Plant Archives Volume 20 No. 2, 2020 pp. 6985-6994 e-ISSN:2581-6063 (online), ISSN:0972-5210

DETECTION AND SEQUENCING OF SEX PHEROMONE-RESPONSIVE PLASMIDS (*CPD*) *GENE* IN *ENTEROCOCCUS FAECALIS* ISOLATED FROM DIFFERENT CLINICAL SAMPLES

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Abstract

Isolation and detection of *E. faecalis* from different clinical samples (urine, stool, vagina swab, blood, pus, wound swab), detection of Sex pheromone-responsive plasmids gene and study the sequencing of *cpd* gene.

Detection of *cpd* gene by using PCR technique, detection of *cpd*gene by using sequencing depending on the next generation sequencing method.

The result was revealed that, 47 isolates were recorded related to *E. faecalis* collected from the following site, 11 isolates (25%) obtained from urine, samples, 10 isolates (21.27%) from stool, 10 isolates (21.27%) from wound, 6 isolates (13.63%) from vagina, 10 isolates (21.27%) from pus. while no bacteria 0(0.0%) were isolated from blood samples the 47 isolates ware subjected to molecular detection method using specific primer based on D-alanine ligase gene as a genetic marker for confirmed isolation of *E. faecalis* by PCR, the results revealed that 47 were positive for PCR. The *cpd* gene is present in all 47 isolates were gave positive result, which include eleven from urine 11/11(100%), ten from stool 10/10(100%), ten from wound 10/10(100%), six from vagina 6/6(100%) and ten from pus 10/10(100%), with long length (782bp).

The results of current study was shown there is more than one mutation in one isolate. Also, the result shown the nucleotide variations which demonstrated the polymorphism of the gene. However, the alignment between three isolates show that the little conservation of *cpd* gene.

Key words: *cpd* gene, PCR, *E. faecalis*, sequencing.

Introduction

Enterococcus is a large genus of lactic acid bacteria of the phylum Firmicutes. Enterococci are Gram-positive cocci that often occur in pairs (diplococci) or short chains and are difficult to distinguish from streptococci on physical characteristics alone (Rajbhandari *et al.*, 2018). Two species are common commensal organisms in the intestines of humans: *E. faecalis* (90-95%) and *E. faecium* (5-10%) (Lebreton *et al*., 2014).

Enterococcal virulence factors can contribute to Enterococcal disease in different ways; by enhancing colonization, adherence and invasion of host tissues, by modulation of the host immunity and by inducing pathological changes in the host associated with increased severity of infection (Chow *et al*., 2011).

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In *E. faecalis*, there are several sex pheromone-responsive plasmids that encode bacteriocins, aggregation substances and a broad range of antibiotic resistance determinants (Hegstad *et al*., 2010). The pheromone peptides (auto inducers) are transported through the ATP-binding cassette transport system. Accumulation of these auto inducers in the extracellular milieu is sensed by their corresponding recipients to regulate conjugation-related mating functions (Ali *et al*., 2017). The regulation and function of the pheromone response process revealed its great complexity and dual role-in plasmid conjugation and modulation of enterococcal virulence. Among other functional modules identified in pheromone plasmids, the stabilization/partition systems play a crucial role in stable maintenance of the plasmid molecule in host bacteria (Hosseini *et al*., 2016). Pheromone-responsive plasmids contribute also to enterococcal phenotype being an important vehicle of antibiotic resistance in this genus. Both types of acquired vancomycin resistance determinants, van'A' and van'B', as well many other resistant phenotypes, were found to be located on these plasmids. They also encode two basic agents of enterococcal virulence, i.e. aggregation substance (AS) and cytolysin. AS participates in mating-pair formation during conjugation but can also facilitate the adherence ofenterococci to human tissues during infection. The second protein, cytolysin, displays hemolytic activity and helps to invade eukaryotic cells. Enterococcal cells are capable of communicating via peptide pheromone encoded by *cpd*, which are secreted by recipient cells to induce the conjugative apparatus of donor cells. In this way they mediate the transfer of pheromone-responsive plasmids, which may carry virulence genes that promote biofilm formation or regulation (Stępień-Pyśniak *et al*., 2019).

Materials and Methods

Patients

A total of 210 samples only 47 isolates of *Enterococcus faecalis*were recovered from clinical samples, this include 11 isolates were isolated from urine sample with UTI patients and 10 isolates were isolated from stool from patients complaining with diarrhea. 10 isolates were isolated from wound, 6 isolates were isolated from vagina from patient with vaginitis and 10 isolates were isolated from abscess. All samples or individual were admitted to Al-Hilla surgical teaching hospital in Al-Hilla city/ Iraq, Babel hospital/ Iraq and Marjan medical city/ Iraq.

Diagnosis of Bacteria

All samples were obtained from patients with UTI, diarrhea, wound infection, vaginitis and abscess was cultured on blood agarand chromogenicagar and the plates were incubated at 37°C overnight. Diagnosis of the bacteria was carried out by biochemical methods (oxidise test, catalyse test, Bile-Esculine Hydrolysis Test, NaCl Tolerance Test, Motility Test) according to Bergy,s Manual for Determinative Bacteriology (Hol *et al*., 1994).

DNA Extraction

DNA was extracted from bacterial isolate according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (UK).

Confirmed detection of *E. faecalis* by PCR technique

To confirmed diagnosis for *E. faecalis* DNA was extracted from all suspected isolates by using the Geneaid DNA extraction Kit. The primer used for the amplification of a fragment gene were listed in table 1.

Molecular Detection of *cpd* Gene by PCR (Polymerase Chain Reaction)

Primer and PCR conditions were used to detect, gene of *cpd* are present in table 1. However, each 25µl of PCR consist of eachup stream and downstream primer (2.5 µl) free nuclease water (2.5 µl), DNA extraction in concentration 0.1µg/ml (5µl) and mastermix (12.5 µl). The polymerase chain reaction amp icon was detected by gel electrophoresison 1.5% agarose gels for 40 min at 70 V.

Detection of *cpd* Gene by Automated Sequencing

According to the results of PCR product, three DNA samples were subjected to sequencing by Macro gene Company/ USA, which give the identity of the genes comprised with the original genes in genebank by blast program which is available at the national center biotechnology information (NCBI).

Results and Discussions

Detection of *E. faecalis* by PCR technique

D-alanine ligase gene is present in *E. faecalis* and this gene is specific for *E. faecalis*. These it can facilitated down stream analyses such as molecular detection. *E. faecalis* is an opportunistic bacterium considered as pathogen for significant infection to human. Increasing research on *E. faecalis* in the past attested. The importance of *E. faecalis* strains with studies on metabolic pathways and analysis on gene. To confirmed diagnosis for *E. faecalis* DNA was extracted from all suspected isolates that previously identified *E. faecalis* by selective media (Chromogenic agar medium) conventional PCR was carried out using these DNA

Table 1: Primer sequence and PCR condition.

Genes	Primer sequence (5'-3')	Size of product bp	PCR condition	Reference
E. faecalis	F: TCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	94ºC-5 min, 30 cycles (94ºC-60s, 55ºC-60s, 72ºC-60s and 72ºC-5min	(Dutka-Malen *et al*., 1995)
cpd	F: TGGTGGGTTATTTTTCAATTC R: TACGGCTCTGGCTTACTA	782	94ºC-2 min, 35 cycles (92ºC-30s, 56ºC-30s, 72ºC-60s and 72ºC-2min	(Eatonand Gasson, 2001)

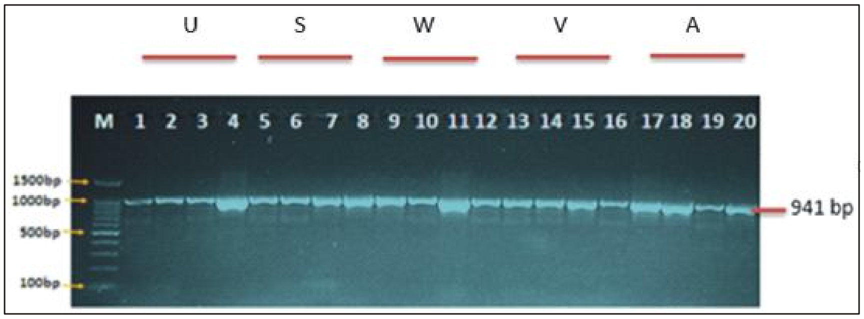

Fig. 1: Agarose gel electrophoresis image that showed the PCR product analysis of D-alanine ligase gene in *Enterococcus faecalis* isolated clinical infection samples. Where M: marker (1500-100bp) and Lane (1-20) showed some positive *Enterococcus faecalis* isolates were showed (1,2,3,4 urine 5,6,7,8 stool, 9,10,11,12 wound 13,14,15,16 vagina and 17,18,19, 20 abscess) at (941bp) PCR product. U=urine sample, S= stool sample W=wound sample, V=vagina sample, A=abscess sample.

samples for the amplification of specific *ddI* primer. The results recorded all isolated 47(100%) were produced the specific 941bp DNA fragment when compared with allelic ladder, as shown in fig 1. A result in this study was disagreement with result obtained by (Khalid, 2016) in Duhok City, Kurdistan Region/Iraq who found that 25 isolates of *E. faecalis* from urine samples were confirmed by successfully amplification of 914bp amplicon of *ddl* gene which used as species specific primer for detection of *E. faecalis*. Nateghian *et al*., (2016) who found that, out of the 200 enterococci studied by multiplex PCR, 180(90%) were identified as *E. faecalis*, also result in this study also disagreement with result obtained by Kafil and Asgharzadeh, 2014) who found that from (100) clinical isolates only (34) isolated *E. faecalis* using specific primer, (López-Salas *et al*., 2013) who detected that (95%) from clinical isolates related to *E. faecalis*. In this study, specific target was obtained and utilized in conventional PCR, which was proven more rapid, convenient and accurate for identification of *E. faecalis*, then previous methods. The results of PCR approach demonstrated that comparative genomic methodology was successful identifying specific target. Identification to the species level using PCR with species-specific primers is a valuable method and can replace complex molecular clustering techniques and conventional microbiological tests that are otherwise necessary to identify species that are difficult to distinguish using phenotypic approaches (Lindenstrauß, 2012).

Detection of *cpd* Gene by PCR

Molecular studies of *cpd* gene was done for all *E. faecalis* isolates by using specific PCR markers. All 47 isolates were gave positive result for *cpd* gene which include eleven from urine 11/11(100%), ten from stool 10/10(100%), ten from wound 10/10(100%), six from vagina 6/6(100%) and ten from abscess 10/10(100%), with long length (782bp) as shown in fig. 2.

The results of this study were agreement with results obtained by (Khalid, 2016) in Duhok City, Kurdistan Region/Iraq who found that out of 25 isolates of *E. faecalis* from urine samples, 24 (96%) was related to this gene. A study of (Belgacem *et al*., 2010) have demonstrated that *Enterococcus* strains that possessed and expressed virulence factors, caused a more serious infection than strains that lacked virulence factors, Gene *cpd* encoding for sex pheromone peptides showed a higher incidence among *E. faecalis*. Other studies also reported higher frequency of this gene among clinical *E. faecalis* isolates (Strateva *et al*., 2016). Aran *et al.,* (2015) determined that, the genetic determinants of aggregation substances were most frequently detected in *E. faecalis*, aggregation substance is a sex pheromone plasmid-encoded surface protein. The presence of the *cpd* genes in all *E. faecalis* isolates. Additionally, production of sex pheromones by *E. faecalis* may favour acquisition of antibiotic resistance and virulence from other enterococci, resulting in increased virulence. Pillay *et al*., (2018) reported that, *E. faecalis* sex pheromone plasmids are one of the most efficient conjugative plasmid transfer systems known in bacteria.

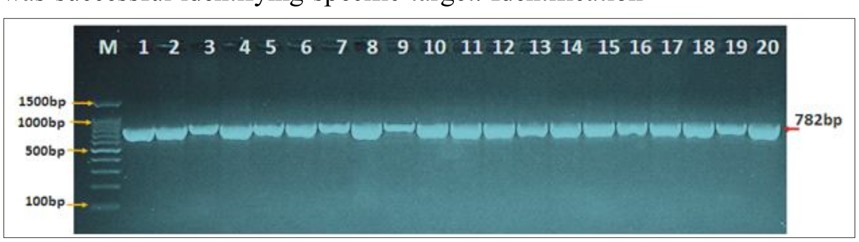

Fig. 2: Agarose gel electrophoresis image that showed the PCR product analysis of *cpd* gene in *Enterococcus faecalis* isolated clinical infection samples. Where M: marker (1500-100bp) and Lane (1,2,3,4,5,6,7,8,9,10,11,12, 13,14,15,16,17,18,19,20) showed positive *cpd* gene were showed at (782bp) PCR product. U=urine sample, S= stool sample W=wound sample, V=vagina sample, A=abscess sample.

Enterococcal cells are capable of communicating via peptide pheromone (e.g. encoded by *cpd*), which are secreted by recipient cells to induce the conjugative apparatus of donor cells. In this way they mediate the transfer of pheromone-responsive plasmids, which may carry virulence genes that promote

Table 2: Sequence information.

Information	9EF	13EF	25EF
Sequence type	DNA	DNA	DNA
Length	753bp	753bp	756bp
Organism	*Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecalis*
Protein	cPD1	cPD1	cPD1

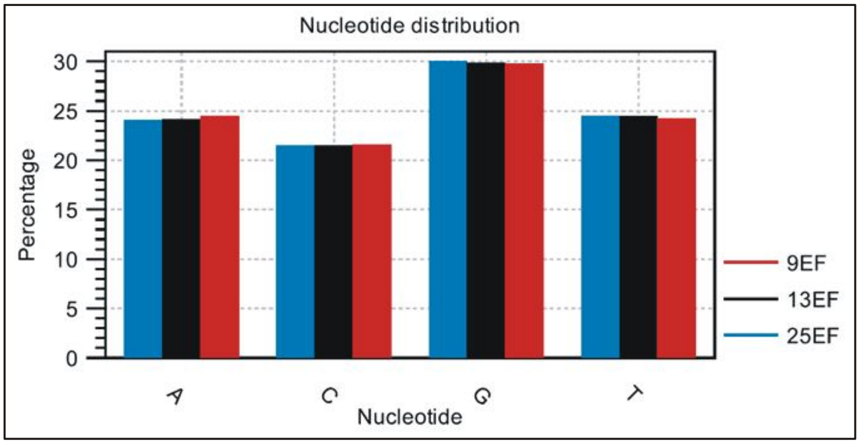

Fig. 3: Histogram of nucleotide distribution.

biofilm formation or regulation. In response to increased cell population densities, important virulence factors in *Enterococcus* spp. (Hashem *et al*., 2017). Detailed analysis of regulation and function of the pheromone response process revealed its great complexity and dual role-in plasmid conjugation and modulation of enterococcal virulence. Among other functional modules identified in pheromone plasmids, the stabilization/partition systems play a crucial role in stable maintenance of the plasmid molecule in host bacteria (Bandyopadhyay, 2018). Plasmid-free strains of *Enterococcus faecalis* excrete a number of small peptide sex pheromones which induce a mating response in strains containing certain plasmids native to this organism. Each individual pheromone is specific for a different plasmid or family of plasmids (Cook, 2012). When plasmid-containing cells are exposed to the specific pheromone, they respond by synthesizing a proteinaceous "aggregation substance," localized to the cell surface, which facilitates the formation of mating aggregates; other functions required for transfer of plasmid DNA are also induced. Acquisition of the plasmid results in the loss of detectable pheromone activity specific for that plasmid (Dunny, 2013).

Table 3: Counts of nucleotides.

Nucleotide	9EF	13EF	25EF
Adenine (A)	181	182	185
Cytosine (C)	162	162	163
Guanine (G)	226	225	225
Thymine (T)	184	184	183
C+G	388	387	388
A+T	365	366	368
GC%	**51.52%**	**51.39%**	**51.32%**

Sequencing of *cpd* Gene

The results of DNA sequencing should firstly examined to confirm the nucleotide sequence and closed relationships with others world strains, test used to confirm was through using NCBI-Blast-query nucleotide, it was perfect program and gave the exact results of identify percent with reference strain (KU311666.1). At a gene level, this study tried to discriminate between closely related strains, *Enterococcus faecalis* (9EF, 13EF, 25EF) for *cpd* gene by using sequencing depending on the next generation sequencing method.

General characteristics of *cpd* gene

In present study, the *cpd* gene sequencing method generated raw paired-end reads. The result of data analysis for the *cpd* gene for three isolates (9EF, 13EF, 25EF) as shown in table 2. It was shown that, the gene size were 753bp, 753bp and 756bp for 9EF, 13EF, 25EF isolates respectively, additionally, the GC% was calculated for each isolate (9EF, 13EF, 25EF) reveled 51.52%, 51.39%, 51.32% respectively. Regarding to the *cpd* gene size, the result of this study noted very slightly difference among three isolates. The results were shown in table 3, fig. 3.

Identification of patterns to nucleotide substitution for *cpd* gene of *Enterococcus faecalis* isolates (9EF, 13EF, 25EF):

This study calculated the base change count on every mutation to identify the type of sequence variation. The

Table 4: Identification of Base substitutions and types of point mutations (TS or TV) between *Enterococcus faecalis* isolates (9EF, 13EF, 25EF) and reference gene (KU311666.1).

Base substitutions	Number (%)	Transition No. (%)	Transversion No. (%)
A\C	15(8.15%)	116 (63)	68 (37)
A\G	57(30.97%)		
A\T	11(5.97%)		
C\G	23(12.5%)		
T\C	59(32.06%)		
T\G	19(10.32%)		
Total	184(100%)		

result of this study revealed that base substitution among studied *cpd* gene of *Enterococcus faecalis* isolates (9EF, 13EF, 25EF) was relative, where the base substitution on the three isolates studied revealed the following base changes: Adenine (A) was substituted with Guanine (G) and Thymine (T) was substituted with Cytosine (C). In more details, the high percentage of A substitution noted as AG substitution with 30.97%, while the higher base substitution count of T nucleotide showed TC substitution with 32.06%. Moreover, the high percentage of C substitution noted as CG substitution as 12.5%. Similarity the most common type of A substitution detected as AC

Fig. 4: Multiple gene alignment and variant detection (base substitution) against reference gene sequence (KU311666.1).

and AT substitution with 8.15% and 5.97% respectively. Also, the percentage of T substitution detected as TG substitution with 10.32%. General base change results were summarized in table 4 and illustrated in fig. 4.

Additionally transition and transversion variants were identified to detect patterns of nucleotide substitution. The results of this study revealed the high rate of transition substitution compared with transversion substitution. The high rate of transition substitution could be related to absence repair mechanism that must revert CT and GA transition. However many of these transitions are implicated in gene expression and virulence. In addition, the presence of high diversity in *E. faecalis* are driven primarily by base substitution mutation. The results of current study was shown there is more than one mutation in one isolate. This displays that the type and location of mutations that were found could lead to a difference in the effect of these mutations and some of these mutations, leading to changes in the genetic code; and then a change in the amino acids at the translation. However, it was documented that the mutation in the sequences of the genes that encode them including deletion or integration of foreign DNA between isolates effect on the sequence composition.

The result shown nucleotide variations which demonstrated the polymorphism of the gene. However, the alignment between three isolates show that the little conservation of *cpd* gene.

High-through put sequencing offers opportunities for understanding bacterial molecular evolution within the host and promise to shed light on the *in vivo* dynamics of bacterial carriage and infection. The role of chance, circumstance and genetics in invasive bacterial disease is let to be determined, but the exhaustive characterization of bacterial genetic version within the host is an important step.

Loss- of function mutation that truncate the amino acid sequence may play an important role in pathogens because point mutation of this sort can quickly effect radical functional change, many mutation as there have no effect on the ability of the protein to work or result in a loss of function. When this occur the bacteria have these mutation are less suited to survive. However, not all mutation lead to significant change in the protein, amino acid can replace another amino acid very similar in term of chemical characteristics and in this case, the protein is still working naturally, or it can happen to replacement of amino acid in a region of the protein that do not significantly affect the secondary structure of function. There were also amino acids encoded by more than one code, which could result in mutation. Additionally, this study noted low rates of deletion, this study presumes that most common type of genetic variation in the studied *cpd* gene is single base mutation rather than deletion mutation.

Acknowledgement

I thankful to Department of Microbiology, college of medicine, University of Babylon, Iraq, for the facilities provided in the completion of the work.

Ethical Approval

Agreement from patients for samplingcollection and carrying out this work isobtained from each patient.

Enterococcus faecalis strain EF9 sex pheromone cPD1 (cpd1) gene, partial cds

GenBank: MT274024.1

LOCUS MT274024 753 bp DNA linear BCT 15-JUL-2020

DEFINITION Enterococcus faecalis strain EF9 sex pheromone cPD1 (cpd1) gene,

partial cds.

ACCESSION MT274024

VERSION MT274024.1

KEYWORDS.

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 753)

AUTHORS Asal,S.S. and Abdul-Lateef,L.A.

TITLE Genetic analysis for Sex pheromone cPD1 in Enterococcus faecalis

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 753)

AUTHORS Asal,S.S. and Abdul-Lateef,L.A.

TITLE Direct Submission

JOURNAL Submitted (01-APR-2020) Microbiology Department, College of

Medicine, University of Babylon, 51001, al-Tibb al-Adli Street,

Hillah, Babil 51001, Iraq

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Sequencing Technology :: Sanger dideoxy sequencing

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481 ctgggccagccgagcatgattctggtggcgctggcgggcctggcgt atctggcgcagggc

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601 gtgagcccgctgatgattgtgtttatgagctttagcagcccggcggg cgtggcgctgtat

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LOCUS MT274025 753 bp DNA linear BCT 15-JUL-2020

DEFINITION Enterococcus faecalis strain EF13 sex pheromone cPD1 (cpd1) gene,

partial cds.

ACCESSION MT274025

VERSION MT274025.1

KEYWORDS.

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 753)

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COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

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GenBank: MT274026.1

LOCUS MT274026 756 bp DNA linear BCT 15-JUL-2020

DEFINITION Enterococcus faecalis strain EF25 sex pheromone cPD1 (cpd1) gene,

partial cds.

ACCESSION MT274026

VERSION MT274026.1

KEYWORDS.

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 756)

AUTHORS Asal,S.S. and Abdul-Lateef,L.A.

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JOURNAL Unpublished

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721 ccgcgtattaaaaaacaggtgcaggaagaactgaaa

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