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Original Article

Antimicrobial Susceptibility Profile of Enterococcus Species Isolated from Companion Birds and Poultry in the Northeast of Iran

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ABSTRACT

Enterococci are Gram-positive facultative anaerobic bacteria commonly found in the gastrointestinal tract of the mammals and birds. These cocci are isolated from urinary tract infections, bacteremia, endocarditis, and burn wounds in humans. The evolution of antibiotic-resistant enterococci raised a problem due to the possibility of the transmission of these organisms between poultry and human. Regarding this, the present study was conducted to evaluate the prevalence of Enterococcus species among companion birds and poultry in the Northeastern of Iran and determine the antibiotic susceptibility profile of enterococci. To this end, oral and cloacal swabs were collected from 150 caged birds. Antibiotic susceptibility profile was determined using the standard disk diffusion method. The results revealed that out of 150 samples, 56 (37.33%) cases contained enterococci. Most of the specimens (25.33%) were Enterococcus faecalis isolated from 6.66% of the samples. Additionally, 2.66% and 1.33% of the samples were contaminated with Enterococcus mundtii and Enterococcus gallinarum, respectively. Furthermore, Enterococcus malodoratus and Enterococcus raffinosus were isolated from 0.66% of the samples. The results revealed that all of the isolates of *E. faecalis* and *E. faecium* were resistant to more than five antimicrobial agents. Most of *E. faecalis* and *E. faecium* isolates showed resistance to Cefazolin, Tiamulin, Flumequine, and Cephalexin. Accordingly, the majority of the isolates had multidrug resistance to the tested antibiotics. In conclusion, the presence of multidrug-resistant enterococci in the birds living close to humans requires thorough observations due to the transmission of these organisms to humans.
Keywords: Enterococci, Bird, Antibiotic resistance

Profil de Sensibilité aux Antimicrobiens des Espèces d'Entérocoques Isolées chez des Oiseaux et des Volailles de Compagnie dans le Nord-est de l'Iran

Résumé: Les entérocoques sont des bactéries anaérobies facultatives à Gram positif communément présentes dans le tractus gastro-intestinal des mammifères et des oiseaux. Ces cocci sont isolés d'infections des voies urinaires, de bactériémies, d'endocardites et de brûlures chez l'homme. L'évolution des entérocoques résistants aux antibiotiques pose un problème en raison de la possibilité de transmission de ces organismes entre la volaille et l'homme. À cet égard, la présente étude a été menée pour évaluer la prévalence des espèces d'entérocoques chez les oiseaux de compagnie et les volailles dans le nord-est de l'Iran et déterminer le profil de sensibilité aux antibiotiques des entérocoques. À cette fin, des frottis buccaux et cloacaux ont été prélevés

chez 150 oiseaux en cage. Le profil de sensibilité aux antibiotiques a été déterminé en utilisant la méthode de diffusion de disque standard. Les résultats ont révélé que sur 150 échantillons, 56 (37,33%) contenaient des entérocoques. La plupart des spécimens (25,33%) étaient des Enterococcus faecalis isolés de 6,66% des échantillons. En outre, 2,66% et 1,33% des échantillons étaient contaminés par Enterococcus mundtii et Enterococcus gallinarum, respectivement. En outre, Enterococcus malodoratus et Enterococcus raffinosus ont été isolés à partir de 0,66% des échantillons. Les résultats ont révélé que tous les isolats d'E. Faecalis et d'E. Faecium étaient résistants à plus de cinq agents antimicrobiens. La plupart des isolats d'E. Faecalis et d'E . Faecium ont montré une résistance à la céfazoline, à la tiamuline, à la fluméquine et à la céphalexine. En conséquence, la majorité des isolats présentaient une résistance multiple aux antibiotiques testés. En conclusion, la présence d'entérocoques multirésistants chez les oiseaux vivant à proximité de l'homme nécessite des observations approfondies en raison de la transmission de ces organismes à l'homme.

Mots-clés: Entérocoques, oiseaux, résistance aux antibiotiques

INTRODUCTION

Enterococci are Gram-positive facultative anaerobic bacteria with a widespread distribution (Kense and Landman, 2011). They are not only commonly found in the gastrointestinal tract of the mammals and birds, but also isolated from the urinary tract infections, bacteremia, endocarditis, and burn wounds in humans (Vankerckhoven et al., 2008). Animal- and human-originated enterococci have genetic similarities and can naturally transmit from animals to human in a frequent manner (Asadian et al. 2016). Antimicrobial-resistant bacteria in the companion birds is considered as a major problem (Marinho et al., 2013). In poultry, enterococci are frequently isolated from endocarditis, arthritis, amyloidosis, and many other disorders (Kense and Landman, 2011). Some studies have provided evidence for the animal-to-human transmission of resistant enterococci. More importantly, there are reports regarding the exchange of resistance genes between poultry and human enterococci (Marinho et al., 2013). The birds, especially ornamental birds, constitute a major area of study on the environmental dissemination and transmission of resistant zoonotic pathogens and commensals to humans (Santos et al. 2013). Enterococci are able to persist in the environment; accordingly, they are frequently isolated from environmental sources, such as the soil and surface waters (Asadian et al. 2016). Moreover, they are not only one of the leading causes of nosocomial infections, but also resistant to the antimicrobial agents commonly used in hospitals more than any other bacteria. Therefore, the identification of the reservoirs of antimicrobial-resistant strains of enterococci needs a special attention (Vankerckhoven et al., 2008). The antimicrobial resistance pattern of Enterococcus species varies depending on the geographical location, as well as the national and local policies regarding the intensity of antimicrobial use. The misuse or overuse of antimicrobial agents is one of the most significant factors resulting in the development and spread of microbial-resistant microorganisms. The distinction between pathogenic and non-pathogenic Enterococcus strains is not simple, especially due to the effective horizontal gene transfer mechanisms (Asadian et al. 2016). Enterococci can also cause food intoxication, especially because of the production of biogenic amines (Asadian et al. 2016). There have been few studies examining the presence of enterococci in birds (Santos et al., 2013), and none have ever been performed in Iran. In order to investigate the possible role of the birds in the transmission and potential dissemination of enterococci, this study was conducted to determine the prevalence, distribution, and antimicrobial susceptibility profiles of different species of enterococci isolated from some ornamental birds and poultry in the Northeast of Iran.

MATERIALS AND METHODS

Sampling. This study was conducted on 150 ornamental birds and poultry, such as canary, rose-ringed parakeet, grey partridge, domestic pigeon, common kestrel, and domestic fowl, from different regions. The birds were both healthy (n=109) and diseased (n=41), diagnosed with respiratory, gastrointestinal, or other disorders (Table 1). Samples were taken from the oral cavity and cloaca by sterilized swabs (2 swabs per bird; i.e., 48 and 102 samples from the diseased and healthy birds, respectively). The swabs were placed into micro tubes containing sterilized phosphate-buffered saline solution, *and then transported to the laboratory immediately.

Isolation of Bacteria. For enterococci isolation, the samples were streaked onto blood agar (Merck, Germany) containing 5% sheep blood, and then incubated at 37.7 °C for 48 h (Ulger et al 2009). Colonies with enterococci morphology were identified at the genus level through cultural characteristics, Gram staining, catalase test, and bile-esculin test. The catalase-negative isolates were purified by several passages on blood agars. In the next step, the isolates showing gamma hemolysis were cultured on bile esculin agar (Merck, Germany) plates, and then incubated at 37 °C for 24 h. Species identification was confirmed by carbohydrate fermentation (Arabinose, Raffinose, Mannitol, Inulin, Sorbitol and trehalose) and polymerase chain reaction (PCR) using specific primers for *E. faecalis* and *E. faecium* isolates (the most frequent isolates).

Polymerase chain reaction confirmation of *E. faecalis* and *E. faecium*. For DNA extraction, one colony of each plate was dissolved in 150 µl of TE (Tris+EDTA) solution, heated at 100 °C for 15 min, frozen at -20 °C for 5 min, and then boiled for another 5 min. Microtubes were centrifuged at 14,000 rpm for 10 min, and the supernatant containing DNA was stored at -20 °C until used. Two pairs of primers were utilized for the confirmation of *E. faecalis* (forward: ATCAAGTACAGTTAGTCTT, reverse: ACGATTC AAAGCTAACTG/ 941 bp) and *E. faecium* (forward: GCAAGGCTTCTTAGAGA, reverse: CATCGTGT AAGCTAACTTC/ 550 bp) using the PCR assay (Jiménez et al. 2013). The amplification of bacterial DNA was performed in a 25 µl final volume containing 12.5 µl of PCR Master Mix 2X (Ampliqon, Denmark). Every reaction contained 10 pmol of the forward and reverse primers and 5.5 µl of DNA template. The PCR reaction was performed in a thermal cycler (Techne, England).

Table 1. Rate of birds positive for Enterococcus species

Common name	Scientific name	Total Samples	Positive sample
Budgerigar	Melopsittacus undulates	5	1 (0.7%)
Canary	Serinus canaria	10	2 (1.4%)
Common kestrel	Falco tinnunculus	10	6 (4%)
Common Myna	Acridotheres tristis	8	3 (2%)
Common raven	Corvus corax	3	3 (2%)
Common starling	Sturnus vulgaris	6	4 (4%)
Domestic fowl	Gallus gallus domesticus	37	18 (12%)
Domestic pigeon	Columba livia domestica	10	3 (2%)
Golden Eagle	Aquila chrysaetos	5	4 (2.7%)
Grey partridge	Perdix perdix	11	0 (0%)
Madagascan manikin	Lonchura Nana	2	1 (0.7%)
Ring-necked Pheasant	Phasianus colchicus	5	0 (0%)
Rose-ringed parakeet	Psittacula krameri	20	10 (6.7%)
Rosy-faced lovebird	Agapornis roseicollis	3	1 (0.7%)
Others	-	15	0 (0%)
Total		150	56

Table 2. Results of antibiotic resistance test of Enterococcus species

Antimicrobial	Percentages (%) of resistant enterococci isolates distributed by species					
	E. Faecalis	*E. Faecium*	*E. Mundtii*	*E. Gallinarum*	*E. Malodoratus*	*E. Raffinosus*
Amoxicillin (25 µg)	8%	30%	50%	0%	0%	0%
Vancomycin (30 µg)	44.7%	40%	50%	0%	0%	100%
Penicillin (10 IU)	63.2%	70%	25%	50%	0%	100%
Cephalexin (30 µg)	94.7%	94.7%	50%	100%	100%	100%
Florfenicol (30 µg)	30.5%	30.5%	50%	0%	100%	100%
Tylosin (30 µg)	80.5%	80.5%	50%	100%	100%	100%
Tiamulin (30 µg)	91%	91%	100%	100%	100%	100%
Enrofloxacin (5 µg)	50%	50%	50%	100%	100%	0%
Streptomycin (10 µg)	84.2%	80%	50%	100%	100%	100%
Doxycycline (30 µg)	81.5%	50%	50%	100%	100%	100%
Neomycin (30 µg)	69.6%	69.6%	25%	100%	100%	100%
Oxytetracycline (30 µg)	87.5%	87.5%	50%	100%	100%	100%
Gentamycin (10 µg)	42.1%	50%	50%	0%	0%	100%
Flumequine (30 µg)	92.8%	92.8%	50%	100%	100%	100%
Ceftriaxone (30 µg)	97.4%	90%	100%	100%	100%	100%
Cefazolin (30 µg)	100%	100%	50%	100%	100%	100%

The PCR process included an initial denaturation of 94 °C for 2 min, followed by 33 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 10 min. The positive controls were kindly provided by Dr. Khoramian's Laboratory. Deionized distilled water was used as negative control. Four microliters of the PCR products were separated on a 1.5% agarose gel (Merck, Germany) and visualized on a transilluminator after ethidium bromide staining.

Antimicrobial susceptibility test. Antimicrobial susceptibility was tested by disc diffusion method following the recommendations of the Clinical and Laboratory Standards Institute (2013). Staphylococcus aureus ATCC 25923 was used as quality control reference strains. The susceptibility of the enterococcal isolates was tested for 16 antibiotics, namely Amoxicillin (25 µg), Vancomycin (30 µg), Penicillin (10 IU), Cephalexin (30 µg), Tiamulin (30 µg), Florfenicol (30 µg), Tylosin (30 µg), Enrofloxacin (5 µg), Streptomycin (10 µg), Doxycycline (30 µg), Neomycin (30 µg), Oxytetracycline (30 µg), Gentamicin (10 µg), Flumequine (30 µg), Ceftriaxone (30 µg), and Cefazolin (30 µg), using the disk diffusion method (Padtanteb Co., Iran).

RESULTS

Out of the 150 samples, Enterococcus species was isolated from 56 (37.33%) cases, 6/48 and 50/102 of which were from diseased and healthy birds, respectively. All oral samples were negative, except for those (positive in both oral and cloacal swabs) of three birds suffering from respiratory illness. Most of the isolates (25.3%) were *E. faecalis.* In this regard, *E. faecium* was isolated from 6.66% of the samples. The results of the PCR confirmed all *E. faecalis* and *E. faecium* isolates (Figure 1). In addition , 2.66% and 1.33% of the samples were contaminated with *E. mundtii* and *E. gallinarum*, respectively. Enterococcus malodoratus and E. raffinosus were isolated from 0.66% of the samples. In this study, the resistance of Enterococcus species isolates to sixteen antibiotic agents was tested (Table 2). All the isolates were resistant to Ceftriaxone. Overall, all the isolates of E. faecalis and *E. faecium* were resistant to more than five

antimicrobial agents. Furthermore, more than 90% of *E. faecalis* and *E. faecium* isolates had resistance to Tiamulin, Flumequine, and Cephalexin. The highest sensitivities were obtained as 79% and 40% among *E. faecalis* and *E. faecium* isolates for Amoxicillin agent, respectively.

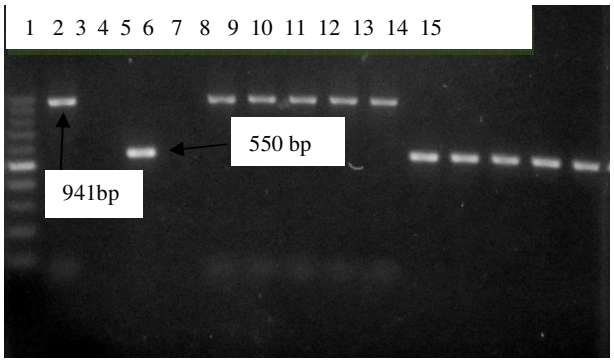

Figure 1. Gel electerophoresis of E. faecalis and E. faecium. From left to right: Lane 1: DNA Ladder (1000bp); Lane 2: Positive control of E. faecalis; Lane 3: Negative Control of E. faecalis; Lane 4: Positive control of E. faecium; Lane 5: Negative Control of E. faecium; Lane 6 to 10: five positive samples of E. faecalis (941bp); Lane 11 to 15: five positive samples of E. faecium (550 bp)

However, the sensitivities of faecalis and *E. faecium* to Vancomycin were determined as 29% and 30%, respectively. Enterococcus gallinarum isolates were totally sensitive to Amoxicillin and totally resistant to Streptomycin, Doxycycline, Ceftriaxone, and Cefazolin. All *E. mundtii* isolates were resistant to Ceftriaxone. In addition, 80%, 50%, and 50% of *E. mundtii* isolates were resistant to Streptomycin, Amoxicillin, and Doxycycline, respectively. Also, 50% of *E. mundtii* isolates were resistant to Vancomycin and Cefazolin. Resistance to Penicillin was observed among 25% of *E. mundtii* isolates. On the other hand, E. malodoratus isolates were completely resistant to Streptomycin, Doxycycline, Ceftriaxone, and Cefazolin. All these isolates were sensitive to Amoxicillin, Penicillin, and Gentamycin. They also showed intermediate resistance to Vancomycin. In addition, *E. raffinosus* isolates were completely resistant to Vancomycin, Penicillin, Streptomycin, Doxycycline, Gentamycin, Ceftriaxone, and Cefazolin.

DISCUSSION

With regard to the importance of enterococci as a pathogen (Hoseini Zadeh et al. 2015), more attention must be paid to identify the carriers of this organism in nature. In the current study, the resistance of enterococci isolated from companion birds to several antimicrobial agents was demonstrated for the first time. The possibility of the bacterial transmission has risen due to the close contact between the birds and humans (Santos et al., 2013). In this study, *E. faecalis* and *E. faecium* were the predominant Enterococcus species identified in the oral and cloacal samples of the birds. Similar results were obtained by other researchers working on faecal enterococci in wild animals (Radimersky et al., 2010). Ruzauskas et al. (2009) reported that 92% of the poultry farm samples were positive for Enterococcus species; however, only 74 strains were selected as non-duplicate isolates. In the mentioned study, the most predominant species were identified as *E. faecium* (38%), *E. faecalis* (17.5%), *E. gallinarum* (12%), and *E. casseliflavus* (12%). However, they failed to identify nine strains because of the weakness of biochemical reactions. Despite a wide variety of species in poultry, *E. faecalis* and *E. faecium* are reported to be still the major isolates (Devriese et al., 2008; Ruzauskas et al., 2009). There is evidence revealing the high susceptibility of these two species to Penicillin (71.6%) (Ruzauskas et al., 2009). Accordingly, in the present study, about 40% of the isolates were sensitive to Penicillin. In a study conducted on chicken's isolates, eight different species were identified. In the mentioned study, *E. faecium* (41.6%) and E. faecalis (41.2%) were the most predominant species, accounting for 82.8% of the total number; however, the isolation rate of E. gallinarum was 2.7% (Hwang et al., 2009). Likewise, in the present study, E. faecalis and *E. faecium* were the predominant Enterococcus species in the oral and faecal samples. Nonetheless, none of the *E. avium*, E. hirae, and *E. durans* species were detected in our study.

Fatholahzadeh et al. (2006) recovered enterococci from the urine specimens of the patients with urinary tract infections from three hospitals in Tehran, Iran. In the mentioned study, 57%, 30%, 6%, 4 3%, 2%, 1%, and 1% of the isolates were identified as *E. faecalis*, *E. faecium*, *E. mundtii*, *E. avium*, *E. hirae*, *E. durans*, and *E. raffinosus*, respectively. Enterococcus faecalis has shown almost no susceptibility to all the tested antimicrobials, except Penicillin (1.4%) and Flavomycin (<24%) (Hwang et al., 2009). Penicillin is the only agent showing relatively strong activity against both E. faecalis and E. Faecium (Hwang et al., 2009). Stramova et al. (2013) found that most of the enterococcal population in animals was dominated by *E. faecalis*, and in some wild-living animals, the predominant strains were *E. faecium* and *E. hirae*. In line with our findings, they reported that *E. faecalis* strains showed the highest antibiotic resistance. Based on the evidence, enterococci obtained from birds have a higher resistance in comparison to those from mammals (Stramova et al., 2013). In a study conducted by Teymourrnejad et al. (2011), out of 422 examined strains of enterococci, most of the isolates contained Vancomycin-resistance genes in their genome. Hoseini Zadeh et al. (2015) processed 200 positive samples of enterococci obtained from the hospitals of Arak, Iran, 82% of which were isolated from urine samples. In the mentioned study, among the isolated enterococci, 44.5%, 71%, and 16% of the cases were resistant to Ciprofloxacin, Tetracycline, and Vancomycin, respectively. In another study, Jackson et al. (2009) isolated *E. faecalis*, *E. faecium*, and *E. hirae* from both dogs and cats. They identified *E. faecalis* and *E. hirae* as the predominant species in the dogs and cats, respectively. In the mentioned study, gentamicin resistance was detected in 79% of *E. faecalis* isolates (all from dogs), while in the present study, it was observed in 70% and 75.7% of *E. faecalis* and *E. faecium* isolates, respectively. Our findings are also in line with those obtained by Jackson et al. (2009) regarding the resistance to Penicillin and Streptomycin. In our study, 2.66% and 0.66% of the isolates were recognized as *E. mundtii* and *E. raffinosus*, respectively. In a study performed by Fatholahzadeh et al. (2006), 8 cases out of 120 Enterococcus isolates were resistant to Vancomycin. In the mentioned study, various Vancomycin-resistant Enterococcus species were isolated, including *E. faecalis* (38%), *E. faecium* (25%), *E. mundtii* (25%), and *E. raffinosus* (13%). In contrast, in our study, 25% of enterococci were resistant to Vancomycin, which is higher than the rate reported by Fatholahzadeh et al. (2006). There is a high prevalence of antimicrobial resistance among enterococcal isolates. Several reports regarding the increasing antimicrobial resistance have provoked significant concerns about untreatable enterococcal infections, including multidrug resistance. Moreover, the possibility of multidrug resistance transmission in bacteria complicates the problem (Santos et al., 2013; Asadian et al., 2016).

In conclusion, the results indicated a high resistance of Enterococcus species to antibiotics, especially Vancomycin and cephalosporin's group. Glycopeptide antibiotics, such as Vancomycin, are the drugs of choice and often the last steps to treat hospital infections caused by multiple drug-resistant Gram-positive bacteria. Therefore, antibiotic resistance in enterococci against glycopeptide is considered a threat for human patients and requires annual surveillance and regular monitoring programs.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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