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Investigation Of Some Genetic Virulence Factors In Enterococcus Faecalis Isolated From Different Clinical Sourcein Kirkuk Governorate Using Polymerase Chain Reaction(Pcr) Technique

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Abstract

The presence of Enterococcus faecalisbacteria was investigated in (260) samples taken from different pathological sources, as (120) samples were collected from persons with urinary tract infection, (80) samples from Stool and (60) samples from infected persons. With infection of the root canal of the tooth, and from different age groups, the isolates were diagnosed by biochemical and culture tests, as well as using the APi-20 strep system and using the Vitek2 device,Then the isolates were diagnosed based on the genotype by molecular diagnosis using the polymerase chain reaction (PCR) technique, and 40 isolates belonging to Enterococci bacteria were obtained, based on the initial culture tests, and 22 isolates by the biochemical tests conducted on them. And 12 isolates belonging to the type Enterococcus faecaliswere obtained by diagnosing them with APi for-20 and Vitek2 system, and after conducting a molecular examination (16SrRNA) on them, it was confirmed that 12 isolates of bacteriaE.faecalis

The results of detecting the ability of the isolates to produce virulence factors showed that all (12) isolates were not producing Van A factor, but (5) isolates (41%) showed their ability to produce Van B factor.

The percentage was distributed as follows: (3) isolates (25%) of tooth root canal infections, (1) isolates (8%) of urinary tract infections, and (1) isolates (8%) of feces.

Key words: virulence factors, Enterococcus faecalis, different clinical sources, polymerase chain reaction.

Introduction

Enterococci bacteria belong to the microorganisms that often inhabit the intestinal lumen as well as the oral cavity and are also found to a lesser extent in the vagina and male urethra in a normal normal commensalism in humans, and these bacteria can cause wide diseases, including infection of the urinary tract, or bloodstream blood stream, abdominal cavity, bill duct, or burns wounds (Gilmore et al., 2002; Jett et al., 1994). E. faecalishas become one of the most common types of enterococci that cause most human diseases because it possesses the majority of virulence factors belonging to this genus. (Koneman et al., 2006). Bacteria have the ability to attach, settle and invade some areas of the body, which gave a good chance for these bacteria to be opportunistic pathogens (Kau, et al., 2005). The importance of these bacteria has also increased, not only for what they cause. It is a serious disease, but also because of its resistance to many antibiotics (Leavis et al., 2006). In the preceding years, many studies showed that mortality These bacteria ranged between (20-68%) before the emergence of cases of resistance to vancomycin, but at the present time the percentage increased to more than 90% after the emergence of strains of enterococcal bacteria resistant to vancomycin (Raad et al., 2005;) Vancomycin resistance enterococci (VRE) (Tenover and McDonald, 2005)) The traditional culture and biochemical methods were used to detect those bacteria that cause infection of different pathological sources, especially dental root canals (molander et al; 1998).

And the use of traditional culture and biochemical methods in diagnosis is very limited, especially in diagnosing E. faecalis because of the difficulty of isolating and diagnosing this bacteria. Therefore, molecular genetic methods were recently used to investigate these bacteria (Siqueira and Rocas, 2005). Therefore, this study came with the aim of the following:

1.Isolation and identification of Enterococcus faecalisbacteria isolated from different pathological sources by culture and biochemical methods, using APi 20 strip system, Vitek2device and PCR.

2.Investigate genetically some virulence factors by using the polymeras

chain reaction (PCR) technquie.

2. Materials and working methods

Collection of samples ,isolation and diagnosis of Enterococcus faecalis

The samples totaling 260 samples were collected from different clinical sources (urinary tract infections, tooth root canal infections, and discharge) from different hospitals and health places in Kirkuk Governorate. The samples were placed on a blood agar medium and incubated for 24 hours at a temperature of 37°C, and then transferred to the selective media represented by Pfizer selective enterococcus agar medium. These samples were diagnosed using the biochemical and culture tests, as well as using the API-20 Strep system, using the Phytec 2 device, and then using the polymerase chain reaction (PCR) technique. In the end, 12 isolates of Enterococcus faecalis were obtained.

Molecular diagnosis of bacteria E. faecalis

DNA Extraction process

The complete DNA extraction kit manufactured by (Intron biotechnology/korea) was used, and the extraction process was carried out according to the company's instructions (Protocol).

Determination of DNA Concentration

According to Promega diagnostic kit Quantus/USA.

Diagnosis of E. faecalisby PCR.

PCR technique was used to diagnose Enterococcal bacteria by using primers with a specialized sequence found in the genetic material of the bacteria, which matches the 16s rRNA gene, as in the following steps.

-Prefixes used in the reaction and their dilution

The primers were converted to (Lyophilil), where they were added in free (ddH2O) to give a final concentration of (100 Pmol/µL) as a stock solution (Stock) and kept at a temperature of (-20 °C). To prepare (10 Pmol/µL) in case Suspend the initial work. (10 µL) of the stock solution is taken and added to (90 µL) of free water (ddH2O) to reach the final volume (100 µL), and this was done according to IDT (Integrated DNA, Technologist, Company, Canada).

Table (1) The specific 16s rRNA initiator sequence of the gene.

Primer	Sequence	Product size
Forward	5'-GGATTAGATACCCTGGTAGTCC-3'	320 base pair
Reverse	5'-TCGTTGTTGCGGGACTTAACCCAAC-3'	

-**Optimum reaction conditions**

Several experiments were conducted to determine the optimal conditions for the reaction, which was carried out in a (Thermal cycler) device, where the program mentioned in Table (2) was applied.

Table (2) Optimal PCR reaction conditions for primers of the efF gene, efR

No.	Phase	Time (Cº)	Time	No. of cycle
1-	Initial Denaturation	94 Cº	3 min	1 cycle
2-	Denaturation -2	94 Cº	45 sec	
3-	Annealing	44 Cº	45 sec	35 cycle
4-	Extension -1	72 Cº	45 sec	
5-	Exten	72 Cº	7 min	1 cycle

Detection of PCR products using electrophoresis

Relay electrophoresis to detect PCR products for 16s rRNA at agarose gel concentration (2%) and voltage of (60) V, and DNA Ladder (100-1000 Pb) is used as a marker. The result of the reaction was positive when pieces of DNA appeared in the gel with a length of (320 bP).

Detection of virulence factors using PCR technique

1.Investigating the Van A gene in Enterococcus faecalisbacteria using PCR technique.

Primer	Sequence	Product
Forward	5'-GGGAAAACGACAATTGC-3'	732 base pair
Reverse	5'-GTACAATGTGGCCGTTA-3'	

Optimum reaction conditions

Several experiments were conducted to determine the optimal conditions for the reaction, which was carried out in a (Thermal cycler) apparatus. Where the program mentioned in Table (4)

Table (4) Optimum PCR reaction conditions for primers of the Van A . gene

No.	Phase	Time (Cº)	Time	No. of cycle
1-	Initial Denaturation	94 Cº	3 min	1 cycle
2-	Denaturation -2	94 Cº	45 sec	
3-	Annealing	44 Cº	45 sec	35 cycle
4-	Extension -1	72 Cº	45 sec	
5-	Extension -2	72 Cº	7 min	1 cycle

Detection of PCR products for the Van A gene using electrophoresis

The PCR products were investigated for the VanA gene using electrophoresis, as the result of the reaction was no longer positive because no DNA fragments appeared in the gel and its length (732 bp).

2.Investigating the VanB gene in Enterococcus faecalisbacteria using PCR technique

Table (5) The specific initiator sequence of the Van B . gene

Primer	Sequence	Product
Forward	5'-ATGGGAAGCCGATAGTC-3'	635 base pair
Reverse	5'-GATTTCGTTCCTGGACC-3'	

Optimum reaction condition

Several experiments were conducted to determine the optimal conditions for the reaction, which was carried out in a (Thermal cycler) apparatus. Where the achievement of the program mentioned in Table (6).

Table (6) Optimum PCR Reaction Conditions for VanB . Gene Primers.

No.	Phase	Time (Cº)	Time	No. of cycle

No.	Phase	Time (Cº)	Time	No. of cycle
1-	Initial Denaturation	94 Cº	3 min	1 cycle
2-	Denaturation -2	94 Cº	45 sec	
3-	Annealing	44 Cº	45 sec	35 cycle
4-	Extension -1	72 Cº	45 sec	
5-	Extension -2	72 Cº	7 min	1 cycle

Detection of PCR products for the VanB gene using electrophoresis

The results of PCR were investigated for the VanB gene using electrophoresis, where the result of the reaction was returned positive when pieces of DNA appeared in the gel and a length of (635 bP).

3.Results & Discussion

Isolation and Identification of E. faecalis

E. faecalisbacteria were investigated in (260) samples taken from different clinical sources. (120) samples were taken from people with urinary tract infections, (80) samples from stool and (60) samples from people with root canal infections The study of collecting isolated samples included both males and females, and the number of females included in the study for urinary tract infections samples was (70), and the number of males was (50), as for stool samples, it was females (44), the number of males was (36), and for inflammatory samples The root canal of the tooth was the number of females (38) and the number of males (22)From different age groups (6-55 years) of patients referred to (Maternity, Women and Children Hospital, Children's Hospital, Public Health Laboratory in Kirkuk, Specialized Dental Center, Specialized Consultation Clinics on Baghdad Road, and Popular Clinics in Hawija Doctors Street).) and in the period between (20-11-2020 to 20-2-2021), and it was possible to obtain (12) isolates belonging to E. faecalis. (6) of them were isolated from feces (50%) and (4) of them were isolated from infections of the tooth root canal and at a rate of (33.4%) and (2) of them were isolated from urinary tract infections (16.6%).

These results are close to the results reached by (Jawad et al., 2010) with regard to .
the stool samples and urine that he mentioned, where he showed that these bacteria are found in a percentage of (45.4%) in the stool, and there is a percentage of (21.1%) in the urine. However, this percentage does not agree with the percentage mentioned by (Uma et al., 2007), who found that these bacteria are present in individuals by (86%) for stool

samples, and by (82%) for urine samples. Other studies indicated that the percentage of E. faecalis bacteria in stool can reach (93.5%) (Forejt et al., 2007).

Molecular diagnosis of E. faecalis by PCR

Molecular Identification of E. faecalis by PCR Technique

All (12) isolates were diagnosed using API 20 strep system and Vitek2 device and isolated from urinary tract infections, tooth root canal infections and feces using polymerase chain reaction (PCR) technique in order to confirm the previous diagnosis and to confirm the relevance of the isolates to E. faecalis. (40) isola tes belonging to Enterococcus bacteria were diagnosed based on the culture and microscopic methods used in the diagnosis, and (22) isolates related to Enterococcus faecaliswere diagnosed based on biochemical methods, and (12) isolates related to E. faecaliswere diagnosed when diagnosed using the system API 20 ,strep

Also, (12) isolates belonging to E. faecaliswere also obtained when diagnosing with the Vitek2 device, which was used for the purpose of comparing the results and confirming the diagnosis. The results of the molecular diagnosis of (12) isolates by polymerase chain reaction (PCR) technology, which depend on the genotypes of the bacteria, showed that they are in complete agreement. With the diagnostic results of the API 20 strep system and the Vitek device. As in Table (7), the differences in the preparation of E.faecalis isolates after diagnosis by culture and biochemical methods, the diagnosis with the API 20 strep system, the Vitek2 device, and molecular diagnostics.

Molecular diagnosis by PCR	**Diagnostics Vitek2**	**Diagnostics API 20 strep**	**Biochemical Diagnosis**	**Diagnosiscultre**	**Diagnosis Preparation Isolates**
6	6	6	12	24	**Stool**
4	4	4	7	11	**Tooth root canal infections**
2	2	2	3	5	**Urinary Tract**

					Injuries
12	12	12	22	40	**Total**

To confirm the diagnosis of bacteria using PCR technique, 16srRNA was used. This initiator binds to the required DNA template or piece and then amplifies the required piece of it to hundreds of thousands or millions of times. So, (12) isolates belonging to E. faecalis were obtained, distributed as follows (6) isolates (50%) of stool samples (Stool), (4) isolates, and (34.4%) of root canal infection samples, (2) Isolated (16.6%) of urinary tract infections .(UTI).

It is noted from the results that E. faecalisis present in a somewhat small percentage compared to the number of samples (260) samples. The results of the molecular diagnosis showed a clear difference from the results obtained using the agricultural and biochemical methods, as a smaller percentage of isolates related to bacteriaE.faecaliswere obtained., but the results of the molecular diagnosis completely agreed with the results of the API 20 strip system and the Vitek2 device, which shows the importance of the molecular diagnosis(Siqueira * Rocas, 2005)

It is also noted from Table (7) that there is a difference between the numbers of enterococci isolates isolated from urine, stool and tooth root canal infections, as the largest number of samples were collected from urine, which amounted (12) samples, but the least number of isolates was obtained, amounting to (2)) isolated, while (80) stool samples were collected, during which the largest number of isolates was obtained, which amounted to (6) .isolates. 4) isolates.

The results of the current study did not agree with what was stated by (Al-Saadi, 2007), as it was shown that the rate of isolates of urine isolates (6.94%). The presence of enterococcal bacteria in the root canals of the tooth by diagnosing it using PCR technique, where it was found that it constitutes (35%). The results of the study also slightly agreed with the results of (Fouad et al., 2005).

Those who proved in their study on the investigation of the presence of enterococci in the root canals of the tooth and the resistance to treatment that E. faecalisis the most common type, and it constituted a percentage (22%). As for fecal isolates and urinary tract infections,

their results were somewhat in agreement with what was reached (Jawad, et al., 2010), who showed that these bacteria are found in a percentage of (45.5%) in stool and (21.1%) in urinary tract infections.

Electrophoresis was carried out in 2% agarose gel, stained with ethidium bromide, at a potential difference of (70) volts and ampere (60 A) for 30 minutes, during which it was checked for the pieces that were amplified by PCR, where the amplified pieces of DNA appeared in bundles in The gel has a length of 320 Pb and was photographed under UV light as in Figure (1).

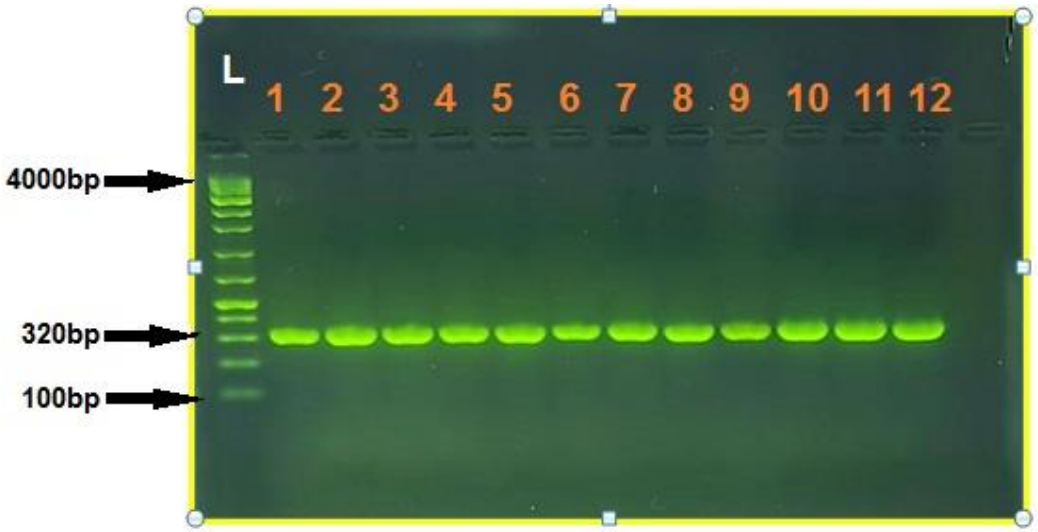

Figure (1) Electrophoresis of the products of molecular diagnostics by PCR in agarose gel with a concentration of 2%, a voltage of 70 volts, an ampere of 60 A and a time of 1 hour

Investigation of the presence of VanA-VanBgenes in isolates of E. faecalis

The results of the investigation for the vancomycinresistance genes, which are VanA, VanBin E. faecalisbacteria (VRE), specifically E. faecalis, showed that the VanA resistance gene was not present in all isolates compared to the VanB gene, which was present in only five isolates, which were distributed as follows (3) isolates of tooth root infections, (1) isolates of The presence of VanA,Bgenes in .urinary tract infections, and (1) isolates from feces Enterococcus faecalis was investigated in (12) isolates from faeces, urinary tract infections and tooth root canal infections, which were diagnosed based on genotypes using PCR technique, where molecular methods based on genotypes were used to investigate. About this gene by using specialized primers to link these genes. The appearance of a band of 732 bp in length when separating the segments that were amplified by PCR is evidence of the

presence of the VanA gene in the isolate and it is positive for the test, and a length of 635 bp is evidence of the presence of the VanB gene, but the current study showed that all isolates did not contain the agent or the VanA gene by 100%. A voltage of 70 volts for an hour and the result of theVanA gene did not appear for any of the isolates in the electrical relay asinFigure(2).

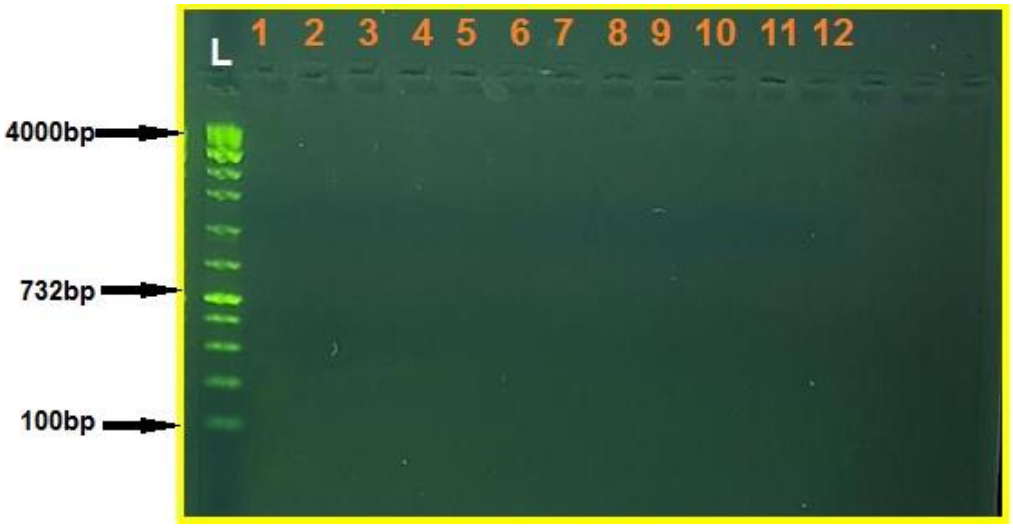

Figure (2) Electrophoresis products of VanA gene in E. faecalisusing PCR.

As for the electrophoresis of the VanB gene in 2% agarose gel, dyed with ethidium bromide dye, at a voltage of 70 volts, for an hour, five isolates were shown, which are as follows: (3) from tooth root canal inflammation (25%) and (1) from Yorn And by (8%) and (1) from feces and by (8%) as a result of DNA cuts that were amplified by PCR, they appeared in the form of bundles with a length of 635 bp, as in Figure (3)..

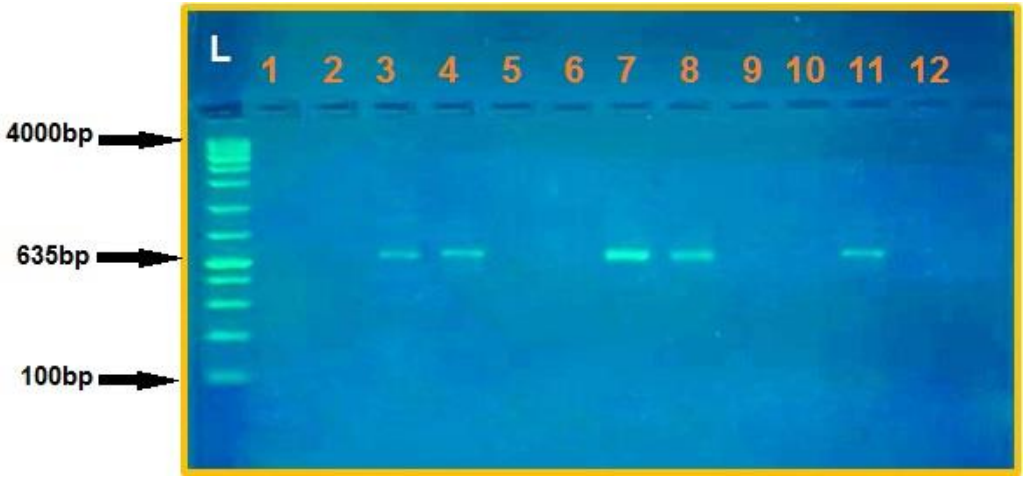

Figure (3) Electrophoresis products of theVanB gene in E. faecalisusing PCR. Positive isolates: 3, 4, 7, 8,11

Table (8) Complete products of DNA extraction and molecular identification of E. faecalisby matching 16s rRNA gene andinvestigation of VanA and VanB genes in Enterococcus faecalisusing PCR technique

Sample	DNA Extraction	PCR Result 16s rRNA 320 bP	PCR Result Van A 732 bP	PCR Result Van B 635 bP
1	+	+	-	-
2	+	+	-	-
3	+	+	-	+
4	+	+	-	+
5	+	+	-	-
6	+	+	-	-
7	+	+	-	+
8	+	+	-	+
9	+	+	-	-
10	+	+	-	-
11	+	+	-	+
12	+	+	-	-

In addition, theVanA/B genes express the Enterococcus faecalistype A and B strains, which possess this gene, which is responsible for their resistance to the antibiotic vancomycin, as there have been many reports around the world in the detection of these genes. It has been detected in various settings as well as among hospitalized and non-admitted persons (Wilcks * Licht 2005).

References

Cogulu. D., Uzel, A., Oncag, O., Aksoy, S. C., &Eronat, C. (2007). Detection of Enterococcus faecalis in necrotic teeth root canals by culture and polymerase chain reaction methods. Eur. J. Dent., 1(4): 216-216.

Forejt, M.; Šimůnek, J.; BRáZDOVá, Z., &Lefnerová, D. (2007). The influence of regular consumption of kefir beverage on the incidence of Enterococcus faecalis in the human stool. ScriptaMedica (BRNO), 80(6): 279-286.

Fouad, A. F.; Zerella, J.; Barry, J. &Spångberg, L. S. (2005). Molecular detection of Enterococcus species in root canals of therapy-resistant endodontic infections. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod., 99(1): 112-118.

Gilmore, M.S.; Coburn, P.S.; Nallapareddy, S.R. and Murray, B.E.; (2002). Enterococcal virulence, PP: 301-354- InGilmore, M.S.; Clewell, D.B.; Coburn, P.M.; Dunny, G.M.; Murray, B.E. and Rice, L.B. (2002). The enterococci: Pathogenesis Molecular Biology, antibiotic resistance, and infection control. ASM press, Washington, D. C.

Jawad, K.; Sameer, F. and Mohammed, S. (2010). Virulence factors of Enterococcus faecalis. Med. J. B., 7:(3-4).

Jett, B.D.; Huycke, M.M. and Gilmore, M.S. (1994). Virulence of Enterococci. Clin. Microbiol. Rev., 7(4): 462-478.

Kau, A. L.; Martin, S. M.; Lyon, W.; Hayes, E.; Caparon, M. G., & Hultgren, S. J. (2005). Enterococcus faecalis tropism for the kidneys in the urinary tract of C57BL/6J mice. Infect. Immun., 73(4): 2461-68.

Koneman, E.W.; Allen's S.P.; Janda, W.C. (2006). "Color Atlas and Textbook of Diagnostic Microbiology" 6th ed. Lippincott-Willams and Wilkins Publishers, Philadephia, USA.

Leavis, H. L.; Bonten, M. J., & Willems, R. J. (2006). Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. Curr. Opin. Microbiol, 9(5): 454-60.

Molander, A.; Reit, C.; Dahlen, G., &Kvist, T. (1998). Microbiological status of root-filled teeth with apical periodontitis. Int. End. j., 31(1): 1-7.

Raad, I. I.; Hanna, H. A.; Boktour, M.; Chaiban, G.; Hachem, R. Y.; Dvorak, T., Lewis, R. & Murray, B. E. (2005). Vancomycin-resistant Enterococcus faecium: catheter colonization,

esp gene, and decreased susceptibility to antibiotics in biofilm. Antimicrob. Agents. Chemother, 49(12): 5046-50.

Siqueira, J. F., &Rôças, I. N. (2005). Uncultivated phylotypes and newly named species associated with primary and persistent endodontic infections. J. Clin. Microbiol., 43(7): 3314.

Tenover, F. C., & McDonald, L. C. (2005). Vancomycin-resistant staphylococci and enterococci: epidemiology and control. Current Opinion in Infect. Dis., 18(4): 300-305.

Uma, ch.; Shamma, M. and Yadav, A. (2007). Antimicrobial susceptibility patterns of common and unusual enterococcus species isolated from clinical specimens. J. Infect. Dis. Antimicrob. Agents, 24(2): 55-62.

Wilcks, A., Andersen, S. R., & Licht, T. R. (2005). Characterization of transferable tetracycline resistance genes in Enterococcus faecalis isolated from raw food. FEMS microbiology letters, 243(1): 15-19.