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Distribution of Some Virulence Genes among *Enterococcus Faecalis* Isolates from Urine Samples

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ABSTRACT

Background: *Enterococcus faecalis* is one of the human and animal gastrointestinal flora. They are very robust so that they can live in humid, salty, or acidic environments. Understanding the link between enteric and extra-enteric *E. faecalis* strains is interesting as it is widely distributed throughout a variety of different habitats.

Objective: This research was carried out to investigate the percentage presence aggregation substance, cytolysin and hyaluronidase genes (*asa-1, cyl A, hyl*) in the *E. faecalis* isolates from UTI samples via the method of Polymerase Chain Reaction (PCR).

Materials and Methods: In the current study, collected 64 Samples during the period from November 2021 to January 2022 at Medical City Hospital in Baghdad city, included 18 men and 46 women. The patients ages were ranged from (17-54) years. Urine samples cultured on Pfizer specific *Enterococcus* media, a selective medium for the isolation and growth of *Enterococcus spp,* and then molecular detection was done to detect the *E. faecalis* gene and all three-virulence gene.

Results: Results demonstrated that the proportion of isolation for *E. faecalis* was 48%, while the percentage of *cyl* and *asa-1* genes was 100% and 97% respectively, although *hyl* gene was not detected in any isolates of *E. faecalis* isolated from urine samples.

Conclusion: Virulence genes may be prevalent is some isolates and absent from other depending on many factors such as the source of isolation.

Keywords: Virulence gene, PCR, Urinary tract infections.

INTRODUCTION

Bacterial infections continue to be a serious issue because of the considerable effects they have in public and medical settings. Enterococci are one category of microorganisms linked to opportunistic infections, notably in medical settings. Urinary tract infections, bacteremia, endocarditis, wound infections, and intra-abdominal infections are only a few examples of enterococci-associated illnesses. Since enterococci infections have significant negative effects on mortality, morbidity, and economic costs, they have been a growing source of worry. According to estimates, enterococci infections, particularly those caused by vancomycin-resistant bacteria, increased by 1.4 to 3.5 times the likelihood of death, major surgery, admission to the critical care unit, length of hospital stays, and release to a long-term care facility [(1)].

Enterococcus faecalis is one of the human and animal gastrointestinal flora. They even reside in the mouth and the vagina. They are very robust so that they can live in humid, salty, or acidic environments. Additionally, *E. faecalis* frequently inhabits soil and water. Understanding the link between enteric and extra-enteric *E. faecalis* strains is interesting as it is widely distributed throughout a variety of different habitats[(2)].

Enterococcus faecalis bacteria are often described as Gram-positive bacteria that are seen individually, in pairs, or in short chains under the microscope. That are facultative anaerobes and non-motile and typically non-hemolytic, with colonies on blood agar measuring around 1-2 mm in diameter. Lactic acid is produced by Enterococci as a result of fermentation with no gas production. Catalase and oxidase reactions in *Enterococcus* are negative. So capable of growing in 6.5% salt medium with a pH range of 4.8 to 9.6 and an ideal temperature range of 35 to 37°C [(3)].

Infections of the urinary tract are among the most common diseases; 150 million people worldwide are affected by it each year. The most frequent cause of these diseases is bacteria, the majority of cases (70–80%) Uropathogenic *Escherichia coli* (UPEC) is the primary causative agent, followed by *Staphylococcus, Klebsiella spp., Enterococcus spp.,* and numerous other pathogens [(4)].

A UTI can occur anywhere in the Urinary tract. It includes (urethritis, cystitis, pyelonephritis, epididymitis', prostatitis, per nephritis), and it is considered as one among the most common infectious diseases that are widely seen among all age groups of individuals [(5)].

Virulence factors play a role in pathogenesis by mediating adherence colonization and invasion into host tissues, and the production of toxin and enzymes, all of which can increase the severity of infection. There are several virulence factors, includes cytolysin, hyaluronidase, gelatinase, aggregation substance, enterococcal surface protein, and others [(6)].

Objective:

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This research was carried out to investigate the percentage presence aggregation substance, cytolysin and hyaluronidase genes (*asa-1*, *cyl A*, *hyl*) in the *E. faecalis* isolates from UTI samples via the method of Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Study Samples:

During the period of November 2021 to January 2022, urine samples were collected to shed light on the role of virulence factors of *E. faecalis* in pathogenic of urinary tract infections. The study samples include:

A. Bacterial isolation and identification

Urine samples were collected from (64) patients with UTI 46 women and 18 men age ranged from (17-54 year) mean age (35.1 ± 5.2 year). Sterilized plastic cup containers were used to collect and send urine samples to the lab. Where they were centrifuged and the supernatant discarded, while the pellet was cultured on Pfizer specific Enterococcus media, a selective medium for the isolation and growth of *Enterococcus spp.*, was used to inoculate all samples, and they were then incubated anaerobically for 24 hours at 37°C. The isolates colonies shape, size, and color were all examined. In order to confirm the identification of *Enterococcus spp*. Isolates.

B. Molecular detection

Genomic DNA micro extraction kit (Geneaid, China) was used to extract DNA from pure bacterial colonies following the manufacturer's instructions.

Nanodrop was used for detecting the concentration of the extracted DNA with the intent of detecting the quality of samples for downstream applications. Accurately followed by the genotyping detection to *E. faecalis*, *asa-1*, *cyl* and *hyl* genes using PCR technique.

C. Polymerase chain reaction (PCR):

1. Primers Selection

For this investigation, the primers shown in Table (**1**) were used.

Table (1): The primers and their sequences used in conventional PCR

Gene	Sequence 5 → 3	Size (bp)
E. Feacalis [7]	**F:** TCAAGTACAGTTAGTCTTTATTAG **R:** CGATTCAAAGCTAACTGAATCAGT	940
Cyl A [8]	**F :** ACTCGGGGATTGATA GGC **R:** GCTGCTAAAGCTGCGCTT	688
Asa-1 [8]	**F:** GCACGCTATTACGAACTATGA **R:** TAAGAAAGAACATCACCACGA	375
Hyl [8]	**F:** ACAGAAGAGCTGGAGGAAATG **R:** GACTGACGTCCAAGTTTCCAA	276

2. PCR Amplification

The PCR premix, primers, and extracted DNA were thawed at 4ºC. And the contents were vortexed briefly to bring them to the bottom of their tubes. A 25-ul PCR mixture was prepared, consisting of 5 ul of PCR premix, 1 ul of each primer (forward and reverse), 3 ul of DNA template, and the remaining 15 ul of sterile deionized distilled water.

The thermocycler PCR device was used to amplify DNA in accordance with the PCR protocol after briefly mixing the PCR reaction tubes (Table **2**).

Table (2): Program PCR amplification of *E.feacalis, Asa-1, Cyl A, Hyl* genes

gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	ycle
E.Feacalis	94ºC For 4 min.	94ºC For 40 sec.	56ºC For 40 sec	72ºC For 40 sec	72ºC For 5 min.	30
Cyl A	95ºC For 2 min.	95ºC For 30 sec.	49.5ºC For 30 sec	72ºC For 2 min	72ºC For 10min	35
Asa-1	94ºC For 7 min.	94ºC For 1 min.	56.5ºC For 1 min	72ºC For 1 min	72ºC For 7 min.	30
Hyl (1)	94ºC For 15 min.	94ºC For 1 min.	56ºC For 1 min	72ºC For 1 min	72ºC For 10min	30

3. Agarose Gel Electrophoresis

To detect DNA by electrophoresing 1.5% agarose gel with ethidium bromide staining for 50 minutes at 75 volts, the positive E. faecalis gene result was confirmed. Additionally, the agarose gel was photographed by using (UV) ultraviolet transilluminator.

Statistical analysis

System-SAS (2012) application was used to calculate the qualitative data, which were presented as frequency and percentage.

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Ethics approval:

The Ethics Committee at the Department of Biology (University of Baghdad) approved the study protocol (Reference: CSEC/1021/0102) in October 30, 2021.

This work has been carried out in accordance with The Code of Ethics of the World Medical Association (CSEC/1021/0102) (Declaration of Helsinki) for studies involving humans.

RESULTS

Isolating and identifying *Enterococcus faecalis* was the major goal of the sample collection.

All of the bacterial isolates were identified as Gram-positive cocci with ovoid or spherical shapes that were clustered singly, in pairs, or in short chains under the microscope (Figure **1**).

On Pfizer Selective Enterococci medium, the colonies were spherical, grey, and 2mm in diameter, with a black point in the middle and black zones surrounding the colony (Figure **2**).

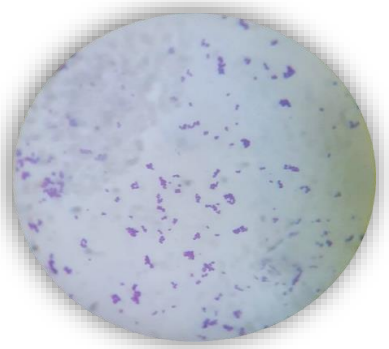

Figure (1): *Enterococcus spp*. under microscope.

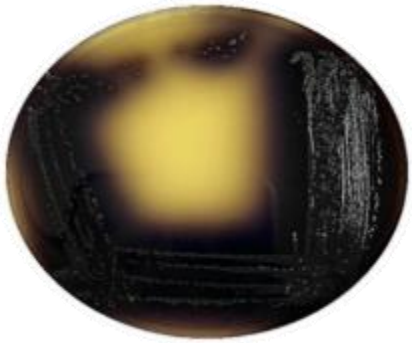

Figure (2): *Enterococcus spp*. on Pfizer Selective Enterococci medium.

The results of PCR detecting the specific gene of *E. faecalis* revealed that 31 (48.43%) isolate from urine samples was positive for *E. faecalis* gene. when compared to the DNA ladder, the present study found that *E. faecalis* gene band was detected at 940 bp (Figure **3**). There was no smear which may have indicated DNA degradation, and the bands were sharp, single and not dispersed.

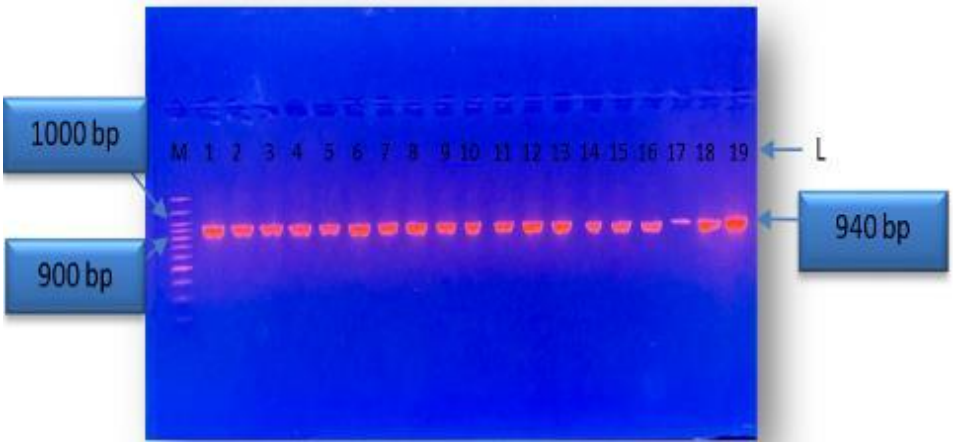

Figure (3): PCR-amplified product electrophoresis in 1.5 % agarose gel for *E. faecalis* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l00bp Ladder. Lane L 1-19: positive bands of 940bp.

Molecular identification of *cyl*, *asa-1* and *hyl* genes:

The virulence factor genes *cyl*, *asa-1* and *hyl* in *E. faecalis* were detected in 31 isolates of the urine samples. When compared to the DNA ladder, the present study found that *cyl* and *asa-1* genes band was detected at 688bp and 375bp respectively (Figure **4, 5**). There was no smear which may have indicated DNA degradation, and the bands showed sharp, single and not dispersed. Whereas the *hyl* gene was not detected in any isolate of *E. faecalis* from urine samples (Figure **6**).

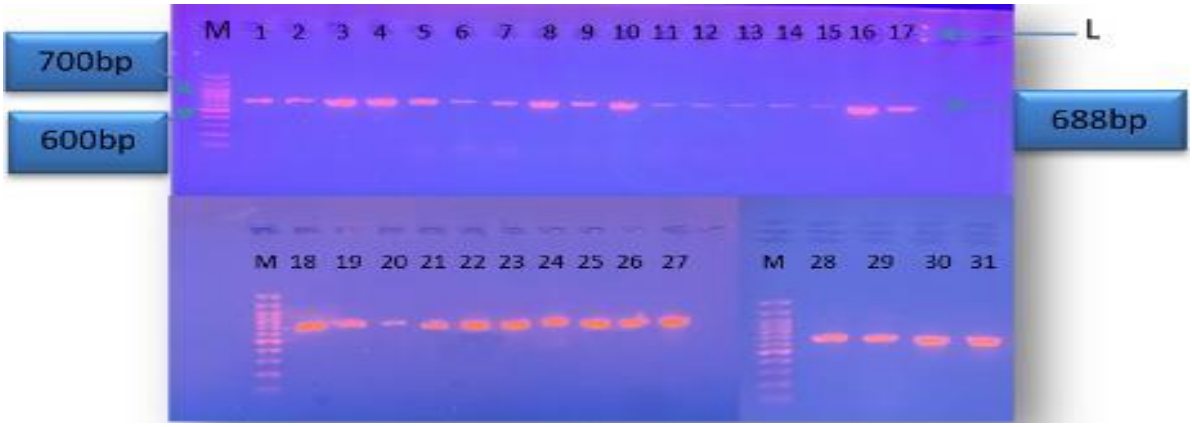

Figure (4): PCR-amplified product electrophoresis in 1.5 % agarose gel for *cyl* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l00bp Ladder. Lane L 1-31: positive bands of 688bp.

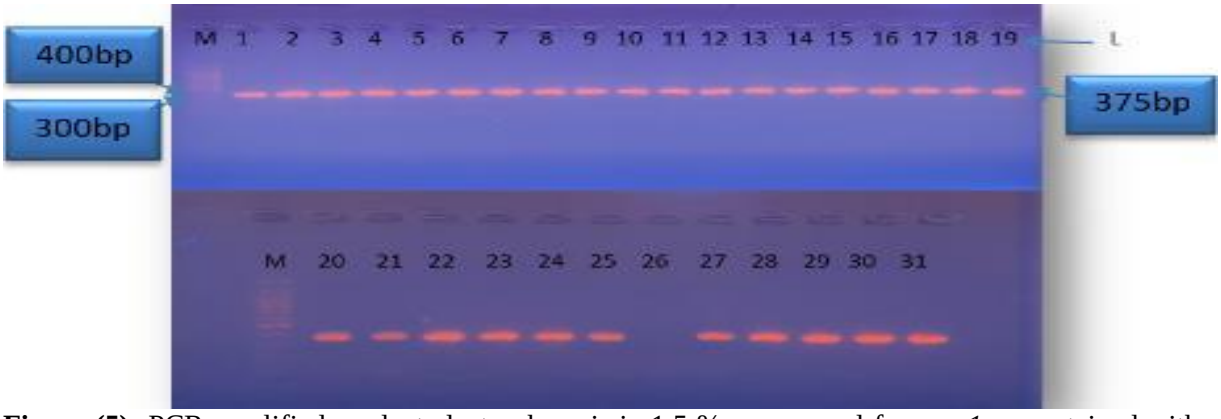

Figure (5): PCR-amplified product electrophoresis in 1.5 % agarose gel for *asa-1* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l00bp Ladder. Lane L 1-25 and 27-31: positive bands of 375bp, And 26: negative result for *asa-1* gene

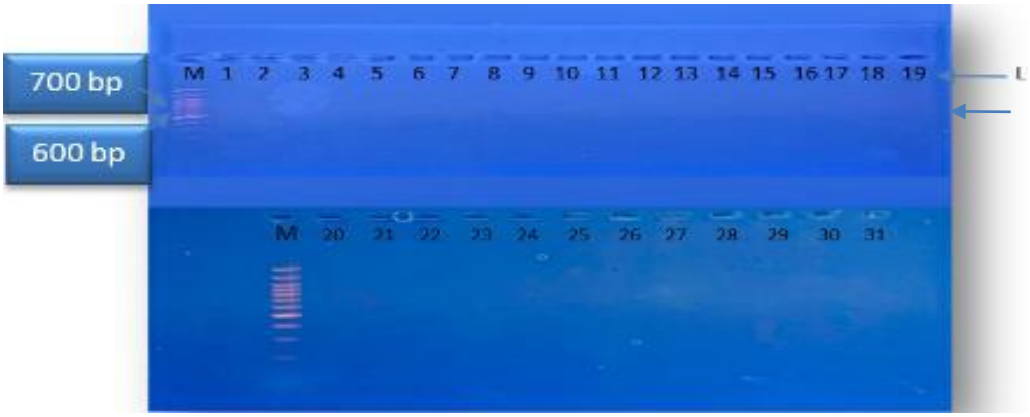

Figure (6): PCR-amplified product electrophoresis in 1.5 % agarose gel for *hyl* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l00bp Ladder. Lane L 1-30: negative bands of 605bp.

Table (3): percentage distribution of *cyl*, *asa-1* and *hyl* gene among *E. faecalis* isolates from urine samples

Gene	Positive No. (%)	Negative No. (%)	Total (%)
cyl	31 (100)	0 (0.0)	31 (100)
Asa-1	30 (96.77)	1 (3.23)	
hyl	0 (0)	31 (100)	

Note: *(p<0.01)

DISCUSSION

The results of PCR detecting the specific gene of *E. faecalis* revealed that 31 (48.43%) isolate from urine samples was positive for *E. faecalis* gene. Studies targeting the *E. faecalis* gene were also detected. **Hossein** [9] found that 56.12% of the sample had *E. faecalis* gene and **Haider and May** [10] found that 46.6% of sample its *Enterococcus faecalis*. The small differences between isolation percentages perhaps as a result of the quantity of specimens, the locations of each, and the various procedures used for identification.

The result showed that 31 (100%) isolates were positive for the *cyl* gene, 30 (96.77%) isolates positive for the *asa-1* gene while all the isolates were negative for the *hyl* gene (Table **3**). Also, from the result previously mentioned showed that (30) isolates had two genes (*cyl* and *asa-1)* together. Another studies carried out by **Hashem *et al.*** [11], **Coskun** [12] and **Samani *et al.*** [13] showed that 45%, 16.3% and 25% had cyl gene, respectively. The result of present study showed similarity to the result of study performed by **Nasaj *et al.*** [14], who showed that 97% of *E. faecalis* isolates harbored *asa1* gene, the result of this study was very as close compared to our result.

Another studies performed by **Jahansepas *et al.*** [15] and **Ghalavand *et al.*** [16], who reported that 74% and 79% of *E. faecalis* isolates harbored *asa-1* gene, respectively.

Other study done by **kafil *et al.*** [17] demonstrated that 1.1% had *hyl* gene, which is close to the result of this study. Another study carried out by **Mobarez and kafil** [18] showed that 17.35 had *hyl* gene, which disagree with the result of this study.

The surface expression of the aggregation substance promotes adherence to the host tissues, controls the body's natural defense against enterococci-caused disease, and so increases the virulence of many opportunistic infections. Additionally, **Bhatty *et al.*** [19] revealed in their investigation that the aggregating substance considerably increases the development of biofilms in vitro.

Cytolysin (also hemolysin) is bactericidal to other Gram-positive bacteria and has hemolytic effects on humans, neutrophils and macrophages are lysed by cytolysin, which makes them immune system-evading [12].

Hyaluronidase mostly comprises of degradative enzymes that act on hyaluronic acid and is linked to tissue injury, and it makes it easier for Enterococci to disseminate their toxins into host tissue. The persistence of *Enterococci* may be aided by these virulence factors [18].

CONCLUSION

Virulence genes may be prevalent is some isolates and absent from other depending on many factors such as the source of isolation.

Conflict of interest: Nil
Source of funding: Nil

REFERENCES

1. **Zinatul H, Ulil H, Suhartoo S (2022):** Distribution of multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens in the Zainoel Abidin General Hospital, Banda Aceh, Indonesia. Biodiversitas, 23(10):5043-5049.
2. **Veljović K, Popović N, Terzić V *et al.* (2015):** Environmental waters as a source of antibiotic-resistant *Enterococcus* species in Belgrade, Serbia. Environ Monit Assess., 187(9):1–15.
3. **Atiyah A, Alkhafaji H (2020):** Isolation of *Enterococcus* Species from Food Sources and Its Antibacterial Activity against *Staphylococcus Aureus.* Iraq J Sci., 61(12): 3164-3171.
4. **Öztürk R, Ahmet M (2020):** Epidemiology of urological infections: a global burden. World J Urol., (38):2669–2679.
5. **AL-Hamdani H, AL-Hashimy A (2020):** Molecular detection of urec, hpma, rsbaand mrpa genes ofproteus mirabilis urinary tract infection in patient with rheumatoid arthritis. Iraq J Agricul Sci., 51(Special Issue): 245-251.
6. **Strateva T, Atanasova D, Savov E *et al.* (2016):** Incidence of virulence determinants in clinical *Enterococcus faecalis* and *Enterococcus faecium* isolates collected in Bulgaria. Braz J Infect Dis., 20(2): 127-133.
7. **Shahi F, Hamidi H, Khoshnood S *et al.* (2020):** Virulence determinants and biofilm formation in clinical isolates of *Enterococcus*: A cross-sectional study. J Acute Dis., 9(1): 27-32.
8. **Nergis A, Taşkın E (2019):** Is there any association between antibiotic resistance and virulence genes in *Enterococcus* isolates? Med Sci Discov., 6(12):310-315.
9. **Hossein S, Ashraf M (2015):** Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. J King Saud Univ Sci., 27(4): 312-317.
10. **Haider S, May T (2014):** Isolation and Identification of Vancomycin-Resistant *Enterococcus Faecalis.* Iraq J Sci., 55(4B):1811-1816.

11. **Hashem Y, Khaled A, Ramy K (2021):** Phenotype–Genotype Correlations and Distribution of Key Virulence Factors in *Enterococcus faecalis* Isolated from Patients with Urinary Tract Infections. Infect Drug Resist., 14: 1713–1723.
12. **Coskun U (2019):** Investigation of the relationship between virulence factors and antibiotic resistance of Enterococci isolates. Cell Mol Biol., (65)2: 14-17.
13. **Samani R, Tajbakhsh E, Momtaz H *et al.* (2021):** Prevalence of Virulence Genes and Antibiotic Resistance Pattern in *Enterococcus faecalis* Isolated from Urinary Tract Infection in Shahrekord, Iran. Rep Biochem Mol Biol., 10(1): 50–59.
14. **Nasaj M, Mousavi S, Hosseini S *et al.* (2016):** Prevalence of virulence factors and vancomycin-resistant genes among *Enterococcus faecalis* and *E. faecium* Isolated from clinical specimens. Iran J Public Health, 45(6):806–813.
15. **Jahansepas A, Aghazadeh M, Ahangarzadeh R *et al.* (2017):** Occurrence of *Enterococcus faecalis* and *Enterococcus faecium* in Various Clinical Infections: Detection of Their Drug Resistance and Virulence Determinants. Microb Drug Resist., 24(1):76-82.
16. **Ghalavand Z, Alebouyeh M, Ghanati K *et al.* (2020):** Genetic relatedness of the *Enterococcus faecalis* isolates in stool and urine samples of patients with community-acquired urinary tract infection. Gut Pathogens, 12(1):1-11.
17. **Kafil H, Mobarez A, Moghadam M (2013):** Adhesion and virulence factor properties of Enterococci isolated from clinical samples in Iran. India J Pathol Microbiol., 56(3), 238-242.
18. **Mobarez A, Kafil H (2015):** Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. J King Saud Univ Sci., 27(4), 312-317.
19. **Bhatty M, Cruz M, Frank K *et al.* (2015):** *Enterococcus faecalis* pCF10 encoded surface proteins PrgA, PrgB (aggregation substance) and PrgC contribute to plasmid transfer, biofilm formation and virulence. Mol Microbiol., 95(4):660-677.