The investigation of prevalence, vancomycine resistance and slime factor production of enterococci isolated from chicken carcasses

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Geliş Tarihi / Received: 08.07.2011, Kabul Tarihi / Accepted: 23.11.2011

Summary: The aim of this study was to investigate the prevalence, vancomycin resistance and slime factor production of *Enterococcus* spp. in chicken carcasses consumed in Samsun province, north of the Turkey. For this purpose, 123 chicken carcasses were analyzed by direct culture technique on Slanetz and Bartley Medium and, a total of 92 *Enterococci* spp. were isolated from 41 (33.3%) out of the 123 samples and identified phenotypically. All enterococci isolates were confirmed at the genus level by a single PCR targeted tuf gene using *Enterococcus* specific primers. To identify these enterococci as either being *E.faecalis* or *E.faecium* and to detect vancomycin resistance, a multiplex PCR based on the amplification of ddl and van (van A, B, C1/2, D, E and G) genes were performed. While 39 (42.4%) and none of these isolates were identified as *E.faecalis* and *E.faecium*, respectively, and the remaining 53 isolates (57.6%) were identified as *Enterococcus* spp. except from *E.faecalis* and *E.faecium*. vanA, vanB, C1/2, vanD, vanE, vanG genes were not detected in any of the isolates by this multiplex PCR. To detect slime factor production, Congo Red Agar Method was used and slime factor production was not detected in any of the isolates. In conclusion, *E.faecalis* isolates from chicken carcasses in Samsun Province of Turkey do not constitute a potential risk to the public health for vancomycin resistance and slime factor production.

Key words: Chicken carcass, Enterococci, PCR, slime factor production, vancomycin resistance.

Tavuk karkaslarında *Enterococcus* spp. prevalansı ile vankomisin dirençliliği ve slime faktör üretme yeteneklerinin araştırılması

Özet: Bu çalışma, Samsun İli'nde tüketime sunulan tavuk karkaslarındaki *Enterococcus* spp.'nin prevalansı, vankomisin dirençliliği ve slime faktör oluşturma yeteneklerini belirlemek amacıyla yapıldı. Bu amaçla, Slanetz ve Bartley besiyerinde direkt kültür tekniği ile 123 adet tavuk karkası analiz edildi. Bu örneklerin 41'inden (%33.3) izole edilen toplam 92 adet suş fenotipik olarak *Enterococcus* spp. olarak identifiye edildi. Tüm *Enterococcus* spp. izolatları, tuf genini hedefleyen PCR ile cins düzeyinde doğrulandı. Bu izolatların *E.faecalis* veya *E.faecium* olup olmadığını ve vankomisin dirençliliklerini belirlemek üzere, ddl ve van (van A, B, C1/2, D, E ve G) genlerinin amplifikasyonuna dayalı multipleks PCR gerçekleştirildi. Suşlardan 39 (%42.4) adedi *E.faecalis* olarak doğrulanırken, hiç bir suş *E.faecium* olarak identifiye edilmedi. Geri kalan 53 (%57.6) izolat ise *E.faecalis* ve *E.faecium* dışındaki Enterokok türleri olarak değerlendirildi. van A, B, C1/2, D, E ve G genleri, hiçbir izolatta belirlenmedi. Slime faktör üretimini belirlemek üzere Kongo Kırmızısı içeren Agar yöntemi kullanıldı ve hiçbir izolatta slime faktör üretimi belirlenmedi. Sonuç olarak, Samsun İli'nde tavuk karkaslarından izole edilen *E.faecalis* izolatlarının, vankomisin dirençliliği ve slime factor oluşturmaları yönünden halk sağlığı için potansiyel bir risk oluşturmadığı görülmüştür.

Anahtar kelimeler: Enterokok, slime factor üretimi, PCR, tavuk karkası, vankomisin direnci.

Introduction

Enterococci are Gram-positive, facultative anaerobic bacteria that live as part of the natural flora in the intestinal tract of animals as well as humans (15). Therefore, for a long time, enterococci were considered to be unimportant from the medical point of view and also for food industry, but later the bacteria have emerged as important nosocomial pathogens of concern, causing a variety of infections. Therefore, the enterococci are not regarded as primary pathogens but due to their ability to acquire high-level resistance to multiple antibiotics including aminoglycosides, ampicillin, tetracyclines, macrolides, chloramphenicol and vancomycin they have emerged as nosocomial pathogens worldwide. Among the antibiotic resistances, vancomycin resistance is of particular concern because of treat-

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ment difficulties and the potential for this plasmid-mediated resistance trait to be transferred to other microorganisms. Of the 24 enterococcal species identified up to now, especially *E.faecalis* (85-90% of isolates) and *E.faecium* (5-10% of isolates) are the most important ones for nosocomial infections in humans (15) and the most prevalent species in foods (6, 14, 17).

Due to the heavy use of growth-promoting drugs in food animals, the antibiotic resistance in enterococci of animal origin has increased and also resistant enterococci have spread in the human population (5). This resistance can be both intrinsic that present in almost all the strains of enterococci or acquired. Cross resistances exist in related antibiotics therapeutically used in human or animal medicine such as avoparcin (similar to vancomycin, teicoplanin), virginiamycin (similar to quinupristin/dalfopristin), spiramycin, tylocin (similar to erythromycin) and avilamycin (similar to evernimisin) (23). Vancomycin, a glycopeptide antimicrobial agent has been used to treat Gram positive infections in humans. Up to date six glycopeptide resistance phenotypes; VanA, VanB, VanC, VanD, VanE and VanG, have been described in enterococci. It has been reported that they could be distinguished on the basis of the level, inducibility, and transferability of resistance to vancomycin and teicoplanin as well as associated resistance genes such as vanA, vanB, vanC, vanD, vanE, and vanG (23). Generally, the vancomycin resistance is related to inhibition of cell wall synthesis by binding to peptidoglycan precursor and inhibition of following transglycosilation modified traget of glycopeptid. The vanA genotype is the clinically most important one and widespread in enterococci. VanA resistance has been characterized as high-level, inducible and transferable. The second important genotype is vanB (23). The vanB resistance is inducible low-level vancomycin resistance. VanA resistance is usually plasmid borne but is now known to be encoded on a transposon (Tn1546) that may pass to the chromosome. VanB resistance is usually chromosomal and is occasionally transferable from chromosome to chromosome on a transposon (29). Both VanA and VanB resistance are seen most commonly in *E.faecium* and *E.faecalis*. VanD, VanE, and VanG have been reported to detect in single isolates of *E.faecium* and *E.faecalis*, respectively, until now. A constitutive low-level vancomycin resistance type, vanC genotype has been seen in some *E.gallinarum* strains (26). In the late 1980s, vancomycin-resistant enterococci (VRE) were first detected in humans as specific pathogens (35). Later, molecular evidences have indicated that in Europe and other countries around the world, food-producing animals are the likely reservoir of one type of VRE, namely *Enterococcus faecium* strains with the vanA antibiotic-resistance gene (25, 27, 38). This case could be associated with the use of the vancomycin-related glycopeptide (vancomycin and teicoplanin), avoparcin (an analogue of the glycopeptides) as prophylactic or growth promoter in animal production (2, 38). The ability of VRE to transmit to humans via the food chain have led to the decision of banning avoparcin in the European Union since 1997 (38) and Turkey since 1999 (1). Decreases in the prevalence of VRE in animals, meat products and humans have been observed after a relatively short period of time from the banning of avoparcin use in several European countries (10, 22).

Slime factor variously termed as biofilm, capsule or glycocalyx is an extracellular polymeric substance (EPS) produced by the some microorganism and play an important role in the attachment and colonization of organ or food-contact surfaces (24). The EPS also supports cell to cell bacterial contacts by means of a multilayered biofilm. Essentially, biofilm formation is a dynamic process and different mechanisms are involved in their attachment and growth. Bacteria in biofilms are generally more resistant to environmental stresses than their free-living bacteria. Therefore, EPS appear to be significant virulence factors for some bacteria such as enterococci. Like other Gram-positive microorganisms, enterococci are able to produce biofilms on abiotic surfaces and increasing their high innate resistance to antibiotics. The initial step in the colonization of surface and biofilm formation is bacterial adherence to the biomaterial. It has been reported that biofilm formation ability of enterococci is highly and significantly associated with the presence of esp gene (32).

The widespread and indiscriminate use of antibiotics in human and veterinary medicine and in livestock breeding has lead to a spread of antibiotic resistance (AR) among both pathogenic and commensal microorganisms. The same AR genes have been also isolated from human and food strains. Therefore, antimicrobial resistance constitutes

a major threat to public health in many countries due to the persistent circulation of resistant bacteria in the environment and the possible contamination of water and food such as poultry meats (30). Biofilm production has been also reported in some enterococcal infections. However, there are limited reports about the prevalence of vancomycin resistance and biofilm production of enterococci isolated from poultry in Turkey.

Therefore, the aim of this study was to investigate the occurence, vancomycine resistance and slime factor produciton of *Enterococcus* spp. namely clinically important species, *E.faecalis* and *E.faecium*, in chicken carcasses consumed in Samsun province, Turkey.

Materials and Methods

Sample collection: A total of 123 chicken carcasses were collected from different markets and butcher shops in the Samsun Province in North of the Turkey, between 2008 and 2009. These samples were analyzed immediately after procurement.

***Enterococcus* spp. isolation and identification:** For the isolation, the whole carcass was transferred into a sterile polyethylene stomacher bag and rinsed with 225 ml 0.1% (wt/vol) of peptone water (Bacteriological peptone, Oxoid, Basingstoke, England) for 1-2 min (rinse method). Following, the carcass was removed aseptically and the remaining rinsate in the bag was cultured for microbiological analysis after 10-fold serial dilutions (up to 10-5) for each sample in sterile peptone water (PW). Subsequently, each of these dilutions was inoculaed onto Slanetz and Bartley Medium (Oxoid CM 377) by spread plating technique (100 µl) and, the plates were incubated aerobically at 37°C for 24-48 h. (31). *Enterococcus* spp. colonies (bright red coloured colonies or pale colonies with bright red coloured centre) were selected and subcultured onto Triptone Soy Agar (CM 131, Basingstoke, England) plates to identify the isolates at the genus level using biochemical tests (10).

DNA extraction: In this study, the DNAs used for PCR analysis were extracted using boiling method.

Table 1. The oligonucleotide primers used in the study

Target gene	Primer names		Oligonucleotide sequences		Amplicon size (bp)
ddl	DD13	F	5'-CACCTGAAGAAACAGGC-3'	*E. faecalis*	476
	DD3-2	R	5'-ATGGCTACTTCAATTTCACG-3'		
ddl	FAC1-1	F	5'-GAGTAAATCACTGAACG-3'	*E. faecium*	1091
	FAC2-1	R	5'-CGCTGATGGTATCGATTCAT-3'		
tuf	ENT1	F	5'-TACTGACAAACCATTCATGATG-3'	*Enterococcus* spp.	112
	ENT2	R	5'-AACTTCGTCACCAACGCGAAC-3'		
vanA	EA1	F	5'-GGGAAAACGACAATTGC -3'	VAN A	732
	EA2	R	5'- GTACAATGCGGCCGTTA -3'		
vanB	EB3	F	5'-ACGGAATGGGAAGCCGA -3'	VAN B	647
	EB4	R	5'- TGCACCCGATTTCGTTC -3'		
vanC1/2	EC5	F	5'- ATGGATTGGTAYTKGTAT-3'*	VAN C	815/827
	EC8	R	5'- TAGCGGGAGTGMCYMGTAA -3'*		
vanD	ED1	F	5'- TGTGGGATGCGATATTCAA -3'	VAN D	500
	ED2	R	5'- TGCAGCCAAGTATCCGGTAA -3'		
vanE	EE1	F	5'- TGTGGTATCGGAGCTGCAG -3'	VAN E	430
	EE2	R	5'- ATAGTTTAGCTGGTAAC -3'		
vanG	EG1	F	5'- CGGCATCCGCTGTTTTTGA -3'	VAN G	941
	EG2	R	5'- GAACGATAGACCAATGCCTT -3'		

Confirmation of the *Enterococcus* spp. by PCR analysis: For determination of *Enterococcus* spp. at genus level, extracted DNA was PCR-amplified using primers that target the tuf gene (elongation factor EF-Tu). For this purpose, Ent1 and Ent2 primers were used and amplification procedures were performed according to (20) (Table 1). *E.faecalis* ATCC 29212 and *S.aureus* ATCC 29213 were used as positive and negative control strains, respectively.

Species specific identification and determination of vancomycin resistance by multiplex PCR analysis: A multiplex PCR was performed to identify *E.faecium* and *E.faecalis* and detect the presence of van genes in these species. The primers for the amplification of species-specific D-Ala:D-Ala ligase genes (ddl genes) and van genes (vanA, van B, vanC1/2, van D, van E and van G) are presented in Table 1. These primers were selected and amplification was conducted as described by Depardieu et al. (9), after amplification, the DNA fragments were separated by agarose gel electrophoresis and visualized under ultraviolet (UV) light. *E.faecalis* ATCC 29212 and *E.faecium* ATCC 19434 were used as control strains.

Slime production: Slime production assay was performed by cultivation of *Enterococcus* spp. isolates on Congo Red agar (CRA) plates containing 0.8 g/l of Congo red dye and 50 g/l of saccharose (16). Isolates were streaked on the CRA plates and incubated at 37°C for 24-48 hrs. Slime production was evaluated observing the rough black (slime positive) or red (slime negative) colonies on CRA.

Results

In the present study, a total of 92 *Enterococcus* spp. were isolated from 41 (33.3%) out of the 123 samples by direct culture on Slanetz and Bartley agar and verified by single PCR targeted tuf gene (Figure 1). Specifity was confirmed on positive and negative control strains. As expected, no band was observed for the negative control strain (*S.aureus* ATCC 29213) but the expected size of PCR products (476 bp) was observed for the positive control strain (*E.faecalis* ATCC 29212).

In multiplex PCR performed to identify enterococci either being *E.faecalis* or *E.faecium* and to detect vancomycin resistance, the ddl gene specific for *E.faecalis* was detected in thirty-nine of 92 isolates (42.3%) and they were identified as *E.faecalis*, but no band was found specific for *E.faecium* (Figure 2). The remaining isolates (57.6%) were evaluated as *Enterococcus* spp. except from *E.faecalis* and *E.faecium*. vanA, vanB, vanC1/2, vanD, vanE, vanG genes were also not detected in any of the isolates. None of the isolates was found slime positive on CRA.

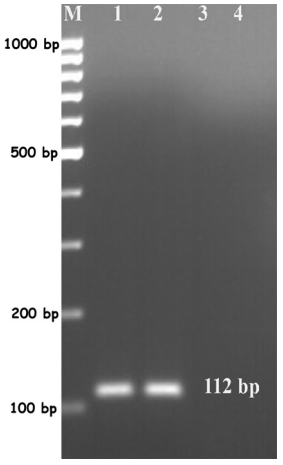

Figure 1. The single PCR results targeted tuf gene for the detection of *Enterococcus* species **M:** Marker; **lanes1-2:** *Enterococcus* species isolated from chicken carcasses

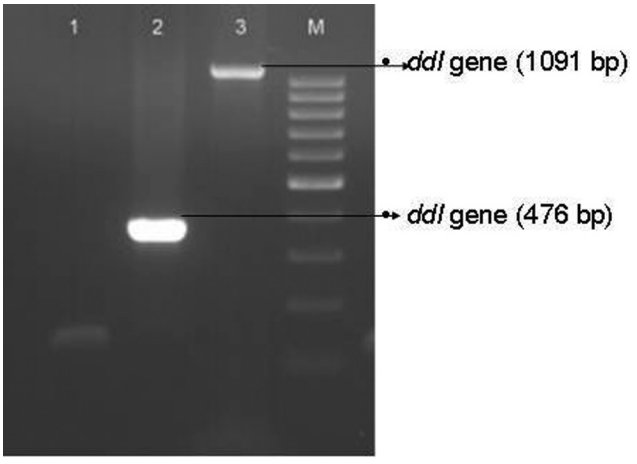

Figure 2. The multiplex PCR results targeted ddl genes for the detection *E.faecalis* and *E.faecium* and vanA,B,C1/2,D,E and G for the detection vancomycine resistant *Enterococcus* species **M:** Marker; **lane 2:** ddl gene (476 bp) for the detection of *E.faecalis* ATCC 29212; **lane 3:** ddl gene (1091 bp) for the detection of *E.faecium* ATCC 19434

Discussion

Enterococci have been considered as low pathogenic bacteria that infect persons with special predispositions such as immunocompromised patients. However they have been reported to be able to cause different infections, even life-threatening infections such as bacteremia or endocarditis. The *Enterococcus* genus comprises more than 20 species and *E.faecalis* and *E.faecium* are the most common species in foods (6, 14, 17, 23). Similiarly in several studies, different predominances of *Enterococcus* species, especially *E.faecalis* and *E.faecium* isolated from various poultry sources have been reported. Similar to later reports, in the present study, *Enterococcus* spp. was isolated from 33.3% of chicken carcasses and *E.faecalis* was found as the most prevalent species (42.3%). However *E.faecium* did not detected in any of the samples. The remaining isolates (57.6%) were evaluated as *Enterococcus* spp. except from *E.faecalis* and *E.faecium*. These differences in the predominance of *E.faecalis* and *E.faecium* in the poultry sources from the different areas in the world could be due to several factors such as geographic area, numbers of the analyzed sample and isolation methods.

It has been reported that enterococci including also the isolates from foods had a broad spectrum of natural or acquired antibiotic resistance. Two prerequisites for acquired antibiotic resistance are 1) the genetic potential by bacteria (mutations or acquisition of resistance genes from donor cells) and 2) the antibiotic selective pressure. Vancomycin, a glycopeptide antibiotic, is an important alternative for treatment of infections caused by multiple resistant enterococci as well as other Gram positive bacteria. The acquisition of resistance against this antibiotic and other glycopeptides results in dramatical decrease of the therapeutic possibilities in enterococcal infections. Thus, when considered in the medical point of view, acquired resistance against glycopeptides has a special concern (23).

Vancomycin resistant enterococci (VRE) had been first reported in UK by Uttley et al. (33), in Turkey, the first VRE isolation had been reported by Vural et al. (36), in Antalya province. Following the first isolation of VRE outside the healthcare settings, from sewage treatment plants in 1993 (4), VRE has been isolated from livestock feces and uncooked chicken samples purchased from retail outlets (5). VRE have also recovered from manure samples from pig and poultry farms in Germany (21) and (11) have found VRE in the feces or intestines of other farm animals and pets, including horses and dogs. After these findings, it has been suggested that there was a relationship between the recovery of these organisms and the use of avoparcin, a glycopeptide antimicrobial drug used as a livestock feed additive in many European countries (21), in an epidemiological study (2), it has been documented that there was an association between the use of glycopeptides in animal production as feed additives and the occurrence of vancomycin resistant Enterococci especially *E.faecium* and *E.feacalis* species with high level resistance to vancomycin in farm animals included poultry and pigs. This association has been most thoroughly investigated for avoparcin-VRE association. The resistant bacteria have spread between animals in the farm environment after the selection of resistant bacteria in food-animals by the antibiotic growth promoters (AGPs). As a consequence of the AGPs use the propagation of food animal reservoir of resistant bacteria which constitute a potential risk for spreading to humans by food intake and animal contact has occurred. Both the spreading of the resistant bacteria from animals to environment and the presence of these bacteria in food chain have been considered as key determinants for spreading to humans (37). Because the vancomycin resistance gene clusters have been found as similar or identical in enterococci of human and animal origin (38), VRE and vancomycin resistant determinants have been considered to be able to spred from animals to humans (37). After the revealing of the avoparcin-vancomycin resistance association, the use of all AGPs including avoparcin and the classes used also in human medicine has been banned in 1997 by European Union. In Turkey, the use of avoparcin and some other feed additives has also been banned by government in 1999 (1). Denmark and Germany had already forbidden the use of avoparcin in 1995 and 1996, respectively. After the ban, the prevalence of VRE in poultry decreased from >80% in 1995 to <5% in 1998 in Denmark, from 100% in 1995 to 25% of samples tested by 1997 in Germany. The prevalance of VRE has also been decreased in faecal samples of healthy persons, from 12% in 1994 to 3% in 1997 (3, 22). Pantosti et al. (28), have reported that the prevalence of VRE in poultry meats decreased from 15%

to 8% in Italy after the avoparcin ban. Lemcke and Bülte (27), have reported the percentage of vanA-VRE isolates from poultry in Germany, Netherlands and France as 14%, 13% and 9%, respectively. Lauderdale et al. (25), have isolated 39 VRE from 28 of 30 chicken carcasses in Taiwan. In Turkey, VRE has been found 13-14% by Çelik (7), in various animal sources. In the results of another study performed in 2005, the isolation percent of VRE from poultry has been reported as 0.25% (34). Kasimoglu-Dogru et al. (19), have reported that no *Enterococcus* isolates from Ankara province in Turkey detected as VRE phenotypically and vanA and vanB genes could not be found in any of these isolates. Similarly, in this study, no VRE was found among the isolates from chicken carcasses and meat samples by multiplex-PCR. This case may associated with the effects of avoparcin (and/or other feed additives) ban. Although the studies have showed that the termination of AGP use resulted in dramatic reduction in occurrence of VRE in food animals, it has been reported that these reductions have not led to disappearance of the strains completely and the resistant strains might be still present in the farm environment, food animals and even in the foodstuffs in low level (37). Thus, although the results of this study in Turkey show the absence of VRE in chicken carcasses, it should not be considered that VRE are not appeared or isolated from various poultry sources anymore.

Slime production and biofilm formation also determined in both *E.fecalis* and *E.faecium* which are the most common enterococci species have been suggested as virulence determinants of clinical isolates (12). Slime factor plays an important role for adhesion and colonization of organ surfaces or food-contact surfaces. If the microorganisms from food-contact surfaces are not completely removed, they may lead to biofilm formation and also increase the biotransfer potential. Also, biofilm formation may lead to food spoilage, contamination and significant economic losses. For these reason, biofilms are an important reservoir of microbial contamination. In addition, if the biofilm bacteria are pathogens, then biofilms pose a serious public health risk (24). It has been reported that enterococci in biofilms are more resistant to antibiotics than planktonically growing enterococci. Therefore, this factor has a great importance for food industry as well as clinical importance.

Çiftci et al. (8), have reported that 60% of *Enterococcus* strains were found as slime positive and 13.43% vancomycin resistant enterococci were obtained from chicken artritis. They also reported that slime factor productions of enterococci were found as 59.7%. In another study (13), the production of biofilm (slime) has been observed mainly in *E.faecalis* isolates from various clinical sources but only non-numerous strains has formed strong biofilm. However, Gomes et al. (18), have reported that none of the different food isolates presented moderate or strong ability to form biofilm on abiotic surfaces. Similarly, in this study, none of the enterococci isolated from chicken carcasses and meat samples had not ability to form biofilm.

In conclusion, for the moment, Enterococci isolated in this study in Samsun province do not constitute a potential risk for the concern of vancomycin resistant enterococcal infections in humans. Similarly, the slime factor in enterococci isolated from chicken carcasses and meat samples in this study do not pose a hazard for the public health and food industry including poultry slaughterhouse. However, because the completely eliminating of VRE among farm animals including animal origin foods needs for a long time, chicken materials should be screened regularly for especially *E.faecalis* and *E.faecium* which may have a potential vancomycin resistance risk.

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