Prevalence and molecular analysis of linezolid-resistant enterococci in clinical specimens in a tertiary care hospital.

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Contents

FIGURES

TABLES

ABSTRACT

Prevalence and molecular analysis of linezolid-resistant enterococci in clinical specimens in a tertiary care hospital.

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Objectives: *Enterococcus* species are the main opportunistic infectious bacteria showing intrinsic or acquired resistance to various antimicrobial agents. Linezolid has been playing an important role in severe infections caused by enterococci due to the spread of vancomycin resistance since the 1990s, but further attention is needed because linezolid-resistant enterococci are in epidemic in recent years. In this thesis, we tried to analyze the prevalence and resistance mechanisms of linezolid-resistant enterococci isolated from a domestic tertiary care hospital.

Methods: Non-duplicated *Enterococcus faecalis* (n=47) and *E. faecium* (n=205) isolated from clinical specimens in a domestic tertiary care hospital in 2019-2020 were collected. Antimicrobial susceptibility was tested with the VITEK® 2 system, and the linezolid minimum inhibitory concentration

was tested by agar dilution method and analyzed according to the Clinical and Laboratory Standards Institute standards. The mechanism of linezolid resistance was analyzed using multiplex-PCR and whole genome-sequencing.

Results: 25.53% (n=12/47) *E. faecalis* and 2.44% (n=5/205) *E. faecium* were resistant to linezolid. Compared to linezolid-susceptible *E. faecalis,* linezolid-resistant *E. faecalis* were 30-40% more likely to show resistance to other antimicrobials. The proportion of linezolid non-susceptible *E. faecalis* strains (29.8%) was higher than that of *E. faecium* (2.4%), but linezolid non-*susceptible E. faecium* had overall higher MICs. 75.0% (15/20) of the LNSE isolates presented a single resistance mechanism while 4 LNSE co-harbored *poxtA* and *cfr(D)* genes. The linezolid resistance determinants were connected with their susceptibility: the *optrA* gene was responsible for a MIC of 8μg/ml; *cfr(D)* and *poxtA* gene complex mainly resulted in a linezolid-intermediate phenotype; G2576T mutation caused a most significant raise in linezolid MIC. Isolates with similar genetic information emerged in groups.

Conclusion: In this study, the linezolid resistance rate was higher than that of previous reports, and resistance mechanisms were becoming diversified, indicating that constant surveillance for linezolid resistance is needed.

Keywords: enterococci, linezolid resistance, *optrA*, *poxtA*, whole genome-sequencing

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1. INTRODUCTION

Enterococcus species have been recognized as one of the major pathogens for hospital-acquired infections for decades, with *Enterococcus faecalis* and *Enterococcus faecium* being the most frequent enterococcal species isolated from human clinical specimens. They most commonly infect the urinary tract, bloodstream, endocardium, surgical site wounds and implants. Data from Korean nosocomial infections surveillance system [1] has shown that *Staphylococcus aureus* has been the most common cause of central line-associated blood stream infection (CLABSI) over the years, while *E. faecium* steadily contribute to about 10% of the cases. In catheter-associated urinary tract infection (CAUTI), *E. faecium* and *E. faecalis* both have a place in the list of the pathogens.

Although they have great clinical significance, resistance to several commonly used antimicrobial agents is a remarkable characteristic of these 2 species. They

are intrinsically resistant to a broad range of antibiotics such as low-level aminoglycosides, β-lactams, and lincosamides. In addition, they can also acquire plasmids and other genetic elements that harbor antimicrobial-resistant genes [2], making them well known as intractable multi-drug resistant pathogens. Especially in immunocompromised patients, multi-drug resistant enterococci are the rising causes of death [3]. Linezolid, the first oxazolidinone antibacterial agent approved for commercial use by the US Food and Drug Administration (FDA) in 2000, has been served as a useful therapeutic option for various gram-positive infections, particularly in the intensive care units (ICUs) [4].

Recently, the increase in vancomycin-resistant enterococci (VRE) leaves it a poorer prognosis and fewer available choices for treatment [5]. Linezolid, the first oxazolidinone antibacterial agent approved for commercial use by the US Food and Drug Administration (FDA) in 2000, has been showing great therapeutic potential in vancomycin-resistant cases [6] and serving as the last-resort therapeutic option for various gram-positive infections.

However, linezolid-resistant enterococci (LRE) have continuously been reported over the years [7]. Linezolid resistance is reported to be attributed to mutations in the 23S rRNA binding site (e.g., G2576T, G2447U, and G2504A) [8]. Alterations in ribosomal proteins L3 and/or L4 are also responsible for decreased linezolid susceptibility [9]. Besides, the acquisition and horizontal transfer of plasmid-mediated resistance genes (e.g., *cfr*-like, *optrA*, and *poxtA* genes) were also described as mechanisms leading to linezolid resistance.

The *cfr* gene encodes a 23S rRNA methyltransferase and confers cross-resistance to oxazolidinones, phenicols, lincosamides, pleuromutilins, and streptogramin A antibiotics ($PhLOPS_A$ phenotype) [10], [11]. Till now, several members of *cfr*-like

genes have been found: *cfr(B)*, *cfr(C)*, *cfr(D)*, and *cfr(E)*, sharing a certain degree of similarity in their genetic sequences. The *optrA* gene, encoding an F lineage of the ATP-binding cassette (ABC) protein superfamily, causes resistance to oxazolidinones and phenicols by ribosomal protection. It was initially discovered in China from animal and human isolates [12] and then widely reported in the American continent [13] and Europe [14], [15]. Recently, a novel gene *poxtA*, sharing a 32% identity with the *optrA* gene, was identified in a clinical MRSA strain in Italy [16] and also reported in *Enterococcus* species worldwide [17]–[19], including a 10-year survey in Korea [20]. The *poxtA* gene, sharing a similar working mechanism to the *optrA* gene, encodes a ribosome protecting protein, thus confers a decreased susceptibility to phenicols, oxazolidinones, and tetracyclines [16].

The resistance genes are commonly detected worldwide, but are still rarely reported in Korea [21]–[23]. However, there's still an urgent need to investigate the mechanisms and prevalence of them due to the continuous clinical use of linezolid and the possibility of susceptibility/intermediate turning into resistance. This study focused on the prevalence of clinical linezolid-non-susceptible *Enterococcus* (LNSE) and corresponding mechanisms in Severance hospital in Seoul, South Korea, and characterized the genetic information of the resistance determinants.

2. MATERIALS AND METHODS

1.*Clinical strain collection and species identification*

All the non-duplicated strains initially identified as *E. faecalis* and *E. faecium* from clinical samples were collected from April 2019 to July 2020 in Severance hospital.

Retrospectively, the isolates were recovered from the stock, striped to blood agar plates (BAP), and then cultured under 35℃, ambient air for 24h. Pure bacterial colonies were obtained after another overnight subculture. Single colony was picked and identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) instrument microflex® LRF. Only those that have been reconfirmed as *E. faecalis* or *E. faecium* were selected as research subjects.

2. *Antimicrobial susceptibility profile*

Antimicrobial susceptibility to commonly used antibiotics linezolid, ampicillin, ciprofloxacin, erythromycin, high-level gentamicin, penicillin G, teicoplanin, tetracycline, tigecycline, and vancomycin was screened by VITEK® 2 Microbial identification system. 3 mL of sterile saline was added into a clear polystyrene 12×75 mm test tube. The homogenous organism suspension was prepared by transferring several isolated colonies from the plates to the distilled water (DW) tube using sterile cotton swabs. The inoculum concentration was adjusted to 0.5 McFarland standard and then combined with VITEK 2 Compact Identification GP cards. Place the cassette in the Filler box press the start button. The results were then displayed on the computer connected to the instrument.

3. *Agar dilution*

The distribution of linezolid MICs was reconfirmed by agar dilution. *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were served as QC strains. A series of Mueller-Hinton agar (MHA) plates containing the antimicrobial agent to be tested in increasing concentrations (0.5-32mg/L) were prepared. Colonies were picked by sterile cotton swabs and then suspended to the DW. Turbidity was adjusted to

0.5 McFarland standard. After inoculating, the plates were incubated under 35°C, ambient air for 16h. MIC was interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria except for tigecycline, which was not included in the CLSI guideline and therefore interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline.

4. *Multiplex-PCR*

Target DNA was extracted from bacterial cell by boiling. Amplification of the optrA gene and the poxtA gene was performed as previously described [24]. The optrA gene was screened with primers 5′-TACTTGATGAACCTACTAACCA and 5′-CCTTGAACTACTGATTCTCGG, producing a 422bp amplicon. The poxtA gene was screened with primers 5′-AAAGCTACCCATAAAATATC-3′ and 5′-TCATCAAGCTGTTCGAGTTC-3′, producing a 533bp amplicon. The forward and reverse primers of each gene are mixed in a ratio of 1:1 to reach a final concentration of 20pmol. 1μl mixed primer solution of each gene and 1μl target DNA was added to AccuPower® Pfu PCR PreMix. Then add DW to make the final volume 20μl. PCR cycle conditions were: initial denaturation 96°C 2min, followed by 30 cycles of 96°C 30s, annealing 50°C for 30s, extension 72°C 30s, and final extension 72°C 5min. The amplification products were further confirmed by agarose electrophoresis.

5. *Whole-genome sequencing and bioinformatics analysis*

Bacterial DNA was extracted using GenElute™ Bacterial Genomic DNA Kits. The steps of the experiment are consistent with the official protocol, with several modifications: 1, Instead of harvesting the cells from broth media, take 1 loop bacterial colonies from BAP and wash the cells with PBS buffer. Then spin under

13000rpm for 5min once; 2, Pipetting rather than vortexing in step five; 3, Add 70µl DW rather than 200µl Elution Solution. The sequencing library was prepared using Twist Library Preparation EF Kit according to protocol "Enzymatic Fragmentation and Twist Universal Adapter System". Sequencing was performed on the Illumina NovaSeq 6000. MLST was identified by pubMLST (https://pubmlst.org/). Relative resistance gene was analyzed using ResFinder 4.1 (http://cge.cbs.dtu.dk/services/ResFinder) [25]. 23S rRNA mutation was identified by LRE-finder 1.0 (https://cge.cbs.dtu.dk/services/LRE-finder) [26]. Genome annotation was performed by RAST 2.0 (https://rast.nmpdr.org/rast.cgi) [27] and visualized by Easyfig (https://mjsull.github.io/Easyfig/) [28]. The location of each resistance gene was determined by mlPlasmids [29].

3. RESULTS AND DISCUSSION

1. *Antimicrobial susceptibility test*

A total of 252 non-duplicated isolates identified as *E. faecalis* (47/18.6%) and *E. faecium* (205/81.4%) were recovered for further experiments (Table 1). E. faecalis showed a high resistance rate to tetracycline (70.21%). On the contrary, low resistance was found in vancomycin (2.13%), teicoplanin (2.13%), and tigecycline (0.0%). In *E. faecium*, resistance to penicillins, erythromycin, and ciprofloxacin was considerably high, reaching above 97%. Compared to linezolid-susceptible *E. faecalis,* linezolid non-susceptible *E. faecalis* were 30-40% more likely to show resistance to other antimicrobials. *E. faecium* showed an overall distinct higher resistance rate to glycopeptides, with vancomycin-resistant *E. faecium* accounting for 82.93% of the population. The resistance pattern of *E. faecalis* in this study was similar to a poultry-related study in Korea [30], only with a slight increase, but we

had a much higher resistance rate in penicillins, vancomycin, erythromycin, tetracycline, and ciprofloxacin in *E. faecium*.

Table1. Resistance rate of enterococci with different linezolid susceptibility

Antimicrobials	*E. faecalis*			*E. faecium*		
	LZD-S (n=32)	LZD-NS (n=15)	Total (n=47)	LZD-S (n=200)	LZD-R (n=5)	Total (n=205)
Ampicillin	3 (9.37)	0	3(6.38)	198 (99.00)	5 (100)	203(99.02)
Penicillin G	8 (25.00)	0	8(17.02)	198 (99.00)	5 (100)	203(99.02)
HL-Gentamicin	11 (34.37)	10 (66.67)	21(44.68)	102 (51.00)	2 (40.00)	104(50.73)
Erythromycin	14 (43.75)	12 (80.00)	26(55.32)	195 (97.50)	5 (100)	200(97.56)
Ciprofloxacin	9 (28.12)	10 (66.67)	19(40.43)	199 (99.50)	5 (100)	204(99.51)
Tetracycline	19 (59.37)	14 (91.67)	33(70.21)	30 (15.00)	3 (60.00)	33(16.10)
Tigecycline*	0	0	0	1 (0.50)	0	1(0.49)
Vancomycin	1 (3.12)	0	1(2.13)	166 (83.00)	3 (60.00)	169(82.44)
Teicoplanin	1 (3.12)	0	1(2.13)	78 (39.00)	3 (60.00)	81(39.51)

*Interpreted according to EUCAST guideline.

LZD-S = linezolid-susceptible; LZD-NS = linezolid-non-susceptible; LZD-R = linezolid-resistant.

The distribution of linezolid MIC was summarized in table 2. No strain was inhibited below 1 μg/ml, and no *E. faecalis* isolate had a MIC value over 8 μg/ml. The proportion of linezolid non-susceptible *E. faecalis* strains (29.8%) was higher than that of *E. faecium* (2.4%), but linezolid non-susceptible *E. faecium* tend to have higher MICs.

Table 2. Linezolid MIC and resistance mechanisms of LNSE

MIC (μg/ml)	0.5	1	2	4	8	16	32
***E. faecalis* (n=47)**	0	2 (4.25)	30 (63.83)	3 (6.38)	12 (25.53)	0	0
optrA (n=12, 25.53%)					12 (100)		
poxtA +cfr(D) (n=3, 6.38%)				3 (100)			
***E. faecium* (n=205)**	0	10 (4.88)	190 (92.68)	0	3 (1.46)	1 (0.49)	1 (0.49)
optrA * (n=1, 0.49%)					1 (100)		
cfr(D) * (n=1)					1 (100)		
poxtA +cfr(D) (n=1)					1 (100)		
G2576T (n=1)							1 (100)
unknown mechanism (n=1)						1 (100)	
Total (n=252)	0	12 (4.76)	220 (87.30)	3 (1.19)	15 (5.95)	1 (0.40)	1 (0.40)
optrA (n=13, 5.16%)					13 (100)		
poxtA +cfr(D) (n=4, 1.59%)				3 (75.00)	1 (25.00)		
cfr(D) * (n=1, 0.40%)					1 (100)		
G2576T (n=1)							1 (100)
unknown mechanism (n=1)						1 (100)	

* resistance gene located on the chromosome.

2. *Resistance determinants identification*

All 252 strains were screened for *optrA* and *poxtA* genes by multiplex-PCR. All the LNSE with or without a positive result in PCR were subjected to whole-genome sequencing. The results of PCR and WGS were highly consistent. Results were given in table 2. Generally speaking, 75.0% (15/20) of the LNSE isolates presented single resistance mechanism: 86.7% (13/15) were *optrA*-positive, 6.7% (1/15) had a G2576T mutation, and 1 *E. faecium* isolate carried a *cfr(D)* gene on its chromosome. 4 of the LNSE carried 2 resistance determinants at the same time, and the genes that both of them carried were *poxtA* and *cfr(D)*. Till now, there has been no *cfr*-carrying clinical enterococcal isolates reported in Korea, but one study revealed the *cfr* gene's existence in 2 chicken meat origin *E. faecalis* isolates [31], indicating the possibility of foodborne transmission. Outside of South Korea, co-location of *optrA* and *cfr* has been reported in human *E. faecium* isolates in Ireland [32] and Italy [33], providing an evidence of their prevalence worldwide.

To be more specific, the *optrA* gene was responsible for a MIC of 8μg/ml in both species, while 3 of 4 isolates that co-harbored *cfr(D)* and *poxtA* gene only caused a linezolid-intermediate phenotype. G2576T mutation was only detected in *E. faecium*, and this mechanism resulted in a most significant raise in linezolid MIC. The strong effect of G2576T mutation in improving MIC value has also been reported by Cho et al. [22]: Two clinical linezolid-resist isolates that had sore G2576T mutation in 23S rRNA exhibited a linezolid MIC over 64 mg/L. Although various resistance mechanisms were found in *E. faecium*, the last 1 LNSE isolate (EFM 118) remained unexplainable.

3. *Genetic context of different resistance determinants*

Resistance gene-harbored contigs were obtained from whole-genome sequencing and aligned by reference *optrA* gene (NCBI Reference Sequence: NG_048023.1), *poxtA* gene (NCBI Reference Sequence: NG_063824.1), and *cfr(D)* gene (NCBI Reference Sequence: NG_067192.1). The contigs were divided into several groups according to their features.

All the *poxtA*-positive isolates co-harbored a *cfr(D)* gene (Figure 1a). The resistance gene-containing regions of EFM 130, EFA 39, EFA 25, EFA 36 were highly identical. The *poxtA* gene is the same sequence as the one that was first discovered in an Italian clinical *S. aureus* isolate [16], but the upstream and downstream components of the gene were very different. IS1216 in *S. aureus* AOUC-0915 was hypothetically responsible for the translocation of *poxtA*-containing segment while no typical insertion sequence was found in our study. Another *cfr(D)*-harboring contig was from EFM 86. The *cfr(D)* gene was the same as those in other isolates. A Guanine Monophosphate (GMP) synthase gene was located right downstream of the *cfr(D)* gene (Figure 1b). Although a chicken-meat

related research has revealed the existence of the *cfr* gene in Korea [31], the clinical isolates that co-harbor a *cfr* gene and a *poxtA* gene was first described in this study.

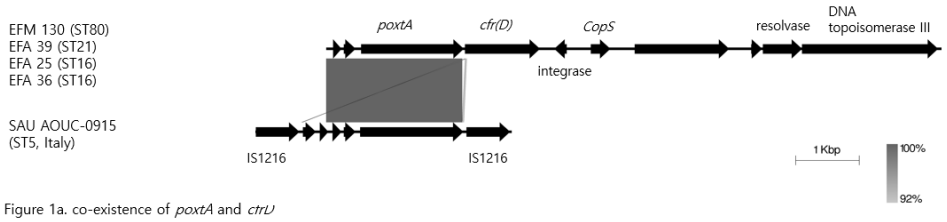

Figure 1a. co-existence of *poxtA* and *ctrU*

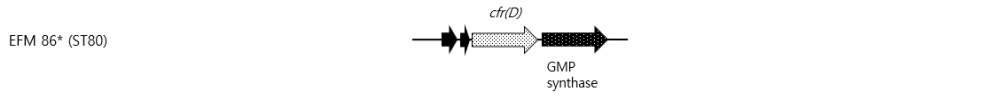

Figure 1b. a *cfrD* harboring strain

Two *E. faecalis* plasmid segments and one *E. faecium* chromosomal region had a ferredoxin-coding gene downstream of the *optrA* gene (Figure 2). In a surveillance study, a human origin *E. faecalis* isolate EFA 838523 from Malaysia also shared a similar genetic information with them [34]. A *fexA* gene, which mediates resistance to chloramphenicol and florfenicol was located 742bp upstream the *optrA* gene and a 23S rRNA(adenine(2058)-N(6))-dimethyltransferase gene *ermA* was detected 1417bp away.

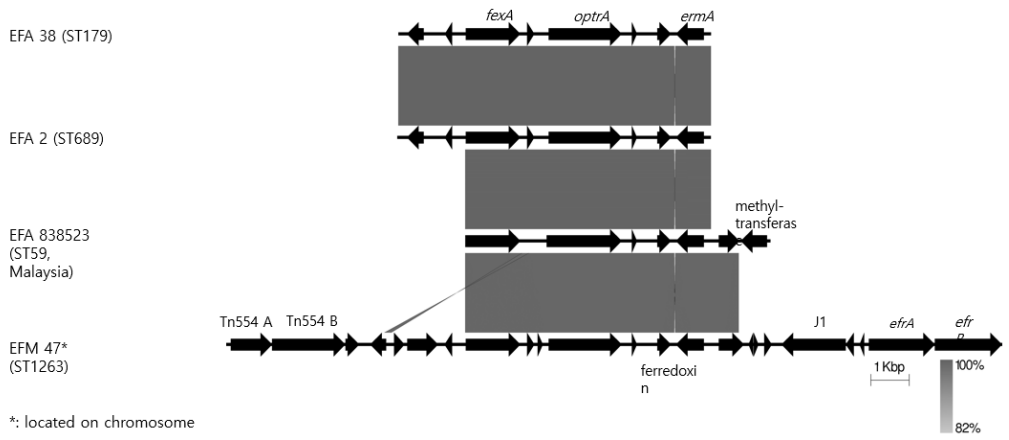

Figure 2. *optrA* with a ferredoxin-coding gene downstream.

E. faecalis EFA 11 carried a *impB* gene. Similar genetic components were also found in a Korean foodborne *E. faecalis* isolate [35] and a Chinese human-origin *E. faecalis* isolate [36] (Figure 3). In the *impB* to *optrA* region, the similarity of the 3 isolates reached 100%, suggesting the spread of resistance determinants between countries and a possibility of foodborne transmission.

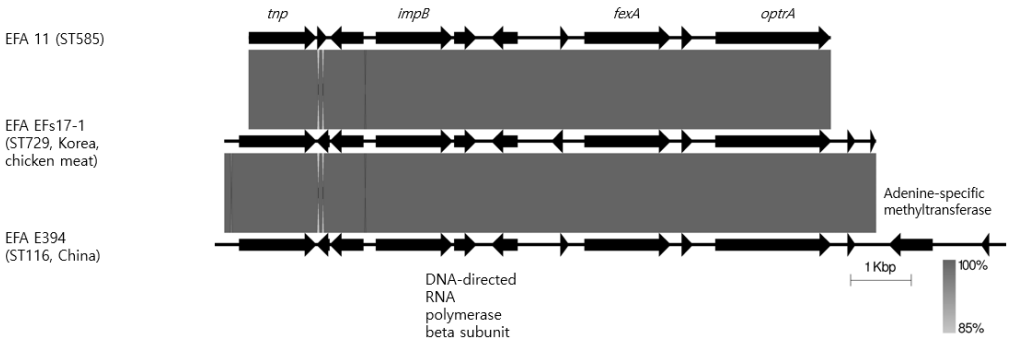

Figure 3. *optrA* with *impB* gene upstream.

Other nine *optrA*-carrying *E. faecalis* shared more than 69 percent of the same genetic environment with each other (Figure 4). The contigs were started with Tn554 components, followed by *fexA*, *optrA*, ribonuclease J1 gene, and two heterodimeric efflux ABC transporter gene *efrA* and *efrB*. Slightly different from other isolates, EFA 14 and EFA 5 both had an around 900bp insertion between the Tn554 and the *fexA* gene.

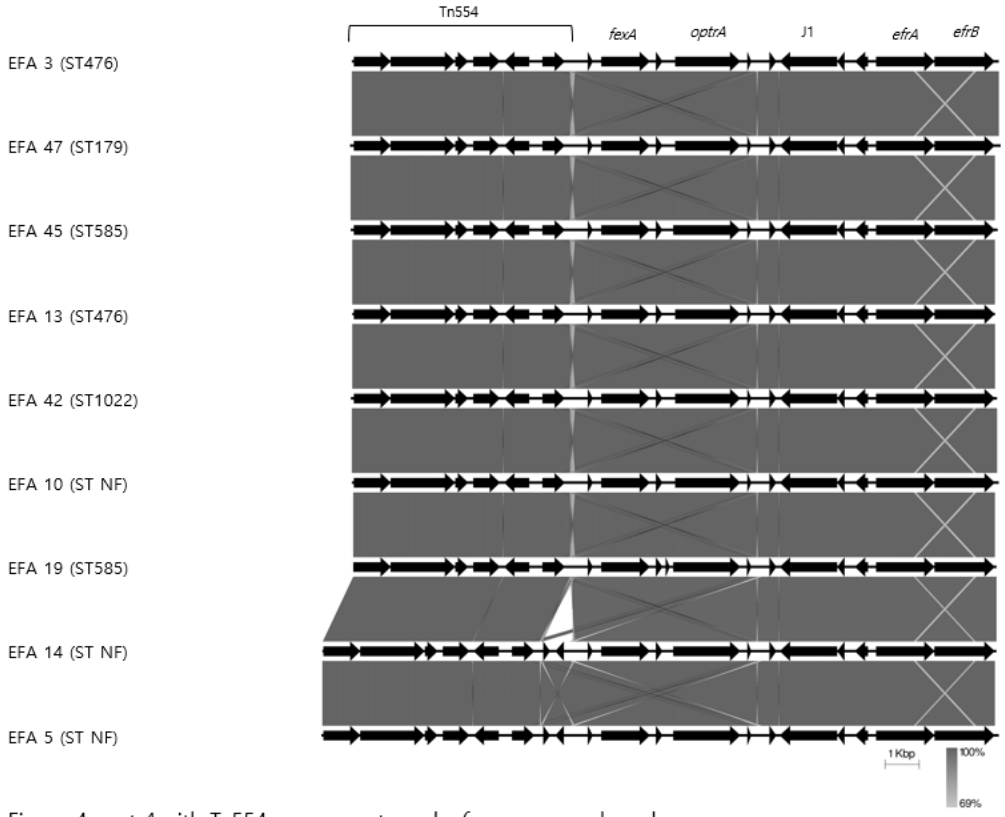

Figure 4. *optrA* with Tn554 components and *efr* gene on each end.

To have a detailed inspect at Tn554 and Tn554 containing gene fragments, EFA 3 and EFM 47 were compared with a reference Tn554 sequence (GenBank accession no. X03216.1) (Figure 5). Original Tn554 has six open reading frames, being

composed of three transposase genes *tnpA*, *tnpB*, and *tnpC*, the spectinomycin-resistance gene *spc*, the erythromycin resistance methylase encoding gene *ermA*, and an S-adenosylmethionine(SAM)-dependent methyltransferase gene *met* [36], [37]. EFM 47 had the classical Tn554 structure, but they were not very similar in sequence. One possible explanation is that the resistance gene-containing fragment was obtained in the past few generations, and such a result was produced after continuous gene rearrangement.

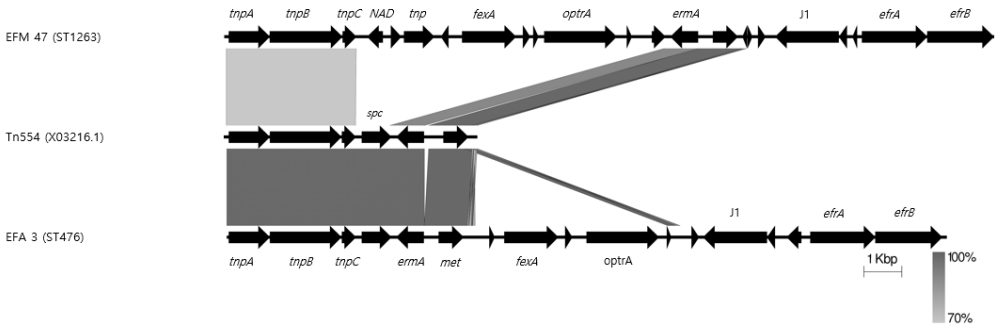

Figure 5. Tn554 and Tn554 containing gene fragments.

Although previous review has summarized that, before 2012, the main oxazolidinone resistance mechanism in both *E. faecalis* and *E. faecium* was G2576T mutation in 23S rRNA [8], a recent study shows that *optrA* has now gradually become the dominant sole mechanism from 2014 to 2016 ([34]), and this is consistent with our results.

4. CONCLUSION

To the best of our knowledge, the *cfr* gene was once described in a chicken-meat related research in Korea, so this study is the first discovery of the *cfr* gene co-harboring a *poxtA* gene upstream in Korean clinical isolates, as well as the first systematic investigation to interpret the molecular genomics of linezolid-resistance

determinants. What's more, the similarities of genetic context between Korean clinical isolates and isolates from other countries/resources indicate the spread of resistance determinants between countries and a possibility of foodborne transmission. A constant surveillance for LNSE monitoring is needed.

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ABSTRACT IN KOREAN

국내 3 차 진료 병원의 임상 검체에서 linezolid 내성 장구균의 유병률 및 분자 분석.

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목적: 장알균은 다양한 항균제에 자연 또는 획득 내성을 보이는 주요 기회 감염균이다. 1990 년대부터 확산된 반코마이신 내성으로 장알균에 의한 중증 감염에서 리네졸리드는 중요한 역할을 하고 있으나, 최근 리네졸리드에 내성인 장알균이 보고되고 있어 주의가 필요하다. 본 논문에서는 국내 1 개 3 차 의료기관에서 분리된 리네졸리드 내성 장알균의 특성과 내성 기전을 분석하였다.

방법: 2019-2020 년에 국내 1 개 3 차 의료기관에 내원한 환자의 임상 검체에서 분리된 *Enterococcus faecalis* (n=47) 및 *E. faecium* (n=205)을 중복 없이 일련 균주로 수집하였다. 항균제 감수성은 VITEK® 2 시스템으로 시험하였고, 리네졸리드 최소억제농도 (Minimum inhibitory concentration)는 한천희석법으로 시험하고 Clinical and Laboratory Standards Institute 기준에 따라 해석하였다. 리네졸리드 내성 기전은 Multiplex-PCR 및 whole genome-sequencing 을 사용하여 분석하였다.

결과: *E. faecalis* 및 *E. faecium* 의 리네졸리드 내성률은 각각 25.5% (n=12/47) 및 2.4% (n=5/205)로 *E. faecalis* 의 내성률이 높았다. 리네졸리드 내성균주는 감수성 균주에 비해

다제내성균주 비율이 30-40% 높았다. 리네졸리드에 비감수성인 20주의 내성 기전을 분석한 결과, 15주 (75%)는 1개의 내성 유전자 또는 변이를 가지고 있었으며, 4주 (20%)는 *poxtA* 및 *cfr(D)* 유전자를 동시에 가지고 있었고, 1 주에서는 기존에 알려진 리네졸리드 내성 기전을 발견하지 못하였다. 균주별로 보유한 리네졸리드 내성 유전자에 따라서 MIC 분포가 달랐으며, optrA 유전자를 가진 균주의 리네졸리드 MIC 는 모두 8㎍/ml 이었고, *poxtA* 와 *cfr(D)* 유전자를 가진 균주는 모두 linezolid 에 중등도 내성이었으며, G2576T 변이를 가진 균주들이 가장 높은 리네졸리드 MIC 분포를 보였다.

결론: 본 연구에서 리네졸리드 내성률은 기존 보고에 비해 높았으며, 내성 기전도 다양해짐을 보여주고 있어 리네졸리드 내성에 대한 감시와 주의가 필요하다.

핵심되는 말: enterococci, linezolid resistance, optrA, poxtA, whole genome-sequencing