3(2): 75-81 (2017) doi: 10.3153/JFHS17010

Journal of Food and Health Science

E-ISSN 2149-0473

ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

INVESTIGATION OF THE PRESENCE AND VIRULENCE TRAITS OF VANCOMYCIN-RESISTANT *ENTEROCOCCUS* IN WATER SAMPLES

Tülay Elal Muş[1], Figen Çetinkaya[2], Evren Erköse[2]

[1] Department of Food Processing, Vocational School of Keles, University of Uludag, Bursa, Turkey

[2] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Uludag, Bursa, Turkey

Received: 16.01.2017

Accepted: 27.02.2017

Published online: 24.03.2017

Corresponding author:

Tülay ELAL MUŞ, University of Uludag, Vocational School of Keles, Department of Food Processing, 16740, Keles, Bursa, Turkey

E-mail: tulay_elal@yahoo.com

Abstract:

The aim of this study was to determine the incidence of vancomycin-resistant enterococci (VRE) in tap and artesian well waters and to detect the vancomycin resistance genes and virulence genes of the isolates obtained from the samples. For this purpose, 200 samples (119 tap and 81 artesian well waters) were collected from several water supplies during November 2013 and June 2015 period in Bursa province. Seven isolates were recovered from artesian well waters and confirmed as *Enterococcus* by PCR method. *E-test* performed for vancomycin and teicoplanin MIC values indicated that only two isolates had the intermediate-level (8 µg/mL) resistance to vancomycin. No resistance was observed to teicoplanin in any of these isolates by *E-test*. All of 7 isolates were tested for vancomycin resistance genes (*van*A, *van*B and *van*C) and virulence genes (*gel*E, *agg, esp* and *ace*). The results showed that enterococci isolates had no these genes. The present study suggested that the presence of the intermediate level VRE in artesian well waters and, also the waters from environmental supplies near human and animal niches could be play a role as potential reservoirs for enterococci having several types of resistance to vancomycin. Also, vancomycin resistant strains can be possible the spread in environment and also the transmission to human and animals through contaminated water sources.

Keywords: Water, Artesian well water, Enterococci, Vancomycin resistance, Virulence

Introduction

Enterococci are natural inhabitants of humans and animals gastrointestinal tract but are also appeared in the water, soil, plants, and food (Strateva *et al.,* 2016). Besides being the hygiene quality indicator for water, enterococci have been proposed as indicator bacteria for antimicrobial resistance (Boehm & Sassoubre 2014). Vancomycin is a antibiotic, strongly affects Gram-positive bacteria for the treatment of serious, life-threatening infections, when other antibiotic treatment did not work (Varela *et al.,* 2013). Vancomycin resistance in enterococci have been occured all over the world since 1986 (Cetinkaya *et al.,* 2013). Vancomycin resistance was described six phenotypes as *van*A, *van*B, *van*C, *van*D, *van*E and *van*G. *Van*A type strains possess high-levels of resistance to vancomycin and teicoplanin. *Van*B and *van*C genes generate low-level resistance to vancomycin. *Van*C phenotype differs from others to its species-specific characteristic. It has seen in only *Enterococcus casseliflavus* and *E. gallinarum* strains (Messi *et al.,* 2006).

Enterococci is a well known bacteria to have various virulence factors associated with hospital infections. Enterococcal surface protein, encoded by the *esp* gene has been related contributing with colonization of urinary tract, and biofilm formation. The collagen-binding protein gene, *ace*, is involved in attachment and colonization of renal tissue in animal models (Sidhu *et al.,* 2014). The aggregation substance (*agg*) takes a part on adhesion to eucaryotic cells and extracellular matrix proteins. Gelatinase, encoded by *gel*E gene, hydrolyses diverse biological peptides such as gelatin, collagen and casein. Another virulence trait cytolysin (*cyl*) is an extracellular toxin, which lyses array of procaryotic and eucaryotic cells (Buyukyoruk *et al.,* 2014). Due to its antimicrobial resistance and virulence factors, enterococci has been not considered generally recognised as safe (GRAS) bacteria and has been known as emerging pathogen of humans. Enteroccocci plays a potential role in hospital associated infections (Cariolato *et al.,* 2008). *Entrococcus* species has advanced active gene transfer mechanisms for transmission of antibiotic resistance and virulence factor genes by plasmids (Chajecka-Wierzchowska *et al.,* 2016). Habitats such as water, soil and food are considered as possible reservuars of antimicrobial resistance and virulence genes of *Enterococcus* strains (Sidhu *et al.,* 2014).

The presence of enterococci in aquatic environments can lead to infection, when water is utilized for drinking water production, recreational activities, irrigation or shellfish harvesting. Treatment of individual diseases, caused by antimicrobial resistant bacteria, with drugs is trouble. Enterococci have a natural tendency to transmit antimicrobial resistance genes to other bacteria species by mobile genetic elements (Servais & Passerat 2009**)**.

The objective of this study was to estimate the frequency of vancomycin-resistant enterococci (VRE) contamination in tap and artesian well waters from various sources and to investigate its virulence traits and vancomycin resistance gene profiles.

Materials and Methods

Water Sampling

A total of 200 water samples including 119 tap and 81 artesian well waters (unchlorinated) were collected from different sources in Bursa province between November 2013 and June 2015. Seasonal distribution of sample numbers was 31, 60, 39 and 70 in autumn, winter, spring and summer, respectively. Tap waters were taken from taps in public places (university, schools, cemetery, mosque, fountain) and from indoor taps. On the other hand, artesian well water samples were provided from artesian pumps and taps without being connected to public water system and supplied from villages and their neighbourhoods. Samples were taken in 1000 mL sterile glass bottle and transported to the laboratory under refrigerated conditions. All bacteriological analyses were carried out on the same day.

The Isolation and Presumptive Identification of Enterococci

100 mL water sample was shaken well to mix and filtered through membrane filter (pore size, 0.45 µm; diameter, 0.47 mm) and filter page was placed in Enterococcal Broth supplemented with 6 µg/mL vancomycin at 37 ºC for 24 h. A loopful from each enrichment was streaked on Enterococcal Agar supplemented with vancomycin (6 µg/mL) and plates were incubated at 37 ºC for 24 h. Typical black colonies were described as presumptive vancomycin-resistant *Enterococcus* spp., and the isolates were preserved in Brain Heart Infusion broth containing 30% glycerol at –80 °C for further analyses (Cortes *et al.,* 2006).

PCR Analysis of Enterococcus spp. Isolates

DNA extraction was performed using by Chelex 100 (Sigma Aldrich, USA). *Enterococcus* spp. specific primer was used to amplify *tuf* gene during the confirmation procedure of the isolates. The presence of vancomycin resistance phenotyping genes (*van*A, *van*B and *van*C) and virulence trait genes (*gel*E, *ace, agg* and *esp*) in the isolates were investigated. While the *van*A and *van*B genes were detected by multiplex-PCR technique, the detection of the other genes was performed using classical PCR method. The sequence of primers used in this study is summarized in Table 1. Briefly, samples (1 µl) of each extract were amplified in 25 µl of reaction mixture containing 10 mM Tris-HCl, pH 8.9, 22 mM KCl, 1.8 mM $MgCl_2$, 200 µM each of dNTPs, 0.5 mM each primer and 1.25 U of Hot Start Taq DNA polymerase. PCR amplification procedure of each gene was performed by using thermal cycler (Runik SCM 96G) according to description of references shown in Table 1.

Determination of Vancomycin and Teicoplanin MICs

The minimum inhibitory concentrations (MICs) of vancomycin and teicoplanin were determined by *E-test* according to the CLSI guidelines (CLSI, 2014). Each isolate was cultured on blood agar and then a bacterial suspension equal to 0.5 McFarland turbidity standards in Mueller Hinton Broth was prepared and inoculated onto Mueller Hinton Agar plates. After incubation at 35-37°C for 24 h, MICs are measured on the test strip scale where the zone of inhibition intersect the strip. The isolates that had MICs of ≥32 µg/mL were considered resistant for both antibiotics, MICs of 8-16 µg/mL and 16 µg/mL intermediately resistant, and MICs of ≤4 µg/mL and ≤8 µg/mL susceptible to vancomycin and teicoplanin, respectively. *Enterococcus faecalis* ATCC 29212 was used as the control microorganism.

Table 1: List of oligonucleotide primer sequences used in this study

Gene	Product size (bp)	Oligonucleotid sequences (5′-3′)	Reference
tuf	112	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	Ke *et al.,* 1999
*van*A	1030	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	Evers *et al.,* 1993
*van*B	433	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	Handwerger *et al.,* 1992
*van*C	822	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	Dutka-Malen *et al.,* 1995
agg	1553	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	Eaton & Gasson, 2001
esp	432	TTACCAAGATGGTTCTGTAGGCAC CCAAGTATACTTAGCATCTTTTGG	Shankar *et al.,* 1999
*gel*E	402	AGTTCATGTCTATTTTCTTCAC CTTCATTATTTACACGTTTG	Mannu *et al.,* 2003
ace	320	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	Mannu *et al.,* 2003

Results and Discussion

Vancomycin-resistant enterococci are now recognized as a major cause of nosocomial infections. The presence of VREs in aquatic environments results from urban sewage or livestock faecal material contamination (Nam *et al.*, 2013). In the present work, presumptive vancomycin-resistant enterococci isolates were obtained from 11 of 200 water samples. 7 out of these isolates were confirmed as *Enterococcus* spp. by PCR. All of 7 confirmed isolates were obtained from artesian well water samples. None of the tap water samples were observed to be contaminated with vancomycin-resistant enterococci. The samples contaminated with *Enterococcus* spp. were collected from different water supplies in west (3 samples), south-east (2 samples) and south-west (2 samples) sides of Bursa province. The sampling time of *Enterococcus* positive isolates is summarized in Table 2. A study conducted in Turkey showed that 13 (23%) out of 57 enterococci from different soil and water samples, animals, raw vegetables and fruits were of intermediate resistance to vancomycin (Oryaşın *et al.*, 2013). Zdragas *et al.* (2008) reported that 35 vancomycin gene-negative strains from seawater in Northern Greece had low-level vancomycin resistance but not high-level VRE.

MIC quantity survey showed that only 2 out of 7 isolates were resistant in the intermediate level (8 µg/mL) to vancomycin. Therefore, the contamination rate of vancomycin-resistant enterococci was considered to be 2.5% (2/81) in artesian well water samples. On the other hand, one isolate had an MIC value of 6 µg /mL and four isolates to MIC of 4 µg /mL, and also these 5 isolates were regarded as susceptible to vancomycin, which is in accordance with reports by other authors. Said *et al.* (2015) suggested that 85 enterococci isolates from 64 wastewater and 50 surface-water samples was susceptible to vancomycin. Vancomycin-susceptible enterococci strains from waters used for human and animal drinking has also been reported from Portugal during 2006 and 2008 (Macedo *et al.*, 2011). Similarly, no VRE were detected in surface waters by Rathnayake *et al.* (2012), in unchlorinated water samples by Wilson & McAfee (2002) and in river samples, municipal and hospital wastewaters by Servais & Passerat (2009). Conversely, a study performed by Varela *et al.* (2013) demonstrated the detection of vancomycin-resistant enterococci from hospital and urban wastewater samples. Again, the VRE prevalence was recorded as 12.9% in the aquatic environmental samples in Korea by Nam *et al.* (2013) and as 25.6% in superficial water samples by Messi *et al.* (2006). Resistance to teicoplanin was not found in any of the *Enterococcus* spp. isolated in our study (Table 2). Some previously published reports also suggested the susceptibility to teicoplanin of enterococci isolates from drinking waters (Macedo *et al.*, 2011), wastewater and surface water samples (Said *et al.*, 2015) and river samples, municipal and hospital wastewaters (Servais & Passerat, 2009).

Table 2: MIC results of presumptive VRE isolates

			Antimicrobial MICs (µg/mL)	
Sample origin	Sampling time	Sample no	Vancomycin	Teicoplanin
Artesian well water	December 2013	29	4	1.0
Artesian well water	June 2014	133	8	0.50
Artesian well water	July 2014	149	8	1.50
Artesian well water	July 2014	151	4	0.75
Artesian well water	March 2015	163	4	0.125
Artesian well water	April 2015	174	6	1.50
Artesian well water	June 2015	199	4	1.0

All of two intermediate-level vancomycin-resistant enterococci and five vancomycin-susceptible isolates were also analysed for the presence of vancomycin resistance genes (*van*A, *van*B and *van*C) and virulence genes including gelatinase (*gel*E), aggregation substance (*agg*), enterococcal surface protein (*esp*), collagen binding protein (*ace*). The results indicated that neither *van*A, *van*B, and *van*C genes nor *gel*E, *agg*, *esp* and *ace* genes were found in any of the seven isolates. In comparison to our work, studies performed by Nam *et al.* (2013) showed that sixty-three and one of 64 enterococci colonies, which were positive for van genes, had the *van*C-2/3 genotype and the *van*C-1 genotype, respectively. The same authors reported the absence of the *van*A and *van*B types which is in line with our observations. Contrary to our findings regarding virulence genes, Rathnayake *et al.* (2012) noticed the presence of *esp* and *gel*E genes in *E. faecalis* and *E. faecium* water isolates. Recently, *gel*E, *efa*A, *ace* and *asa*1 genes were reported to occur in *Enterococcus* isolates from surface waters (Sidhu *et al.*, 2014). A study made by Messi *et al.* (2006) suggested that 3 (0.7%) isolates from superficial waters belonged to the *van*A, 53 (13.7%) to the *van*B and 43 (11.1%) to the *van*C phenotype.

Conclusion

People contact to water from different sources every day. As well as artesian well water do not drink to people, it is generally used on irrigation in agriculture or washing in particularly rural areas. These play an important role for transmission of enterococci to human hands, skin or stuff. In this way, antibiotic resistance genes and virulence traits in enterococci can carry over big areas. The present study revealed that only two isolates from artesian well waters were intermediately resistant to vancomycin, and none of the isolates were positive for the vancomycin resistance genes (*van*A, *van*B and *van*C) and virulence genes (*gel*E, *ace*, *agg* and *esp*). But still, it must be considered that these water sources could act as a reservoir for resistant bacteria. Prevention efforts against the risk of spread to and transmission of these genetic determinants in the environment must be focused on the prudent use of antimicrobial agents in healthcare and livestock production.

References

Boehm, A.B. & Sassoubre, L.M. (2014). Enterococci as indicators of environmental fecal contamination. In: Gilmore M.S., Clewell D.B., Ike Y. *et al*. (eds), *Enterococci: from Commensals to Leading Causes of Drug Resistant Infection*. Eye and Ear Infirmary, Boston, Massachusetts.

Buyukyoruk, S., Ayaz, N.D., Gencay, Y.E., Beyaz, D. & Koçak, P. (2014). Species distribution, molecular characteristics and vancomycin resistance gene profiles of *Enterococcus* sp. isolates from farmhouse cheeses in western Turkey. *International Journal of Dairy Technoogyl*, 67(1), 103-109.

Cariolato, D., Andrighetto, C. & Lombardi, A. (2008). Occurence of virulence factors and antibiotic resistance in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. *Food Control*, 19, 886-892.

Cetinkaya, F., Elal Mus, T., Soyutemiz, G.E. & Cıbık, R. (2013). Prevalence and antibiotic resistance of vancomycin-resistant enterococci in animal originated foods. *Turkish Journal of Veterinary and Animal Sciences*, 37, 588-593.

Chajecka-Wierzchowska, W., Zadernowska, A. & Łaniewska-Trokenheim, L. (2016). Virulence factors, antimicrobial resistance and biofilm formation in *Enterococcus* spp. isolated from retail shrimps. *LWT-Food Science and Technology*, 69, 117-122.

CLSI (Clinical and Laboratory Standards Institute) (2014). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. M100-S24, Vol. 34, No. 1 (76-79), Wayne PA, USA.

Cortes, C., De la Fuente, R., Contreras, A., Sanchez, A., Corrales, J.C., Ruiz-Santa-Quiteira, J.A. & Orden, J.A. (2006). Occurrence and preliminary study of antimicrobial resistance of enterococci isolated from dairy goats in Spain. *International Journal of Food Microbiology*, 110, 100–103.

Dutka-Malen, S., Evers, S. & Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the

species level of clinically relevant enterococci by PCR. *Journal of Clinical Microbiology,* 33(1), 24–27.

Eaton, T.J. & Gasson, M.J. (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology*, 67, 1628-1635.

Evers, S., Sahm, D.F. & Courvalin, P. (1993). The *van*B gene of vancomycin-resistant *Enterococcus faecalis* V583 is structurally related to genes encoding D-Ala:D-Ala ligases and glycopeptide-resistance proteins *van*A and *van*C. *Gene*, 124, 143-144.

Handwerger, S., Perlman, D.C., Altarac, D. & McAuliffe, V. (1992). Concomitant high level vancomycin and penicillin resistance in clinical isolates of enterococci. *Clinical Infectious Diseases*, 14, 655-661.

Ke, D., Picard, F.J., Martineau, F., Menard, C.H., Roy, P.H., Ouellette, M. & Bergeron, M.G. (1999). Development of a PCR assay for rapid detection of enterococci. *Journal of Clinical Microbiology*, 37(11), 3497-3503.

Macedo, A.S., Freitas, A.R., Abreu, C., Machado, E., Peixe, L., Sousa, J.C. & Novais, C. (2011). Characterization of antibiotic resistant enterococci isolated from untreated waters for human consumption in Portugal. *International Journal of Food Microbiology*, 145, 315-319.

Mannu, L., Paba, A., Daga, E., Comunian, R., Zannetti, S., Dupre, I. & Sechi, L.A. (2003). Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *International Journal of Food Microbiology*, 88, 291-304.

Messi, P., Guerrieri, E., de Niederhausern, S., Sabia, C. & Bondi, M. (2006). Vancomycin-resistant enterococci (VRE) in meat and environmental samples. *International Journal of Food Microbiology*, 107, 218-222.

Nam, S., Kim, M.J., Park, C., Park, F.G., Maeng, P.J. & Lee, G.C. (2013). Detection and genotyping of vancomycin-resistant *Enterococcus* spp. by multiplex polymerase chain reaction in Korean aquatic environmental samples. *International Journal of Hygiene and Environmental Health*, 216, 421-427.

Oryaşın, E., Bıyık, H.H., Başbülbül, G. & Bozdoğan, B. (2013) Antimicrobial susceptibility patterns of environmental and hospital isolations of enterococci in Aydın. *Turkish Journal of Biology*, 37, 514-519.

Rathnayake, I.U., Hargreaves, M. & Huygens, F. (2012). Antibiotic resistance and virulence traits in clinical and environmental *Enterococcus faecalis* and *Enterococcus faecium* isolates. *Systematic and Applied Microbiology*, 35, 326-333.

Said, L.B., Klibi, N., Lozano, C., Dziri, R., Slama, K.B., Boudabous, A. & Torres, C. (2015). Diversity of enterococcal species and characterization of high-level aminoglycoside resistant enterococci of samples of wastewater and surface water in Tunisia. *Science of the Total Environment*, 530-531, 11-17.

Servais, P. & Passerat, J. (2009). Antimicrobial resistance of fecal bacteria in waters of Seine river watershed (France). *Science of the Total Environment*, 408, 365-372.

Shankar, V., Baghdayan, A., Huycke, M.M., Lindahl, G. & Gilmore, M.S. (1999). Infection-derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. *Infection and Immunity*, 67, 193 – 200.

Sidhu, J.P.S., Skelly, E., Hodgers, L., Ahmed, W., Li, Y. & Toze, S. (2014). Prevalence of *Enterococcus* species and their virulence genes in fresh water prior to and after storm events. *Environmental Science and Technolgy*, 48, 2979-2988.

Strateva, T., Atanasova, T., Savov, E., Petroga, G. & Mitov, I. (2016). Incidence of virulence determinants in clinical *Enterococcus faecalis* and *Enterococcus faecium* isolates collected in *Bulgaria. Brazilian Journal of Infectious Diseases*, 20(2), 127-133.

Varela, A.R., Ferro, G., Vredenburg, J., Yanık, M., Vieira, L., Rizzo, L., Lameiras, C. & Manaia, C.M. (2013). Vancomycin resistant enterococci: From the hospital effluent to the urban wastewater treatment plant. *Science of the Total Environment*, 450-451, 155-161.

Wilson, I.G. & McAfee, GG. (2002). Vancomycin-resistant enterococci in shellfish, unchlorinated waters, and chicken. *International Journal of Food Microbiology*, 79, 143-151.

Zdragas, A., Partheniou, P., Kotzamanidis, C., Psoni, L., Koutita, O., Moraitou, E., Tzanetakis, N. & Yiangou, M. (2008). Molecular characterization of low-level vancomycin-resistant enterococci found in coastal water of Thermaikos Gulf, Northern Greece. *Water Research*, 42, 1274-1280.