9% to enrofloxacin, and the authors suggested that antibiotic resistance may be different in different *Enterococcus* species. In the study presented herein, *E. faecalis* isolates showed higher resistance rates to quinupristin/dalfopristin, gentamicin, chloramphenicol, and ciprofloxacin, whereas *E. faecium* isolates showed higher resistance to rifampin (Table 2). Quinupristin/dalfopristin has substantial activity only against *E. faecium* (20). Multidrug antibiotic resistance (resistance to 3 or more antibiotic groups) rates were 54.9% in *E. faecium* isolates, 78.4% in *E. faecalis* isolates, and 47.3% in all enterococci tested, indicating higher rates of multidrug resistance in *E. faecalis* isolates. However, all the isolates tested were sensitive to ampicillin and nitrofurantoin. These results were similar to the research results given by Maasjost et al. (16).

In the present study, resistance to vancomycin was determined in 5 *Enterococcus* isolates (1 *E. faecium* and 4 E. *faecalis*) using the disk diffusion test and agar with vancomycin. However, in the PCR analyses, none of these enterococci was found to have vancomycin resistance genes. Similarly, Usui et al. (14) in their study in which they found low susceptibility to vancomycin (8 mg/L) in 4 isolates could not detect *van*A and *van*B resistance genes in these isolates. Therefore, it is evident that phenotypic tests alone are not sufficient to determine vancomycin resistance and that vancomycin resistance genes should also be identified. On the other hand, this is explained by the possibility of less common strains carrying the genes (*van*D, *van*E, *van*G, *van*L, *van*M, and *van*N). In fact, Getachew et al. (11) is emphasized in this situation.

Avoparcin (vancomycin analogue) using was banned in 1997 in European countries and Turkey. Vancomycin resistance was reported to be decreased in Japan by Usui et al. (14) and in Turkey by Kasimoglu Dogru et al. (13) *van*A and *van*B genes were not detected in any of the 235 *Enterococcus* isolates in this research. Bortolaia et al. (21) in a study they performed in the poultry farms 15 years after prohibition of avoparcin in Denmark, isolated vancomycin resistant *E. faecium* isolates at low fecal concentrations in selective agars containing only 16 µg/mL vancomycin. It was reported that these isolates may be those transmitted from parent animals.

In conclusion, *E. faecium* and *E. faecalis* were common among broiler-derived enterococci and the dominant species was *E. faecium.* Erythromycin and tetracycline resistance

was over 80% in both species, and there were differences between the species in terms of resistance to other antibiotics. Multidrug resistance was higher among *E. faecalis* (78.4%) isolates than it was among *E. faecium* (54.9%) isolates. It was important that lack of VRE among *Enterococcus* isolates from broilers cloacal samples was determined. However, enterococci possess a zoonotic risk to public health by their resistance properties to other antibiotics.

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