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Demonstration of the presence of the InuB gene in environmental materials in Turkey

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Abstract

Aim: Although Enterococcus is among bacteria of normal gut flora in animals, these bacteria are also causative agents of nosocomial infections. *E. faecium* and *E. faecalis* are the most common enterococci isolated from patients. *E. faecium* is intrinsically resistant to many antibiotics but susceptible to macrolides and lincosamides. The most common resistance mechanisms to lincosamides are modification of target by methylases and inactivation of lincosamides by nucleotidyltransferase. In present work mechanism of resistance, transferability of resistance and characterisation of genetic support of lincosamide resistance in *E. faecium* ADU1 which is resistant to macrolides and lincosamides was studied.

Materials and Antimicrobial susceptibilities were determined by agar dilution MICs and disk diffusion methods. Lincosamid inactivation of *E. faecium* ADU1 was tested by Gots' test. Presence of known *erm* and *lnu* genes was checked by PCR. *E.faecalis* JH2-2 was used as recipient strain for conjugation experiment to test transferability of resistance. Chromosomal changes in receptive strains were tested by PFGE. To analyse genetic support of resistance gene invers PCR method was used and amplicon was sequence and homologies were checked with gene bank.

Results. A total of 57 environmental Enterococci was tested for resistance to lincosamide by inactivation and 16% were found to be positive. One of these isolates, ADU1 was studied further. The MICs of ADU1 strain for clindamycin and erythromycin were both > 128 µg/ml. Gots' test showed inactivation of lincosamides by ADU1 strain which was shown to carry *ermB* and *lnuB* by PCR. Conjugation experiments showed co-transfer of lincosamid resistance with erythromycin resistance. No change in chromosomal restriction profile of transconjugants was observed by PFGE which showed possible transfer of resistance by plasmid. A fragment of 2500bp was obtained by invers PCR. Sequence analysis of this fragment showed that partial homology with pEF418 which was found in *Enterococcus faecalis*.

Conclusion. Resistance to lincosamides is conferred by many different nucleotidyltransferases and disseminated among a large spectrum of bacteria genus and species via mobile conjugative genetic elements. *E. faecium* ADU1 was resistant to lincosamides by inactivation due to *lnuB* gene which is carried by conjugative plasmid pEF418. Presence of multiple resistance genes on a transferable plasmid is important for dissemination of antibiotic resistance. Our study is the first study which showed presence of *lnuB* gene in an Enterococcus isolated in Turkey. Large survey studies should be done to evaluate dissemination of lincosamide resistance by inactivation.

Introduction

Macrolide, lincosamide and streptogramine (MLS) antibiotics are considered in the same group of antibiotics, although they differ in their chemical structures, because of their similar mechanism of action. One of the other reasons for these antibiotics to be taken together in the same group is the presence of a common resistance mechanism, methylation of the target of these antibiotics, 23S rRNA of ribosome. However, separate resistance mechanisms to each of these antibiotics can also to be observed, especially by inactivation of these antibiotics (Leclercq, 2002).

Lincosamide antibiotics effectively inhibit protein synthesis in Gram-positive bacteria, while they exhibit lower activity in many Gram-negative bacteria. MLSb (Macrolide, Lincosamide, Streptogramin B) resistance phenotype is one of the types of resistance to lincosamides. This phe-

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notype is usually due to ribosomal methylation. These methylases are encoded by *erm* (erythromycin resistance methylase) genes and act by causing mono or di methylation of adenine at 2058 position of 23S ribosomal RNA (Leclercq and Courvalin, 2002). Ribosomal mutation at 23S rRNA gene or in ribosolam proteins may also confer resistance. Generally, many of the methylase genes that provide resistance to MLSb antibiotics in clinical isolates are carried by plasmids.

One of the common type of lincosamide resistance is due to enzymatic inactivation of these antibiotics (Quiros et al., 1988). Up to date all of the identified enzymes are nucleotidyl transferases which transfer the nucleotidyl group to the lincomycin and clindamycin. The first enzymes were found in *Staphylococcus haemolyticus* BM4610 and *Staphylococcus aureus* BM4611 and these enzymes with 14 amino acid differences were shown to be encoded by genes called *lnuA* and *lnuA'*, respectively (Brisson-Noel et al, 1986). Then, the *lnuB*, *lnuC* and *lnuD* genes were found in *E. faecium* HM1025, *S. agalactiae* UCN36 and *S. uberis* strains, respectively (Bozdogan et al., 1999; Achard et al., 2005; Petinaki et al., 2008). The *lnuE* gene was found in *Streptococcus suis* but also in *E. coli* X1D61 which are naturally resistant to lincosamides (Zhao et al., 2014; Heir et al., 2004). In recent years, the *lnuG* gene was characterized from the *E. faecalis* E531 strain with lincosamide resistant macrolide sensitive phenotype (Zhu et al., 2017).

In the L phenotype due to the presence of the *lnu* gene, which is seen among staphylococci, streptococci, and enterococci, bacteria become resistant to lincomycin but remain susceptible to erythromycin. In this phenotype, the *in vitro* effects of clindamycin are limited and bacteria may remain susceptible to clindamycin, and antibacterial activity is reduced (Leclercq 2002).

In this study, the resistance mechanism, transferability of the resistance and genetic structure of the resistance gene were investigated in *E. faecium* ADU1 strain which is resistant to macrolides and lincosamides.

Materials and Methods

Strains and growth conditions

In our study, 57 enterococcus strains isolated from various environmental sources were used (Oryasin et al., 2013). *Micrococcus luteus* ATCC 9341 strain was used to determine the presence of inactivation. *Enterococcus faecalis* JH2-2 was used as the recipient strain for conjugation assay. Enterococci were incubated at 37 °C and *Micrococcus luteus* at 30°C for 16-18 hours and Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) media were used for this purpose.

DNA extraction and molecular identification

DNA isolation from *Enterococcus isolates* was performed with DNA4PCR extraction kit (R Tech, Turkey) according to the manufacturer's recommended protocol. PCR amplification was performed using universal 16S rRNA primers 27F and 1390R (Table). Template DNA were added to a reaction mix containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 μL of each deoxynucleoside triphosphate (dNTP), 0.4 μM of each primer, and 2.5 U Taq DNA polymerase to complete a final volume of 50 μL. The PCR conditions were: initial denaturation of 4 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 56 °C and 1 min at 72 °C, and a final extension at 72°C for 8 min. The reaction product was visualized on a 1% agarose gel under UV light. The resulting amplicons were purified by PCR clean-up kit (Invitrogen) and then sent to Macrogene sequencing company for 16S rRNA sequence identification. The sequences thus obtained were compared with the NCBI database through BLASTn (http://blast.ncbi.nlm.nih.gov).

Antimicrobial susceptibility testing

The antibacterial susceptibility of *Enterococcus* isolates were measured minimum inhibitory concentration (MIC) by broth dilution (CLSI document, 2012).

Inactivation screening Test

Inactivation of lincosamides was screened by the Gots' test with *Micrococcus luteus* ATCC 9341 as indicator organism. According to this test, a reduction in the zone of inhibition caused by antibiotics was considered to be the expression of the inactivating enzyme tested and the resistance was considered positive by inactivation (Gots, 1945).

Detection of lincosamides genes

PCR was performed for detection of lincosamide inactivation genes *lnuA, lnuB, lnuC,* and *lnuD* with specific primers (Table). Each gene was amplified separately. PCR amplification were done with a reaction mix containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 μL of each deoxynucleoside triphosphate (dNTP), 0.4 μM of each primer, and 2.5 U Taq DNA polymerase to complete a final volume of 50 μL. The PCR conditions were: initial denaturation of 5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, and a final extension at 72°C for 8 min.

Transferability of lnuB gene

Conjugation method was used to determine whether the *lnuB* gene is on a mobile genetic element. *Enterococcus faecalis JH2-2* was used in mating experiments as a recipient strain. Donor strain and *E. faecalis* JH2-2 were incubated overnight in brain heart infusion (BHI) broth. Then, 400 µl of the recipient strain and 600 µl of the donor strain were placed in a sterile eppendorf tube and centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and the pellet was homogenized in 200 µl broth medium, and inoculated onto BHI agar and incubated at 37C overnight. Following day bacterial culture was taken to be spread on the antibiotic medium for selection of transconjugants. *E. faecalis* JH2-2 strain is resistant to rifampicin and fusidic acid and susceptible to erythromycin. The antibiotics used for selection of transconjugants were rifampin (100 μg/ml), fusidic acid (50 μg/ml), and erythromycin (10 μg/ml). Gots' test was performed to see if transconjugant colonies could inactivate lincosamides. The presence of inactivation gene in transconjugants was confirmed by PCR (Bozdogan et al., 1999; Gots, 1945).

Table 1. The primer sequences used in PCR assays.

Primer Name	Primer sequence
20F-*1390R*	AGAGTTTGATCCTGGCTCAG
-	GACGGGCGGTGTGTACAA
lnuA	GTATTAACTGGAAAACAGCAAAG
-	GAGCTTCTTTTGAAATACATGG
lnuB	TACAGCTTGTATGATGTAATGG
	AAGGCTAATTTCTTTTTCAAG
lnuC	GATTAAAGTCTTTCTTGATGG
	AACCAGATTTTCCAATTTGC
lnuD	GAAAATGAGATTGACATTTGG
-	GCATAATAACCTTACGTCATG
INVLINB	GTTCCACAAACAATAGGTAG
-	TAGTGAATAGGAGAGTAACG
Ori-pEF418	ATCGACGATGTTAATATAAG
-	GCTACAATAATCACATCCTG
aadE	GATTTAGTACTTTCTTTAGC
-	GTAGAATCTGGTTCAGATG
pEF418	AGGAATATCAAGTAATTCATGAAAGT
-	ACACCAGTCGAAATGAATTT

Pulsed field gel electrophoresis (PFGE)

After conjugation, erythromycin resistant and lnuB positive *E. faecalis* JH2-2 transconjugants were tested by PFGE to determine whether the transferred mobile elements were plasmids or transposons. Pulsed-field gel electrophoresis of *E. faecalis* JH2-2 and transconjugant *E. faecalis* JH2-2 total-DNA extract digested with SmaI was performed as previously described (Bozdogan et al., 2013).

Characterization of the lnuB carrier genetic element

Based on the *lnuB* gene sequence AJ238249 from the gene bank primers suitable for inverse PCR were designed (INVLINB, Table). Total DNA of *E. faecium* ADU1 was restricted with EcoRI and HindIII enzymes and after restriction fragments were purified by phenol chloroform precipitation and then ligated using T4 DNA ligase enzyme at 16 ^{o}C for 3 hours. After ligation fragment were purified by precipitation and used for PCR amplifications using INVLINB primers for inverse PCR. The PCR conditions were: initial denaturation of 5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, and a final extension at 72°C for 8 min. The DNA sequence analysis was performed for the detection of the genetic element containing the *lnuB* gene from the amplicon obtained by inverse PCR and the results were compared with the GenBank (BLAST) (Ochman et al., 1988).

Results

In our previous study, 57 enterococcal isolates were studied for their antibiotic susceptibilities and resistance mechanisms. It was reported that 38 (67%) of these strains were *E. faecium* (Oryasin et al., 2013). In this study, the ability of lincosamide inactivation of these 57 enterococcal strains from environmental sources were investigated using Gots' test. Out of 57 strains, 9 (16%) were found to be resistant to lincosamide by inactivation of these antibiotics. All 9 isolates positive with Gots' test were *E. faecium* (Figure

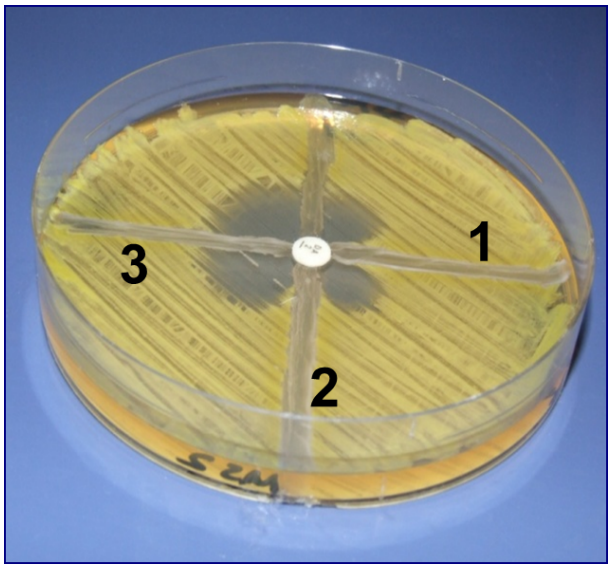

Figure 1. Zones seen as a result of the Gots' test. Clindamycin (DA: 2 µg) was used as the antibiotic disc. *M. luteus* ATCC 9341 control strain was used as 1: *E. faecium* ADU1 trial strain, 2: *E. faecium* MS2 positive control strain and 3: *E. faecium* MS1 negative control strain.

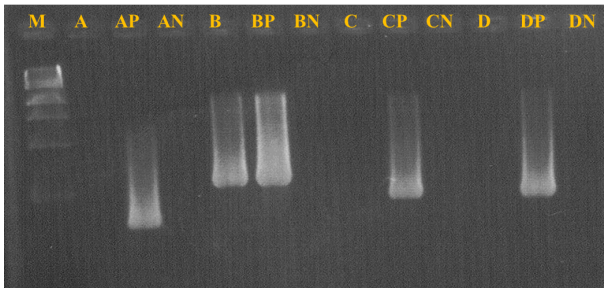

Figure 2. PCR image made with specific primers for lnuA, lnuB, lnuC, lnuD in E. faecium ADU1 strain. M: marker, A: ADU1 strain with *lnuA* primers, AP and AN: positive and negative controls for *lnuA* respectively, B: ADU1 strain with *lnuB* primers, BP and BN: positive and negative controls for lnuB, CP: ADU1 strain with *lnuC* primers, CP and CN: positive and negative controls, respectively, for lnuC, D: *lnuD* primers with ADU1 strain, DP and DN: positive and negative controls, respectively, for *linD*.

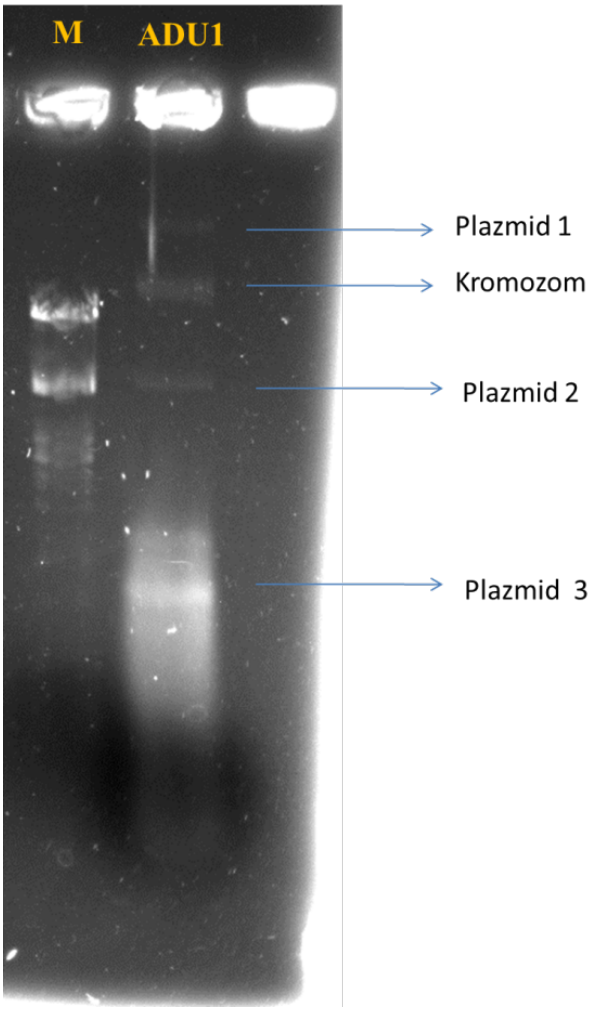

Figure 3. Plasmids observed as a result of total DNA extraction from *E. faecium* ADU1 strain

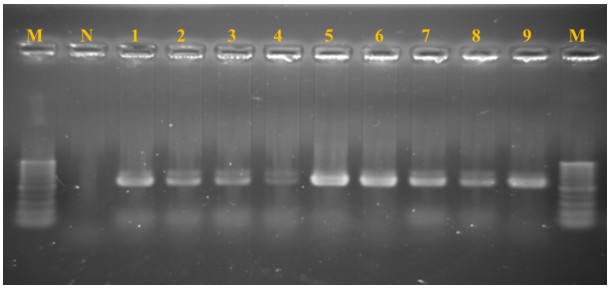

Figure 4. *lnuB* genes and 9 selected transconjugants. M: Marker, N: Negative control, 1,. . . , 9: Transconjugants

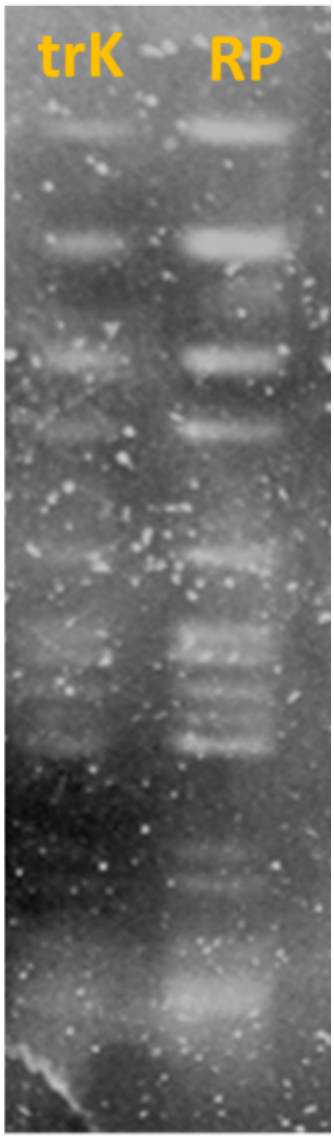

Figure 5. PFGE image of the transconjugant (trK) and recipient (RP) strain

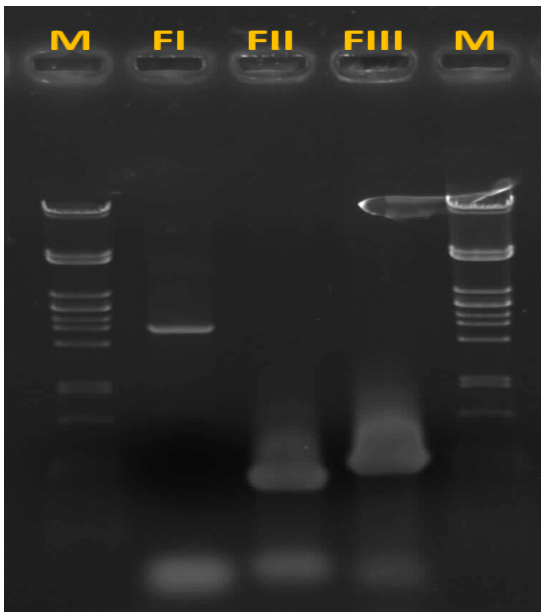

Figure 6. PCR and electrophoresis results for the intermediate regions with primers designed to detect whether the *lnuB* gene is located on the pEF418 plasmid. In the directory indicated by FI, 2000 bp region could be reproduced using ori-pEF418R and aadF primers. ˜+ 590 bp region was replicated using ori-pEF418R and pEF418F primers in the sequence indicated by FII. In the sequence indicated by FIII, ori-pEF418F and pEF418F primers were used and the region of 616 bp was increased

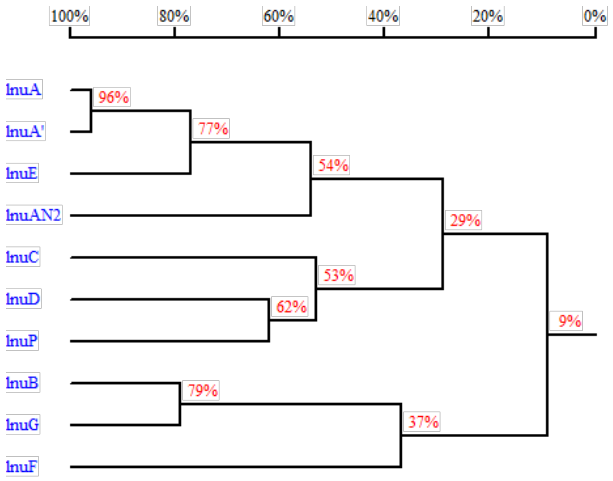

Figure 7. Homology between defined Lnu proteins. The tree was designed using DNAMAN software (Lynnon-Biosoft, Ontario, Canada).

1). Among all enterococci, inactivation resistance rate was 16 % and among *E. faecium* was 24%.

The presence of the lincosamide inactivation genes in 9 enterococcal strains was screened with specific primers *lnuA*, *lnuB*, *lnuC* and *lnuD*. In all enterococcus strains with positive Gots' test, *lnuB* gene was found to be positive and other investigated genes were negative. The *lnuB* positive *E. faecium* ADU1 strain was further studied in terms of phenotypic properties and localization of resistance gene (Figure 2).

MIC values were determined for 10 antibiotics in *E. faecium* ADU1 strain. The strain was resistant to erythromycin (> 128 µg/ml), clindamycin (> 128 µg/ml), ciprofloxacin (4 µg/ml), tetracycline (16 µg/ml) and ampicillin (32 µg/ml), while were susceptible to rifampicin (0.12 µg/ml), vancomycin (4 µg/ml), teicoplanin (2 µg/ml), chloramphenicol (8 µg/ml), and gentamicin (2 µg/ml).

Electrophoresis of total DNA extract showed presence of at least 3 plasmids in *E. faecium* ADU1 strain. One of these plasmids was approximately 30 kb and the other two plasmids were 7 kb and 2 kb. After conjugation with *E. faecalis* JH2-2 9 transconjugants were obtained and all transconjugants were positive for Gots' test for lincosamides. Also all transconjugants were positive for *lnuB* gene by PCR (Figure 4).

Genomic changes in 2 of 9 transconjugants were investigated by PFGE electrophoresis and no obvious band difference was found between transconjugants and recipient strain *E. faecalis* JH2-2. This result indicated a possible gene transfer by a conjugative plasmid which did not change restriction profile of *E. faecalis* JH2-2 genome (Figure 5).

In order to determine the genetic element on which the *lnuB* gene was located, inverse PCR was done. An amplicon of approximately 2500 bases was obtained by inverse PCR after restriction with HindIII and ligation, and no amplicon was obtained after restriction with EcoRI. The amplified fragment was sequenced and compared to GenBank. Accordingly, the genetic element on which the *lnuB* gene in *E. faecium* ADU1 was found to show sequence homology with the pEF418 plasmid (15890 bp) previously identified in *E. faecalis* (accession number: AF408195).

In order to determine whether the *lnuB* gene is located on the pEF418 plasmid, Ori-pEF418 primers were designed to amplify the origin of replication of the pEF418 plasmid. Furthermore, primers were designed to amplify aminoglycoside resistance gene (*aadE*) found in pEF418 (Table) (Garcia-Migura et al., 2007). Using these primer pairs, the presence of intermediate regions on the pEF418 plasmid sequence was investigated. Thus, a 2642 bp intermediate region between 849-3491 was amplified using the Ori-pEF418-R and the aadE-F primers. The 616 bp intermediate region between 103-719 was amplified using the Ori-pEF418-F and the pEF418-F primers. The 590bp intermediate region between 258-848 was amplified using the Ori-pEF418-R and the pEF418-F primers. The homology was in the region between 8354 and 9290 bp, and between 10287 and 11220 bp of pEF418 plasmid. When the plasmid sequence and these regions were overlapped using Clustal Omega (Multiple Sequence Aligment) pro-

gram, it was seen that putative ABC transporter and lincosamide nucleotidyltransferase (*lnuB*) genes were present in between the two sequenced region.

As a result, the *lnuB* gene was found to be located on a variant of pEF418 plasmid and this variant show sequence and size discrepancies with pEF418 plasmid. As shown in Figure 6, the region amplified using ori-pEF418R and aadE-F primers in the FI region should have 2640 bp, while approximately 2000 bp fragment was obtained. It was thought that there might be a deletion in the *orfX*, *orfY* and transposase genes that should be in the amplified region.

Discussion

Lincosamide resistance due to inactivation was first detected in *Staphylococcus haemolyticus* BM4610 and *Staphylococcus aureus* BM4611 isolated from humans. The widespread use of lincomycin for therapeutic purposes, especially in veterinary medicine, can be an important factor in the spread of resistance to lincosamides. The strain *E. faecium* ADU1 used in this study was an environmental isolate from Menderes River, Aydın, Turkey. To the best of our knowledge, resistance to lincosamides by inactivation has not previously been reported in environmental isolates. The presence of this phenotype in environmental samples is very important for show dissemination ability of resistance genes.

In this study, it was shown that 16% of environmental enterococci were resistant to lincosamides by inactivation which is higher than *lnuB* positivity among clinical *E. faecium* isolates [5]. This is the first report showing emergence of this type of resistance among environmental *E. faecium* isolates. All these isolates including *E. faecium* ADU1 carried *lnuB* gene which may indicate that this gene is common among environmental isolates.

In our study, it was seen that the inactivation gene *lnuB* could be transferred to *E. faecalis* JH2-2 strain by conjugation and the transferred lincosamide resistance was with other antibiotic resistance properties. In the later stages of the study, the *lnuB* gene was shown to be on the plasmid and carried with other resistance genes. These resistance genes have been shown to include aminoglycoside and macrolide resistance genes. The sequence study by the inverse PCR method showed that the *lnuB* gene was on the previously sequenced pEF418 plasmid. pEF418, a conjugation transferable plasmid, is thought to be important in the spread of resistance genes.

In our study, differences were observed in the sizes of the fragments obtained by amplifying different regions on the pEF418 plasmid. This showed that the *lnuB* gene was located on a plasmid homologous with the pEF418 plasmid, but was not the same. The *lnuB* gene has been shown to be located on many different conjugative plasmids when it was first identified and in later studies. In a study, the *lnuB* gene was shown on the pY13 plasmid containing the non-conjugative multiple resistance gene cluster in *E. faecium* isolate (Si et al., 2015). The size of the *E. faecium* pY13 plasmid is 28,489 base pairs and p11-27 (28,489 bp) (accession number: KT448818), pE15 (28,490 bp) (accession number: KT448821), p12-7 (28,491 bp) (accession number: KT448817), pD12 (28,491 bp) (accession number: KT448820) and p14-1 (28,492 bp) (accession number: KT448819) plasmids identified in *E. faecalis* were reported to have a very high sequence similarity. In addition, the pY13 plasmid has been reported to have high sequence similarity with the pY15 (15,464 bp) (accession number: KT448822) plasmid containing the *lnuB* gene in *E. gallinarum* isolate (Si et al., 2015). The *lnuB* gene, which provides resistance to lincosamides by inactivation, has been found in different Gram positive bacteria such as *E. faecalis, E. faecium, E. gallinarum, S. lutetiensis, S. aureus*. In addition, studies on the location of the *lnuB* gene in these bacteria have been found to be involved in different plasmids (Almuzara et al., 2013; Lozano et al., 2012).

In the first study that identified *lnuA* gene, it was shown the lnuA gene was carried on the pIP855 plasmid of *Staphylococcus haemolyticus* (Brisson-Noel et al., 1986). Later, the presence of *lnuA* gene has been reported in other bacteria including *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus simulans* (Rosander et al., 2008; Jalilsood et al., 2014; Lüthje et al., 2007). The *lnuA'* gene was first identified in 1988 in *S. aureus* strain (Brisson-Noel et al., 1988). The *lnuC* gene was first characterized in lincomycin resistant *Streptococcus agalactiae* UCN36, and was also detected later in different strains such as *Haemophilus parasuis* (Achard et al., 2005; Chen et al., 2010). The *lnuD* gene was found in moderately lincomycin resistant *S. uberis* UCN42 which was isolated from bovine mastitis (Petinaki et al., 2008). The *lnuE* gene was first found in the *S. suis* strain and was shown to be transmitted by the ISEnfa5-cfr-ISEnfa5 gene segment insertion (Zhao et al., 2014). The *lnuF* gene was found in clinical *E. coli* X1D61 strain, a species which is naturally resistant to lincosamides, and was shown to be transferred by class 1 integron gene cassette (Heir et al., 2004). The *lnuF* gene was also found in a *Salmonella enterica* strain when the genome sequence was made (Accession no: NZ_CP037959.1). The *lnuP* gene was identified in the *Clostridium perfringens* isolate and was shown to be located on pJIR2774, the first conjugative R-plasmid known in these strains (Lyras et al., 2009). In recent years, the *lnuG* gene was reported to be transported by the mobile genetic element Tn6260 located on the *E. faecalis* E531 strain (Heir et al., 2004). The *lnuAN2* gene was found in the genome sequence (Accession no: NZ_JH992940.1) of *Bacteroides oleiciplenus* bacteria.

Responsible for lincosamides resistance by enzymatic inactivation, the genes *lnuA*, *lnuA'*, *lnuAN2*, *lnuB*, *lnuC*, *lnuD*, *lnuE*, *lnuF*, *lnuP* and *lnuG* were identified and carried in different mobile genetic elements in different bacterial groups. Protein sequence homologies of all these identified *lnu* genes were shown in Figure 7. According to the amino acid sequence homologies, possible to divide lincosamide inactivation genes into two main groups as LnuA and LnuB groups: LnuB, LnuG and LnuF are in the LnuB group; there are other inactivation enzymes LnuA, LnuA', LnuAN2, LnuC, LnuD, LnuE and, LnuP and are in the LnuA group. The homology ratio of the LnuA and LnuB proteins, which are the representatives of these groups, in the amino acid sequences is 12% and is quite low.

The presence of lincosamide inactivation genes on mobile elements increases their transferability and spread. The *E. faecium* ADU1 strain expressing LnuB is an environmental isolate. Due to the presence of antibiotic residues in the environment and the widespread use of lincosamides in the treatment of animals, may be the reason for the increased number and variety of bacteria carrying *lnuB*. Mobile and conjugative genetic elements play a major role in the spread of resistance to antibiotics, which are among the top 10 health problems reported by the World Health Organization. Measures to reduce the accumulation of antibiotics in the environment should be taken which will reduce the antimicrobial pressure on environmental bacteria that may decrease spread of antimicrobial resistance.

All samples were taken from environment, no animal or human specimen was used. That's why no application was done for Ethics Committee decision.

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