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Molecular detection and occurrence of vancomycin resistance genes (*van A, B, C1, C2/C3*) among *Enterococcus* species isolated from farm ostriches

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Abstract

Background: Evaluating the prevalence of vancomycin resistance genes (*van* genes) in enterococcal isolates from food-producing animals is an important public health issue because of the possibility of resistance genes spread to human.

Objectives: The present study aimed to determine the occurrence of vancomycin resistance genes among *Enterococcus* species obtained from ostrich faecal samples.

Methods: One hundred and twenty-five faecal samples of apparently healthy ostriches from five different farms were investigated. Genes encoding vancomycin resistance were studied by multiplex-PCR, and susceptibility to six antibiotics was evaluated by disk-diffusion method.

Results: In total, 107 *Enterococcus* spp. isolates were obtained and confirmed by biochemical and molecular tests. *Enterococcus faecium* was the prevailing species (56 isolates of 107; 52.3%), followed by *E. hirae* (24 isolates; 22.4%) and *E. gallinarum* (12 isolates; 11.2%). Of the 107 recovered isolates, 44% harboured at least a type of *van* genes. *van*A, *van*C2/3 and *van*C1 were identified in 34 (31.7%), 13 isolates (12.1%) and 4 (3.7%) isolates respectively. Additionally, four isolates (*E. gallinarum*, *E. rafinosus*) co-harboured the the *van*A and *van*C1 or *van*A and *van*C2/3. *Enterococcus faecium* and *Enterococcus hirae* strains with the *van*A genotype were the most frequent *van*-carrying enterococci from ostrich faecal samples. Among *van*-carrying enterococcal isolates, 23.4% were phenotypically resistant to vancomycin. This study revealed a relatively high prevalence (44%) of *van*-carrying enterococci in ostrich faecal samples.

Conclusions: Results of the present study suggest that ostrich faeces could be considered as a reservoir of vancomycin resistance genes, especially *van*A containing enterococci that could be potentially transferred to human through the food chain.

KEYWORDS
enterococci, ostrich, *van*A, *van*C, vancomycin

1 | INTRODUCTION

Enterococci are commensal bacteria of intestine which colonise human and other mammals, birds, reptiles and insects. These bacteria are considered as important opportunistic pathogens for human causing urinary tract infections, wound infections and endocarditis (Fisher & Phillips, 2009). *Enterococcus faecalis* and *Enterococcus faecium* are the most important species in medicine, the former is the most pathogenic *Enterococcus* species and the latter is of increasing importance due to its more resistance to antimicrobials. The most prevalent species of *Enterococcus* in farm animals are *E. faecium*, *E. cecorum*, *E. faecalis* and *E. hirae* (Nilsson, 2012). Both animal-derived and vegetable foods may be contaminated by enterococci for example by contamination of meat by faecal material at the slaughterhouse and contamination of vegetable by manure or sewage water for fertilisation and irrigation. In addition to potential pathogenicity of some *Enterococcus* species in human, possibility for transmission of genes conferring antimicrobial resistance from these commensals to more pathogenic organisms in human intestine indicate that the public health could be negatively affected by enterococci (Hammerum et al., 2010).

Vancomycin is a glycopeptide antimicrobial which interfere with production of bacterial cell wall resulting in lysis of the bacteria (Courvalin, 2006). This antimicrobial is considered as critically important in human medicine for the treatment of infections caused by *Enterococcus* spp. and methicillin-resistant *Staphylococcus aureus* (MRSA) (WHO, 2018). Additionally, vancomycin can be prescribed for intestinal infections where the poor absorption of vancomycin via digestive tract is advantageous (Nilsson, 2012). The VRE could potentially reveal multiple antibiotic resistances which is an important public health treat that necessitates evaluation of resistance to other antibiotics commonly used in poultry production (Cetinkaya et al., 2000; George et al., 2021; Gonçalves et al., 2010 Marrow et al., 2009). In this regard, tetracyclines, quinolones and macrolids are among the antimicrobial classes which are commonly used in poultry and ostrich production in Iran (Aalipour et al., 2014; Faghihi et al., 2017; Kazeminia et al., 2020). There are also some domestic reports regarding the use of a prohibited antibiotic such as chloramphenicol in food-producing animals in the country (Faghihi et al., 2017; Tajik et al., 2010). Therefore, determination of resistance patterns of VRE isolates for those antibiotics may be beneficial in revealing the level of multiple antibiotic resistances.

Enterococcal resistance to glycopeptides has been studied in previous studies (Eisner et al., 2005; Courvalin, 2006; Gousia et al., 2015). In vivo transfer of vancomycin resistance from vancomycin-resistant enterococci (VRE) of animal origin to enterococci of human origin in the intestine of human has been reported (Lester et al., 2006). Infections with vancomycin-resistant enterococci (VRE) may be associated with increased rate of therapy failure, length of hospital stay and mortality (Patel, 2003). In addition, VRE have been identified as a significant public health hazard because of *van* resistance genes transmission to other organisms especially MRSA to form vancomycin-resistant *Staphylococcus aureus* (VRSA) which is a pathogen that is difficult to treat (Chang et al., 2003). Emergence of VRSA carrying *van* genes have been reported in some countries including Iran (Tacconelli et al., 2013; Yousefi et al., 2017). For these reasons, VRE have been placed in the list of high priority pathogens of World Health Organization (WHO) (Tacconelli et al., 2013).

It has been recognised that modification of antimicrobial target that is substitution of D-Alanyl-D-Alanin termini with D-Alanyl-D-Lactate or D-Alanyl-D-Serine in peptidoglycan structure of bacterial cell wall forms the basis of resistance to glycopeptides. This shift results in a decreased affinity for vancomycin approximately by 1000 and seven times, respectively (Fisher & Phillips, 2009). These modifications are mediated by nine gene clusters which are classified into two categories based on the ligases they encode (George et al., 2021). The first group of genes comprises *van*A, *van*B, *van*D and *van*M which encode for D-Alanyl-D-Lactate ligase. The second group consists of the gene clusters *van*C, *van*E, *van*G, *van*L and *van*N and encodes for D-Alanyl-D-Serine ligase. Among nine *van* gene clusters *van*C is of the intrinsic resistance type and is identified to be mostly frequent in *E. gallinarum*, *E. flavescens* and *E. casseliflavus* strains and other genes (*van*A, *van*B, *van*D, vanE, *van*G, *van*L, *van*M and *van*N) are of the acquired glycopeptide resistance type (Ke et al., 1999; Manero & Blanch, 1999; Wardal et al., 2014). Additionally, *van*A and *van*B are the most prevalent vancomycin resistance gene clusters worldwide and are mainly carried by *E. faecium* (CLSI, 2008; Seo et al., 2005).

Industry of ostrich farming is growing globally due to providing multiple economically beneficial products including meat, leather, egg, eggshell and oil. In Iran, commercial ostrich farming started in 1999 and ostriches are reared mainly for meat (Salari & Hoseini, 2021). According to the data from Ministry of Agriculture (Jihad) around 12.5% of total world ostrich meat is produced in Iran. In recent years especially, there has been an increasing trend toward ostrich meat consumption as a good protein source with nutritional benefits and low cholesterol and high omega-3 poly unsaturated fatty acid content (http://www.iana.ir/fa/tiny/news-23484).

Regarding public health impact of VRE from animal origin which may act as antimicrobial resistance genes reservoirs, evaluating the prevalence of antimicrobial resistance genes in food-producing animals is important. To the best of our knowledge, there are no previous reports on vancomycin resistance genes prevalence in ostrich in Iran, hence, the present investigation aimed to determine the prevalence, and distribution of *van*A, *van*B, *van*C1 and *van*C2/3 genes among *Enterococcus* species from farm ostrich faecal samples.

2 | MATERIAL AND METHODS

2.1 | Isolation and identification of enterococci

In order to isolate *Enterococcus* species, 125 faecal samples of apparently healthy ostriches from five different farms (25 samples per farm) in various parts of Tehran and Semnan provinces were collected. Swabs from faecal samples were put in Amies transport medium, cooled in an icebox and immediately transported to the laboratory. In the laboratory, samples were incubated at 37°C for 18–20 h in buffered peptone water. Then the sample were streaked on Bile esculin azide

TABLE 1 Primer sequences and target genes used in multiplex-PCR

Gene target	Primer sequence (5'–3')	Product size (bp)	Reference
*van*A	F-ATTGCTATTCAGCTGTACTC	559	Seo et al. (2005)
	R-GGCTCGAGTTCCTGATGAAT		
*van*B	F-AACGGCGTATGGAAGCTATG	467	Seo et al. (2005)
	R-CCATCATATTGTCCTGCTGC		
*van*C1	F-GGCATCGCACCAACAATGGA	902	Seo et al. (2005)
	R-TCCTCTGCCAGTGCAATCAA		
*van*C2/3	F-TTCAGCACTAGCGCAATCG	663	Seo et al. (2005)
	R-TCACAAGCACCGACAGTCAA		

agar (BEAA, Merck, Germany) plates and incubated at 37°C for 24–48 h. The suspect isolates were identified to the genus level by Gram staining, catalase test, oxidase test, blackening of Bile esculin azide agar, growth on 5% Sheep blood agar with non-haemolytic or alpha haemolytic colonies, culture on brain heart infusion broth at 10°C, 45°C, and with 6.5% NaCl, and then were identified to the species level by motility test, production of yellow pigment, and sugar fermentation tests (L-arabinose, mannitol, sorbitol, raffinose and sucrose) (Manero & Blanch, 1999; Teixeira et al., 2015).

2.2 | DNA extraction, PCR and detection of van genes by multiplex PCR

Enterococcal isolates were subjected to DNA extraction using DNA extraction kit (Cinnagen, Iran) according to the manufacturer's guidelines. The extracted DNA was stored at −20°C until test time. Biochemically identified enterococci were confirmed by polymerase chain reaction (PCR) by using of a primer pair Entero-F (5'-TACTGACAAACCATTCATGATG-3') and Entero-R (5'-AACTTCGTCACCAACGCGAAG-3') with amplicon size 112 bp according to Ke et al. (1999). The final concentrations were as follows: 2.5 μl of 10X PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 1 U *Taq* polymerase, 0.2 μM of each primer and 2 μl of DNA templates and adjusted to 25 μl by adding of molecular grade water (Cinnagen, Iran). PCR conditions were as follows: initial denaturation at 94°C for 3 min, 33 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 20 s and extension at 72°C for 40 s followed by a final extension step at 72°C for 5 min. The *Enterococcus fecalis* (University of Tehran Collection) containing the *van*A gene was used as the positive control in all experiments.

Extracted DNA was amplified by a multiplex-PCR with primers specific for *van*A, *van*B, *van*C1 and *van*C2/3 genes that previously described by Seo et al. (2005) (Table 1). The final concentrations in PCR reactions were as follows: 2.5 μl of 10X PCR buffer, 2 mM $MgCl_2$, 250 μM dNTP, 1 U *Taq* polymerase, 0.6 μM of each primer and 3 μl of DNA templates and the reaction volumes were adjusted to 25 μl with sterile molecular grade water. The PCR conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 57°C for 45 s and 72°C for 1 min. A final extension step was carried out at 72°C for 5 min. Amplified product obtained using Techne thermocycler (UK) were analysed by electrophoresis on 1% agarose gel and DNA bands were visualised by staining with ethidium bromide.

2.3 | Antimicrobial susceptibility test

Susceptibility of enterococcal isolates to six antibiotics (vancomycin, chloramphenicol, erythromycin, tetracycline, ciprofloxacin and ampicillin) was assessed by the disk diffusion method which were conducted and interpreted according to CLSI guideline (CLSI, 2008). The intermediate strains in the primary susceptibility test, were assessed by minimal inhibitory concentration (MIC) test for vancomycin by microdilution Muller–Hinton broth method according to the guidelines. Strains were considered as resistant, intermediate and sensitive to vancomycin when MIC was ≥32, 8–16 and ≤ 4 μg/ml respectively as recommended breakpoints of vancomycin for enterococci (CLSI, 2017).

2.4 | Statistical analysis

The antimicrobial susceptibility results between two groups of *van*-positive and *van*-negative strains were compared by chi-square method to check if the difference in susceptibility/resistance was statistically significant ($p \leq 0.05$) or not.

3 | RESULTS

3.1 | Isolation, molecular and biochemical identification of enterococci

One hundred and seven *Enterococcus* isolates were obtained from 125 faecal samples in five ostrich farms. All of the isolates were positive for 112 bp amplicon size in the PCR method specific for the genus, *Enterococcus* (Figure 1). The species diversities included seven *Enterococcus* species. *Enterococcus faecium* was the prevailing species (56 isolates of

TABLE 2 The frequency of vancomycin resistance (*van*) genes in *Enterococcus* species

		van genes		
Enterococcus species	No. of isolates	*van*A	*van*C1	*van*C2/3
E. faecium	56	15 (26.8%)	-	2 (3.5%)
E. hirae	24	10 (41.6%)	-	2 (8.3%)
E. gallinarum[†]	12	4 (33.3%)	4 (33.3%)	5 (41.6%)
E. faecalis	7	4 (57.1%)	-	-
E. avium	4	-	-	1 (25%)
E. raffinosus[‡]	3	1 (33.3%)	-	3 (100%)
E. durans	1	-	-	-
Total	107	34 (31.7%)	4 (3.7%)	13 (12.1%)

[†]Three isolates of *E. gallinarum* were positive for both vanA and vanC1.
[‡]One isolate of *E. raffinosus* was positive for both vanA and vanC2/3.

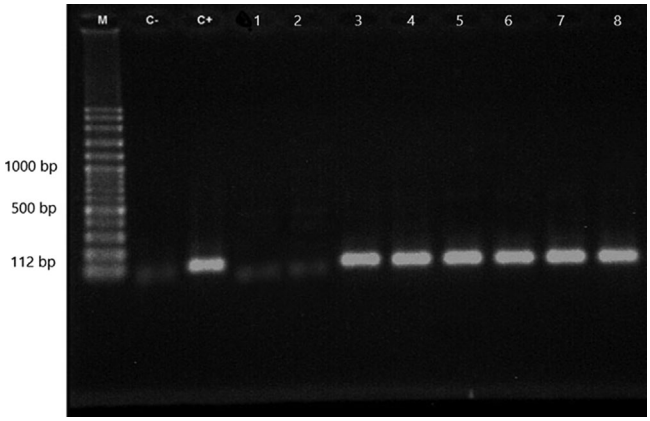

FIGURE 1 Gel electrophoresis for PCR product specific for the genus *Enterococcus*. Lanes M: 100 bp molecular weight marker; C^-: negative control; C^+: positive control; 1 and 2: negative strains; 3–8: positive strains (112 bp).

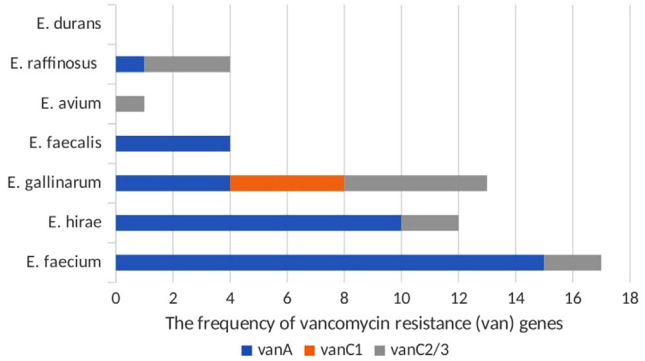

FIGURE 2 The frequency of vancomycin resistance (*van*) genes in *Enterococcus* species

107; 52.3%), followed by *E. hirae* (24 isolates; 22.4%) and *E. gallinarum* (12 isolates; 11.2%) while other species including *E. fecalis* (7 isolates; 6.5%), *E. aviam* (4 isolates; 3.7%), *E. raffinosus* (3 isolates; 2.8%) and *E. durans* (one isolate; 0.9%) were the least frequent species.

3.2 | Detection of the van genes by multiplex-PCR

The prevalence of vancomycin resistance genes is presented in Table 2 and Figure 2. As shown in the table, *van* genes were detected in 47 out of 107 (44%) enterococcal isolates from faecal samples. Of these positive samples, 34 isolates were associated with the *van*A, 13 isolates with *van*C2/3, and 4 isolates with *van*C1 (Figure 3). No *van*B containing enterococci were detected in the faecal samples of ostriches. The gene *van*C1 but not *van*C2/3 was found only in *Enterococcus gallinarum* isolates (Figure 2). Three isolates of *Enterococcus gallinarum* harboured both *van*A and *van*C1 and one isolate of *Enterococcus raffinosus* carried *van*A and *van*C2/3 at the same time.

3.3 | Antimicrobial susceptibility

The results of antimicrobial susceptibility test for enterococcal isolates with and without *van* genes are presented in Table 3 and Figure 4a and b, respectively. Among 107 enterococcal isolates, 11 isolates showed resistance to vancomycin and 33 isolates considered as intermediate (reduced susceptibility to vancomycin) in antimicrobial susceptibility test. Statistical comparison indicated that the rates of sensitivity and resistance to vancomycin were significantly different between enterococcal strains with and without *van* genes ($p < 0.05$) (Table 3). The most commonly observed antimicrobial resistance was found against tetracycline which was seen in 40% and 35% of the enterococcal isolates with and without *van* genes respectively. Enterococcal strains with *van* genes showed a significantly higher intermediate resistance against tetracycline as compared with none-*van* harbouring strains ($p < 0.05$). All except one enterococcal isolates were found to be susceptible to ampicillin. Of 33 isolates which demonstrated intermediate sensitivity to vancomycin by disc diffusion method, 20 strains (60.6%) showed the actual intermediate sensitivity and the remaining was found to be sensitive to vancomycin according to the CLSI breakpoints; therefore, no VRE was found using the MIC test in the intermediate group.

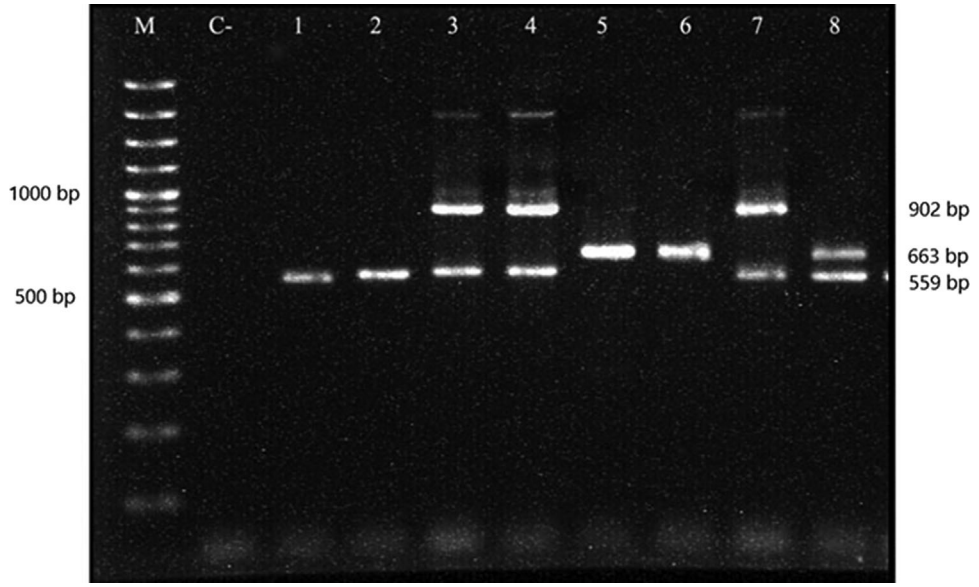

FIGURE 3 Gel electrophoresis of amplified *van*A, *van*C1 and *van*C2/3 by the multiplex-PCR test. Lanes M: 100 bp molecular weight marker; C⁻: negative control; 1 and 2: *Enterococcus hirae van*A; 3, 4 and 7: *Enterococcus gallinarum van*A+ *van*C1; 5 and 6: *Enterococcus gallinarum van*C2/3; 8: *Enterococcus raffinosus van*A+ *van*C2/3.

TABLE 3 Antimicrobial resistance rate for enterococcal isolates with and without *van* genes

	Isolates with *van* genes ($n = 47$)			Isolates without *van* genes ($n = 60$)		
Antimicrobial agent	**Sensitive**	**Intermediate**	**Resistant**	**Sensitive**	**Intermediate**	**Resistant**
Vancomycin	**6 (12.7%)***	**30 (63.8%)**	**11 (23.4%)**	**57 (95%)**	**3 (5%)**	**0**
Chloramphenicol	34 (72.3%)	12 (25.5%)	1 (2.1%)	44 (73.3%)	14 (23.3%)	2 (2.3%)
Erythromycin	19 (40.4%)	18 (38.2%)	10 (21.2%)	38 (63.3%)	17 (28.3%)	5 (8.3%)
Tetracycline	21 (44.6%)	**7 (14.8%)**	19 (40.4%)	38 (63.3%)	**1 (1.6%)**	21 (35%)
Ciprofloxacin	29 (61.7%)	15 (31.9%)	3 (6.3%)	36 (60%)	18 (30%)	6 (10%)
Ampicillin	47 (100%)	0	0	59 (98.3%)	0	1 (1.6%)

*According to the statistical analysis the cells that significantly differs ($p \leq 0.05$) from the comparison group are shown in bold.

4 | DISCUSSION

Monitoring antimicrobial resistance rate in commensal bacteria provides evaluation of the occurrence and development of resistance patterns over time. Previous works have indicated that resistance genes can be transferred to different *Enterococcus* spp. and other bacteria such as *Staphylococcus* through conjugation (Jennes et al., 2000; Ke et al., 1999). Food-producing animals could serve as a reservoir of antimicrobial-resistant bacteria for human and transmission of vancomycin resistance has been previously shown by molecular analysis of *van*A (Van den Bogaard et al., 2002).

Our study revealed that enterococcal strains were detected in 85% of ostrich faecal samples with *E. faecium*, *E. hirae* and *E. gallinarum* (52.3%, 22.4% and 11.2%, respectively) recovered as the top three prevalent species. Our results were in accordance with those by Laukova et al. (2016) that reported *E. hirae* and *E. faecium* as the most frequent species in faecal samples of ostriches (73.9% and 21.7% respectively). *E. gallinarum* was also isolated from the ostrich duodenum in a previous study (Jennes et al., 2000). Distribution of enterococcal species in ostrich faecal samples has been shown to be comparable to the species that usually detected in broiler chickens or other food-producing animals (Laukova et al., 2016).

According to our study a relatively high prevalence (44%) of *van*-carrying enterococci in ostrich faecal samples was found. This is the first report regarding determination of *van*-carrying enterococci in apparently healthy ostriches in Iran. According to the results of this study, *van*A gene which is associated with acquired mechanism of vancomycin resistance was detected in 72.3% and genes corresponded to intrinsic mechanism of vancomycin resistance (*van*C1 and *van*C2/3) were found in 36.1% of total *van*-carrying enterococcal isolates. In addition, four isolates of enterococcal strains harboured both the *van*A and *van*C1 or *van*A and *van*C2/3. In a study, seven isolates of

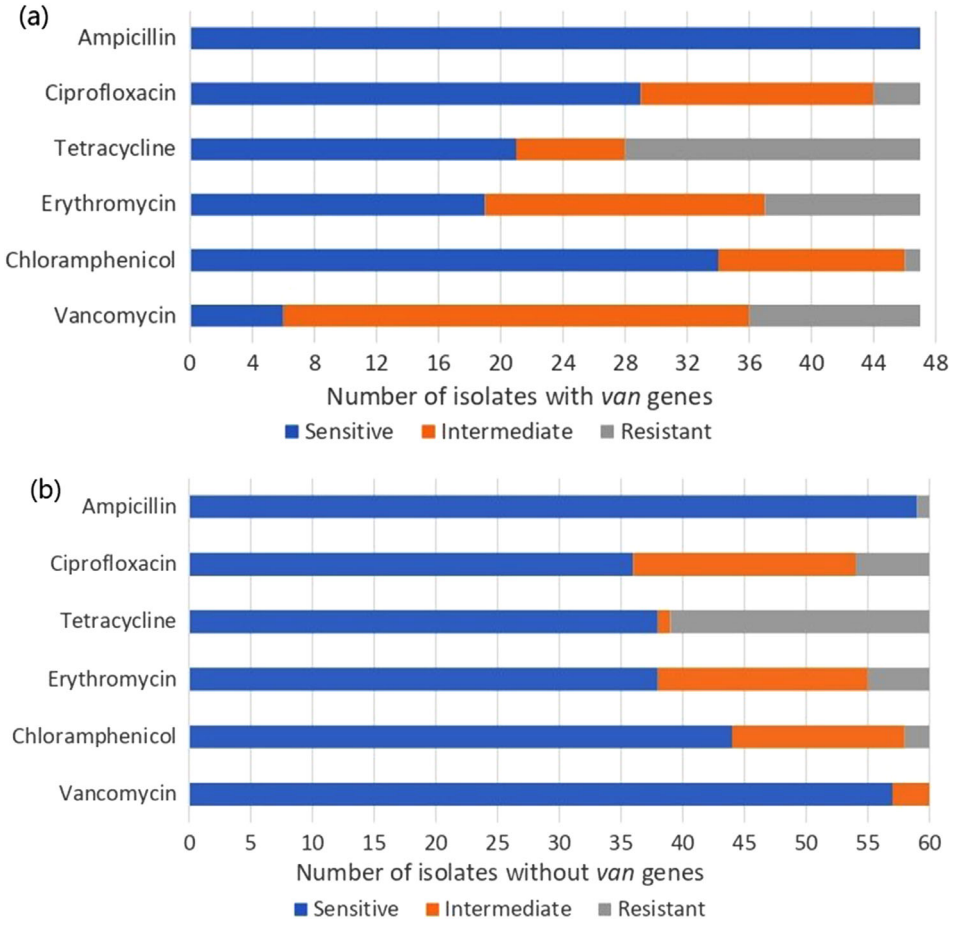

FIGURE 4 Antimicrobial resistance rate for enterococcal isolates with (a) and without (b) *van* genes

vancomycin-resistant enterococci from poultry meat, bovine meat and eggs which carried the *van*A and *van*B genes at the same time were detected (Gousia et al., 2015). Our results indicated that *E. faecium* and *E. hirae* strains with the *van*A genotype were the most frequent *van*-carrying enterococci from ostrich faecal samples. High prevalence of *E. faecium* with *van*A gene has been described previously regarding Norwegian and Danish poultry farms (Borgen et al., 2001; Heuer et al., 2002). In a similar study in Portugal, Gonçalves et al. (2010) detected VRE including *E. durans* with *van*A and *E. gallinarum* with the intrinsic *van*C1 genotypes in 7 out of 54 (13%) tested faecal samples of ostriches. *E. faecium* with *van*A mediated resistance was frequently reported in broiler chickens (Eisner et al., 2005).

A meta-analysis on *van* genes frequencies among vancomycin-resistant enterococcal isolates in Iran revealed that 80%–86% of resistant enterococci belonged to genotype *van*A and 14%–20% of resistant strains were of genotype *van*B (Moghimbeigi et al., 2018). According to our results, 23.4% phenotypically vancomycin-resistant enterococcal isolates were found among *van*-carrying strains (equivalent to total VRE prevalence of 10.2%). Avoparcin (a gycopeptide) usage as a growth promoter in farm animals in the past decades may have selected vancomycin resistance trait among enterococci because discontinuing its use resulted in decrease of *van*A frequency in *E. faecium* of animal origin (Aarestrup, 2000; Guerrero-Ramos et al., 2016; Hammerum et al., 2010). Although, the use of antimicrobials as growth promoters is not approved in Iran, the misuse of these agents frequently reported by the farm owners. Occurrence of VRE in farm ostriches in such conditions may be explained by co-selection for VRE as a consequence of the use of some other antimicrobial classes especially macrolides which are commonly administered to farm animals including poultry and ostriches for therapeutic purposes. Since the genes encoding macrolide resistance and vancomycin resistance are located on the same plasmid, co-selection by using of macrolids was described to be a mechanism for the development of glycopeptide resistance (Javadi et al., 2021; Nilsson, 2012). In the present study, although not statistically significant but higher percentage (21.2%) of resistance against erythromycin (a macrolide antimicrobial) in *van*-carrying enterococci compared with only 8.3% of erythromycin resistance in none *van*-carrying enterococci may indicate that the linkage of resistance to macrolids and glycopeptide could be a factor for observation of enterococci harbouring vancomycin resistance genes ($p < 0.1$). In a previous study, using of the macrolide tylosin in pigs was suggested to co-select for vancomycin-resistant enterococci (Aarestrup, 2000). All of VRE isolated from different poultry, pork and beef meat preparations in Spain were shown to be resistant against erythromycin (Guerrero-Ramos et al., 2016). It has been reported that VRE strains isolated from chicken meat had high-level resistance to erythromycin and tetracycline (Song et al.,

2005). All the enterococcal isolates in the current study showed sensitivity to ampicillin. This could be due to low β-lactamase production of enterococci as showed by Song et al. (2005). Gonçalves et al. (2010) reported that 85.7% of VRE isolates including *E. durans* and *E. gallinarum* obtained from ostriches showed resistance to tetracycline. Similarly, in the present study, the most antimicrobial resistance in enterococcal isolates especially in the isolates with *van* genes was found against tetracycline. Tetracyclines have been frequently used in poultry production in Iran for many years and hence the level of resistance to this antimicrobial is substantial. Also, a high percentage of *E. faecium* (93%) and *E. gallinarum* (73%) which were isolated from Austrian poultry were found to be resistant to tetracycline (Eisner et al., 2005).

5 | CONCLUSION

Evaluation of *van* genes prevalence in enterococcal isolates from the intestinal tract of food-producing animals is important for monitoring the development of antibiotic-resistant bacteria that could be passed to human by the food chain. Results of the present study revealed that ostrich faeces should be considered as a possible reservoir of vancomycin resistance genes, especially acquired type (*van*A) in enterococci that could be potentially transferred to other bacterial species.

AUTHOR CONTRIBUTIONS

Sara Mirzaie: methodology; writing – original draft. Isa Faghiri: investigation. Mahdi Askari Badouei: conceptualisation, investigation, methodology. Seyed Madani: methodology.

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CONFLICT OF INTEREST

Authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Any further available data will be provided upon a resonable request.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, regarding research and publication, have been adhered to and the appropriate ethical review committee approval has been received. Since only the faecal samples from farm animal has been collected, the signed consent was not necessary in this research.

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.1010.

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