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ORIGINAL ARTICLE

Emergence of *vanA* and Sequencing in Vancomycin resistance *Enterococcus faecalis* Isolated from Urinary Tract Infections

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ABSTRACT

Background*: E. faecalis is a common microbe causing urinary tract infections in Najaf city, Iraq, having resistance to many antibiotics.* ***Objectives****: The present research aimed to identify vanA gene presence and sequence in E. faecalis for detection of vancomycin resistance.* ***Methodology:*** *From 140 specimens collected from UTI patients, we identefied 21 Enterococcus isolates using bacteriological tests, among them 15 (71.4%) were E. faecalis detected by using Vitek-2 system and molecular diagnosis using ddl gene of E. faecalis. The remainder isolates belong to other Enterococcus species.* ***Results****: In this study, total isolates were vancomycin resistant VRE using MIC technique. Molecular studies detected 12 (80%) of E. faecalis isolates which carry vanA gene. Also, the study showed that the reference isolate has three mutations and an identity 97-100% among vanA of the strains that include a number of Gram positive bacteria such as Staphylococcus aureus and Clostridium difficile, and other species of Enterococci.* ***Conclusion****: The incidence of vanA gene has developed great challenges, indicates that E. faecalis were reservoirs of vanA gene which may be transmitted to other bacteria especially Staphylococcus aureus and Clostridium difficile. It is significant for continued checking the spread of VRE and effective approaches that control and limit antibiotic resistance distribution.*

INTRODUCTION

Enterococcus faecalis was naturally present as normal flora in the digestive tract of numerous animals and humans [1]. The host range has enabled them to stay live through a variety of innate defenses of the host [2]. *E. faecalis* inhabits the female genital tract, the intestine, and the oral cavity [3]. *E. faecalis* able to cause biofilm associated opportunistic infections [4,5]. Similarly, quorum sensing besides bacterial enzymes save bacteria in harsh environments [6]. The antibiotics misusing and extensive using affect the resistant strains rate [7]. *E. faecalis* accumulates in significant numbers when treated with many antibiotics. As well as coexisting in kindly relationships with other antibiotics resistant bacteria, which enhances the likelihood of interaction with them leading to acquisition of different resistances that harbor on mobile elements [2].

Vancomycin (VA) is a glycopeptide that inhibits synthesis of the cell wall [8]. The *vanA* gene carried by Tn-1546 transposon, this gene might transpose among plasmids in same or diverse bacterial strains by conjugation [9].

The *vanA* was one of the first category of *van* genes, which encodes the D-Alanyl-D-Lactate ligase. However, a second group encode for D-Alanyl-D-Serine ligase, which reduce affinity to vancomycin about 1000 and 7 times, respectively [10]. The treatment value was regulated through antibiotic nature and plan for treatment [11]. Also, supportive treatment might involve along with the treatment for other secondary infections [12,13]. Controlling VRE dissemination needs detection, instruction about VRE, and control processes for careful vancomycin use, and to avoid VRE spread from person-to-person. The aim of our study is to identify *vanA* gene presence and sequence in *E. faecalis* for detection of vancomycin resistance.

METHODOLOGY

Specimens Collection

140 urine samples have been collected from UTI patients inside sterile containers, then centrifuged for 2 min, and inoculate the sediment into brain heart-infusion broth then incubate for 24 h at 37˚C, then streaked on MacConkey agar, blood agar, and CHromagar™orientation. Then incubation for 24 h at 37˚C for detection of *Enterococcus faecalis* colonies. The Vitek-2 compact system is used to identify *E. faecalis*, including Gram-positive-ID cards, with 64 biochemical tests. Molecular detection by *ddl of E. faecalis* was done.

Agar Dilution Test for Diagnosis of Vancomycin-Resistant *Enterococcus*

The isolates were inoculated in brain heart-infusion agar (BHI) supplemented with a 6 mg/l vancomycin antibiotic depending on CLSI-2023[14] which identify the

lowest concentration of antibiotic that prevent bacterial growth is considered the MIC[11]. All isolates were compared to concentration with the 0.5 McFarland standard and examined for their growth in the medium by adding 10 µl from tested isolates like spots to surface media. Then, totally inoculated petri-dishes should be incubated inside sterile conditions for 24 hours at 37 °C in ambient air. The positive result is absorbed by one to many colonies of bacteria.

Molecular Detection

DNA extraction was done from overnight culture by taking 5 mL from each isolate. The DNA concentration and purity were estimated by a nanodrop spectrophotometer from Nabi-Korea. The DNA purity was 1.4-1.8, and the concentration was 70-120 ng/µL. The reaction comprises 25 µl volume, which contains 5 ml from DNA template, 12.5 from MasterMix 2X (Promega, USA), also contains 2.5 µl forward and 2.5 µl reverse primers shown in Table1, and the volume was complete by nuclease-free water, then placed in the thermal cycler and programmed conditions early with initial denaturation at 94°C for 5 min, subsequently repeated 30 cycles including denaturation at 94°C for 1 min, annealing at 54°C for 1 min, in addition to extension at 72°C for 1 minute, and lastly extension for 10 min at 72°C.

Table 1: Primers have been used in this study

Genes name	Primer sequence (5´- 3´)	Size of PCR product bp	Reference
ddl E.faecalis	F:+ ATCAAGTACAGTTAGTCT R: - ACGATTCAAAGCTAACTG	941	DUTKA-MALEN *et. al.*, [15]
vanA	F:+GGGAAAACGACAATTGC R: -GTACAATGCGGCCGTTA	732	

Consequently, electrophoresis using gel 1.5% agarose and staining with the ethidium bromide aimed at separating PCR products, later imagined by a gel-documentation system. The control positive and control negative (deficient DNA template) were used. The amplicon size can be marked by a molecular-weight marker known as a ladder.

Gene Sequencing

Gene sequencing was performed on PCR products of *vanA* gene, by sending reference isolate for sequencing in macrogen company within South Korea, using Sanger's sequencing technology by the ABI-genetic analyzer. FASTA sequences aimed at PCR products were matched alongside a previous discovered gene from the (National-Center for Biotechnology-Information) NCBI database using BLAST program.

Phylogenetic Tree Analysis

Neighbour Joining considers a method used for estimating the evolution history through revealing phylogenetic distances organized using MEGA11 program.

RESULTS

Identification of *E. faecalis* Isolates

From a total of 140 urine samples, 21 *Enterococcus* species were identified depending on the culture characteristics of the colonies; including pink color (lactose fermenting) on MacConkey, with blue turquoise colonies on the CHromagar™orientation and black color development in bile-esculin agar (Figure 1).

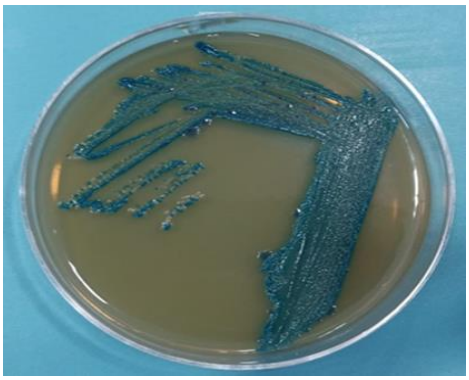

Fig 1: Growth of *Enterococcus* isolates on CHROMagar™ Orientation.

Among them, 15 (71.4%) were *E. faecalis* with specific primer *ddl for E. faecalis* (Figure 2). Besides, the Vitek-2 compact system was used to identify *E. faecalis*, including Gram-positive-ID cards, with 64 biochemical tests.

Detection of Vancomycin Resistance Enterococci Phenotypically and Genotypically

The investigation of the vancomycin susceptibility among *Enterococcus* isolates employing the MIC technique yielded the result that total isolates 15 (100%) were resistant to vancomycin VRE.

Among 15 isolates of *Enterococcus faecalis*, we detected *vanA* gene in 12 (80%) of them as shown in (Figure 3).

Also, Sanger sequence investigation in this study showed that the reference isolate has three mutation, one mutation converts A to P at the position 36, the second mutation converts G to R at the position162, and the third mutation converts V to A at the position 168 (Figure 4).

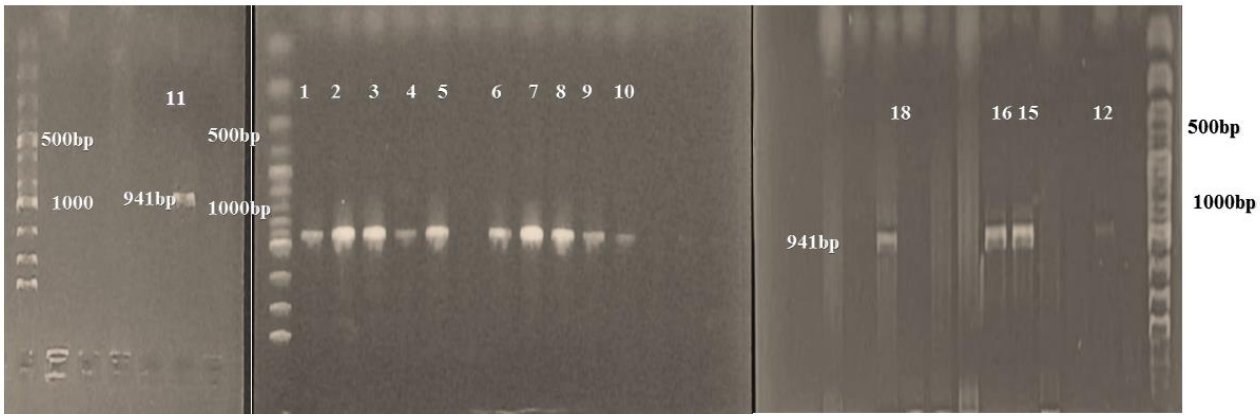

Fig 2: Electrophoresis results from 21 isolates with specific primer *ddl for E. faecalis*, first well for DNA marker, and other wells for positive result at 941 bp. staining with ethidium bromide, and the migration at 60 v. for 80 min.

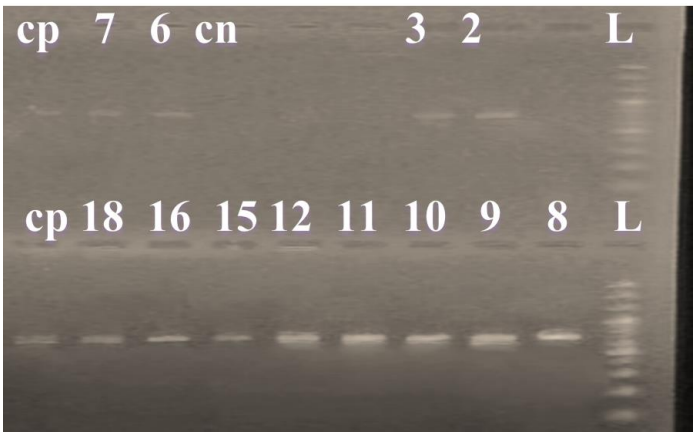

Fig 3: Electrophoresis diagram for amplified products by PCR that extracted DNA from 21 isolates of Enterococcus using a primer specific *vanA*. The first well was molecular size of the DNA marker, while other wells 1,2 to 18 showed positive result products at 732 bp. cp was control positive, while cn was control negative. The products migrated for 80 min. at 60 v. and stained by ethidium bromide.

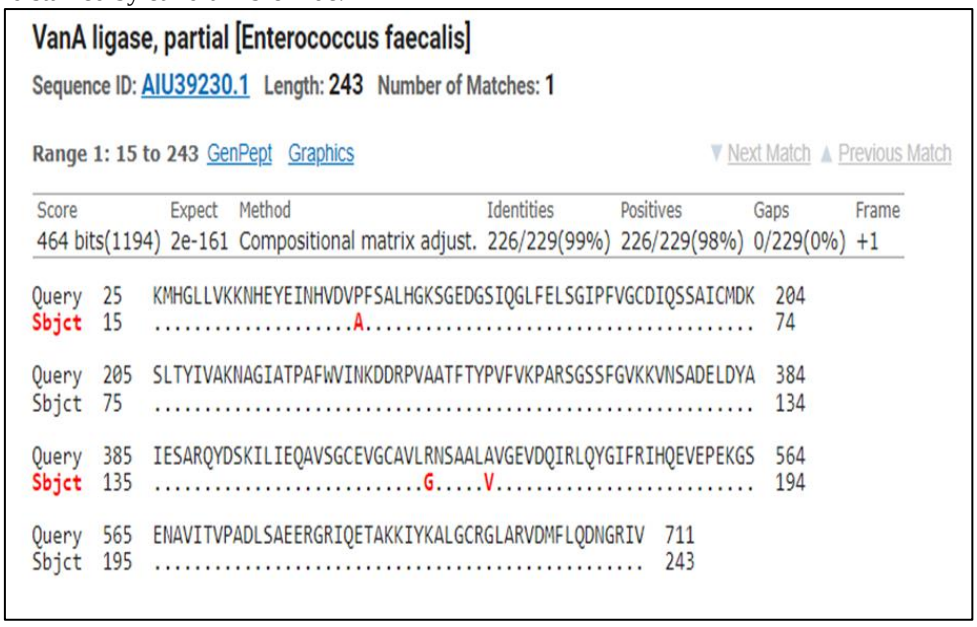

Fig 4: Sequencing for *vanA* gene for the reference isolate.

Phylogenetic Tree

The result of PCR sequencing for reference isolates to *vanA* gene, subsequently alignment with Gene Bank *vanA* sequencing in NCBI. An identity is 97-100% among *vanA* of the strains that include a number of Gram positive bacteria such as *Staphylococcus aureus*, *Bacillus*, *Clostridium difficile*, and other species of Enterococci (Fig. 5). To examine the phylogenetic tree and recognize which strains were closer or distant away from specific strain, depending on branch length and node location. The reference strain (H241223-026 M17 J1 JF.ab11282 our isolates): seemed close to strain WP 196382232.1 D-alanine-(R)-lactate ligase VanA partial *Enterococcus faecalis*. This is obvious from a very short branching between them, signifying that the two strains connect very close shared ancestor. The difference was less than (0.000). While, the farthest from our isolates are QBY26909.1 VanA ligase partial *Clostridioides difficile*. ACU27887.1 D-alanine:D-lactate ligase partial *Staphylococcus aureus*. Both display significant divergence from other strains, which means more genetic variation. Other strains, *Enterococcus faecalis* (HAQ1234526.1 or HAP5099867.1), mostly closer to our strain contrasted to *Staphylococcus aureus* or *Clostridioides difficile*. The isolates in the center part (such as HDT7324923.1) display moderate similarity.

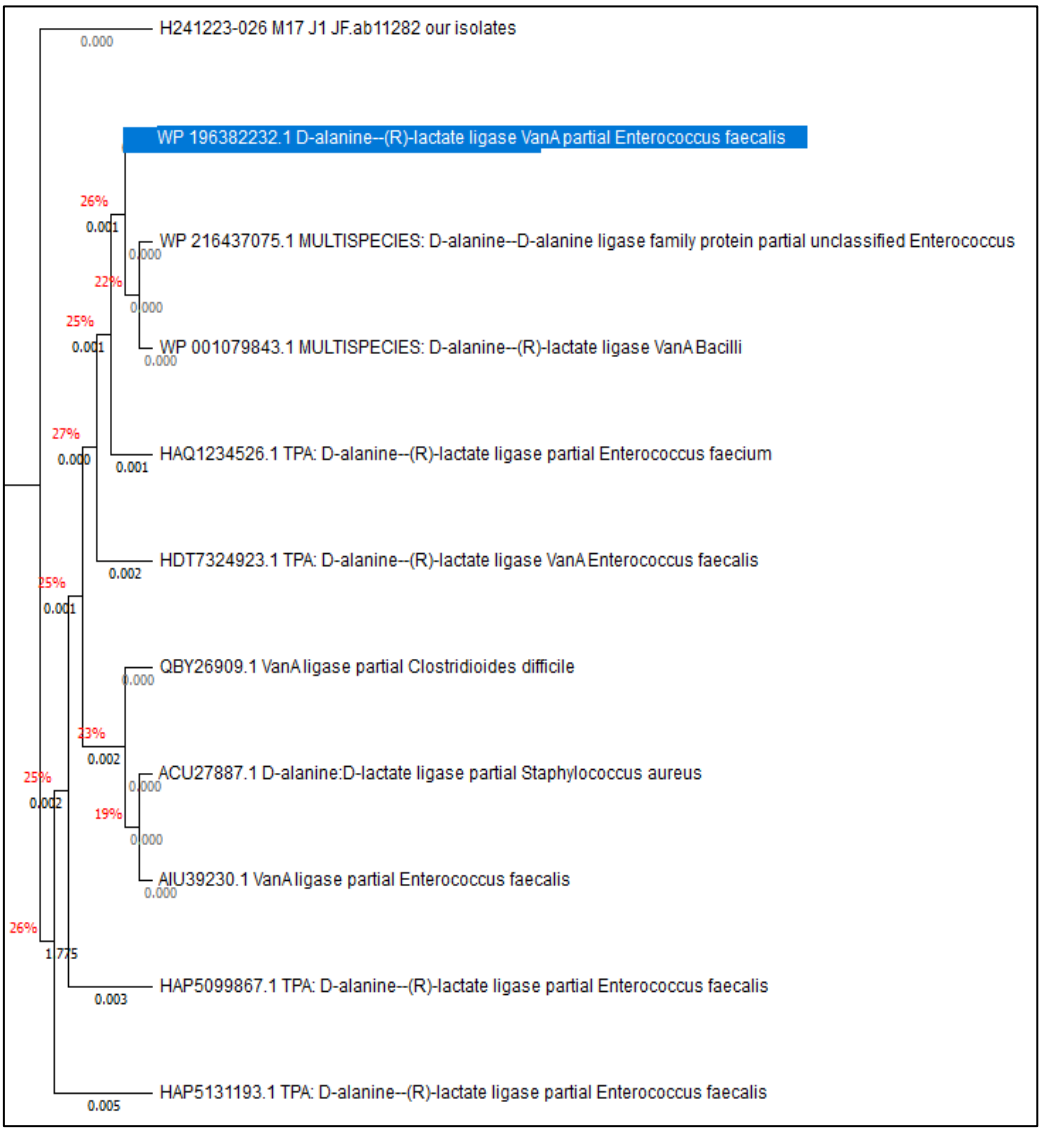

Fig. 5: Phylogenetic relationships flanked by local *vanA* gene for *Enterococcus faecalis* isolates and strains from NCBI through phylogenetic tree by Neighbor Joining technique aimed at historical evolution appreciated. The numbers express a branch lengths that predictable the convergence among isolates, and the percentage displays data coverage.

DISCUSSION

The *Enterococcus* isolates distribution showed a greater percentage of *E. faecalis* 15 (71.4%), while other isolates belong to the rest of *Enterococcus species*. This result agreed with the report of Maia *et al.* [16] displaying 68.75% of *Enterococcus*, which also matched with the search results of Phukan *et al.* [17] where *E. faecalis* has 54 (81%) percent, however the search of Mustafa *et al.* [18] displays that from the total *Enterococcus* isolates 17 (68%) isolates were *E. faecium* via PCR. Instead, Al-Halaby *et al.* [19] demonstrated that 28 (56%) were *E. faecalis*.

Among 15 isolates of *Enterococcus faecalis,* the incidence of *vanA* gene was 12 (80%), It is a problem of concern because of its capacity to transfer genes for vancomycin resistance to other organisms, especially to *Staphlococcus aureus*. This agreed with the report of Mirzaie *et al*., [10] which showed a relatively high prevalence (44%) of van-carrying Enterococci. The study provided by Kadhem [9] agreed with the present study, showing that *E. faecalis* isolates have 12 (84.6%) positive results for *vanA*. The result shown by Phukan *et al.* [17] reports *vanA* gene in 9 (56.25%) of the VRE isolates. Gulhan *et al.,* [20] show that none of *Enterococcus* isolates that are vancomycin resistant phenotypically carry the *vanA* gene.

The MIC technique of Vancomycin resistance revealed that all isolates were resistant to this antibiotic; the resistance might be attributed to an efflux pump or to *vanA* gene or other resistance genes not identified in this research. The present study agrees with the study of Kadhem [9] which showed that from 7 *E. faecalis* isolates has one isolate only sensitive to vancomycin with a percentage of resistant isolates (85.71%). Also, noted that *vanA* resistance was usually inducible through sub inhibitory-concentrations of vancomycin or teicoplanin, and it is related to a 38-40 kD protein production named *vanA* encoded by *vanA* gene. On the other hand, the study of Maia *et al.* [16] showed that 50% of the *E. faecalis* are considered as VRE. However, the result was reported by Phukan *et al.*, [17] were 16 (24%) VRE.

The study of Gorrie *et al*.,[21] agree with the present study that showed point mutations, insertion and deletions sequences initiated in *vanA* displaying a high level of heterogeneity. Also, much remains unidentified and the VanA-containing plasmids vary in molecular epidemiology among regions. Besides, this contributes to the expansion of the vancomycin resistance. While the result of Patel *et al*., [22] have shown 9 from 10 *vanA*-containing isolates organized identical *vanA* sequences of amplicon, and changed from reference *vanA* of B7641strain by only single base pair and single amino acid changed. Also, one isolate developed a *vanA* sequence of amplicon that is identical with the reference strains. Likewise, the evidence shown from 10 *vanA* discovered that 2 isolates were strongly related while the rest were different.

Sanger sequence investigation by Harada *et al*., [23] discovered that *orf1, orf2, vanA, vanR, vanX, vanH,* and *vanZ* were 100% like corresponding genes that were encoded by a prototype Tn1546 portion. But, the *vanS* differed by 3 mutations at positions Tn1546; 4796 T to G, 4808 G to C, and 4855 A to T, leading to 3 determined amino acid alterations in the VanS protein at the positions 50 Leu to Val, 54 Glu to Gln, and 69 Gln to His, respectively. However, *vanY* sequence has one mutation at the position 9280 G to C, and one amino acid changed Val to Leu at position 77. One mutation was detected in the position 5880 between *vanH* and *vanS*.

The result of PCR sequencing of reference isolates to *vanA* gene, Sanger sequence inspects subsequently alignment with Gene Bank *vanA* sequencing in NCBI. The identity of *vanA* with other species of Enterococci and number of Gram positive bacteria such as *Staphylococcus aureus, Bacillus,* and *Clostridium difficile*, indicated that the *vanA* gene was highly conveyed among these bacteria. WP 196382232.1 D-alanine-(R)-lactate ligase VanA partial *Enterococcus faecalis* was the nearest of our isolates. QBY26909.1 (*Clostridioides difficile*) and ACU27887.1 *Staphylococcus aureus* was the farthest.

While the result of Harada *et al*., [23] showed the phylogenetic tree of *vanA* for that study was 100% similar to vanA-genotype *E. faecium* and *E. faecalis* isolated from Japan broiler droppings, Taiwan chicken meat, and *Enterococcus cecorum* isolated from Japan chicken meat GenBank accession number AB663321.

Van Hal *et al*., [24] show a main *vanA* sequence-type may influence the obvious shift from conventional *vanB* dominance in the direction of rising *vanA* dominance. This finding may discuss the result of our study that showed 23 (85.18%) of isolates holding *vanA* gene. Otherwise, our result that show high prevalance of *vanA* may be attributed to the importance of this gene in comparable with other genes of vancomycin resistance [25].

Since vancomycin was the last line for treatment. So, vancomycin resistance at a high percentage was a major problem, and this resistance may be distributed to other bacteria, which was indicated by 97-100% identity among *vanA* in strains that include a number of Gram positive bacteria and other species of Enterococci. The *vanA* gene carried by Tn-1546 transposon, this gene might transpose among plasmids and also the chromosome in same or diverse bacterial strains by conjugation, or other transition methods [26]. Consequently, improving knowledge around genetic factors will be a significant step in explaining the pathogenesis, making prevention achievable [27].

CONCLUSION

Lastly, the VRE dissemination results obtained in our study proposed a great challenge which may transmit resistance to other pathogens, so permanent monitoring of the VRE emergency could provide the appropriate treatment methods.

Ethical Approval Declaration

Ethical Committee at the University of Kufa, college of Medicine, approved the study, with ref H K/1052, and at April 2023.

Assignment

All the participants provided informed consents for inclusion in the study and were assured that all the information provided would be used solely for the purposes of this study and treated confidentially.

Statements and Declarations

Conflict of Interest: "non-financial interests to disclose."

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Author Contributions: Khitam Fadhil Abbas and Zahraa Yosif Motaweq contributed to the research idea and the project; both authors were reading and then approved the final manuscript.

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