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Research Article

DISTRIBUTION OF VIRULENCE FACTORS AMONG VANCOMYCIN RESISTANT *ENTEROCOCCUS FAECALIS* FROM DENTAL ISOLATES

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ABSTRACT

Objective: *Enterococcus faecalis* causing serious infections especially as a nosocomial pathogen was reinforced in many epidemiological studies. Many virulence factors were found to be involved in the pathogenesis of enterococcal infections and understanding of those factors are still limited. The aim of this study was to detect the presence of seven virulence genes in *E. faecalis* isolates from various dental conditions.

Methods: A total of 42 *E. faecalis* isolates that were found to be vancomycin resistant were studied. Identification of the isolates was done by biochemical methods and 16s rRNA and screened for the presence of virulence genes *eep*, *ace*, *asa1*, *asa373*, *enlA*, *fsr,* and *sprE* using PCR.

Results: All the 42 isolates were found to contain at least one and concomitantly up to as many as six virulence genes, with three or four being a common pattern. Most of the strains carried the *ace* gene (95%), and other genes were present at the frequency of 33% to 90% as well and 12% of the isolates carried *eep+ace+asa1+asa373+fsr+sprE* pattern in combination.

Conclusion: From the data, it was observed that with different dental (clinical) conditions both dental caries and gingivitis were found to have various and highest prevalence of virulence factors though all the virulence genes were observed randomly in all the isolates. It should be pointed out that gene silencing could play its part in virulence determinants regardless of mere presence of virulence gene.

Keywords: Virulence factors, Aggregation substance, Enterolysin, Collagen-binding protein, Molecular detection.

INTRODUCTION

The human dental cavity is colonized with large groups of aerobic and anaerobic bacterial species. *Enterococcus faecalis* as a nosocomial pathogen can cause serious infections that are frequently isolated (30-90%) from root canal treated patients [1]. The high prevalence of this species in root canal treated patients evidenced by culturing methods, and molecular detection tools suggested that it may be the reason for most of the endodontic treatment failures. Virulence factors of *E. faecalis* play a key role in its pathogenicity, and some of the most important factors are adherence, aggregation formation, enterolysin/cytolysin, and pheromone secretion [2]. Investigating virulence gene prevalence in *E. faecalis* would be useful in predicting its role in dental infections [3].

Considering virulence genes such as *eep* (enhanced expression of pheromone), *ace* (collagen-binding protein), *asa1* and *asa373* (aggregation substance), *enlA* (enterolysin), *fsr* (quorum sensing system), and *sprE* (serine protease) each playing its critical role in the pathogenesis of *E. faecalis* in dental infections. In this study, the prevalence of virulence genes from dental isolates was investigated, and further study was done to report its importance in various dental infections. Ace protein which is specific for *E. faecalis* that mediates bacterial adhesion and characteristic binding to collagen type proteins plays an important role in pathogenesis [4]. Colonization of *E. faecalis* was associated with *asa* gene and its presence can be stimulated by peptide pheromone (*eep*) from other nearby enterococci [5]. Quorum sensing system, *fsr* was found to regulate both *gelE-sprE* downstream, and both gelatinase (*gelE*) and serine protease (*sprE*) activities were also found in non-*fsr* strains and in natural conditions were gelatinase-negative strains carrying *gelE* were also found [6]. *fsr-gelE* system also found to involve in ace cell surface expression and disruption of *fsr* or *gelE* found to increase collagen adherence of *E. faecalis* [7]. Other studies were also reported virulence factors in *E. faecalis* [9-14]. Considering the reports, we studied the distribution of seven virulence genes in vancomycin resistant *E. faecalis* isolates and compared the data in combination with virulence observed in different clinical conditions. Interestingly, results indicated the combination of virulence determinants in each clinical condition.

METHODS

Identification of isolates

A total of 42 *E. faecalis* isolates from various clinical conditions such as dental caries, chronic periodontitis, gingivitis, grossly dental caries, and periodontal abscess were included in the present study. Samples were collected from the dental science department (Institutional ethics was cleared to use bacterial isolates from human samples). All the stored isolates were recovered using BHI medium. ATCC29212 was used as a positive control for further studies.

Biochemical characterization

Biochemical characterization was performed by ethyl violet azide agar (EVA) and arabinose fermentation test. For EVA test, all the isolates were streaked over EVA agar plates, and after overnight incubation, the presence of greenish white colonies indicated the *Enterococcus* species. Arabinose fermentation test was done by phenol red as an indicator and *Salmonella typhi* as a positive control. Formation of yellow indicated the arabinose utilization.

Molecular studies

All the 42 isolates were subjected to genotypic analysis by 16s rRNA primer sequences, and PCR conditions were retrieved from Sedgley *et al.* [18] and Salah *et al.*, 2008 [15], respectively. Molecular detection of virulence genes such as *eep*, *ace*, *asa1*, *asa373*, *enlA*, *fsr,* and *sprE* were done using sequence-specific primer sequences (Table 1). The PCR products were run on 1.5% agarose gel and visualized under UV transilluminator (UVP upland, USA) and documented using gel

Table 1: Oligonucleotide primers used in this study

S. No.	Gene	Primer sequence	References
1.	*eep*	F: 5'-GAGCGGGTATTTTAGTTCGT-3' R: 5'-TACTCCAGCATTGGATGCT-3'	[16]
2.	*ace*	F: 5'-CTATTGTCAACTTCTGAAAAAG-3' R: 5'-GAGAACTATTGGTGATAAGCG-3'	[4,18]
3.	*asa1*	F: 5'-CCAGCCAACTATGGCGGAATC-3' R: 5'-CCTGTCGCAAGATCGACTGTA-3'	[3]
4.	*asa373*	F: 5'-GGACGCACGTACACAAAGCTAC-3' R: 5'-CTGGGTGTGATTCCGCTGTTA-3'	[3]
5.	*enlA*	F: 5'-TTCTTCTTATTCTGTCAACGCAGC-3' R: 5'-GACTGTGAAATACCTATTTGCAAGC-3'	[16]
6.	*fsr*	F: 5'-AACCAGAATCGACCAATGAAT-3' R: 5'-GCCCCTCATAACTCAATACC-3'	[17]
7.	*sprE*	F: 5'-CAGGTGGTCAATCTGGTTCC-3' R: 5'-CTGCTGGCACAGCGGATA-3'	[15]

documentation system. Single isolate from each gene product was sequenced and submitted to NCBI nucleotide database, and accession numbers were obtained.

RESULTS AND DISCUSSION

Identification and biochemical characterization

Isolates collected (42) from different clinical conditions includes 30 from dental caries, 4 from chronic periodontitis, 4 from gingivitis, 1 from grossly dental caries, and 3 from a periodontal abscess. All the isolates were found to be resistant to vancomycin in earlier studies using antibiotic susceptibility tests and MIC. EVA test results showed that all the 42 isolates were *Enterococcus* species by the formation of greenish white colonies and arabinose fermentations test also indicated that none of 42 isolates were capable of fermenting sugar (pink) with regard to yellow observed in positive control.

Molecular studies

For all the 42 isolates, 16s rRNA was done and confirmed as *E. faecalis* from amplified products (Fig. 1). Further studies on detection of virulence genes *eep*, *ace*, *asa1*, *asa373*, *enlA*, *fsr,* and *sprE* were done and found that 20 isolates (48%) had *eep* gene, 40 had *ace* (95%), 27 had *asa1 (*65%), 14 had *asa373 (*33%), 8 had *enlA* (19%), 34 had *fsr* (81%), and 38 had *sprE* (90%), respectively (Fig. 2). (Accession numbers: *eep* (KT222185), *enlA* (KF020735), *fsr* (KF020737), and *sprE* (KF020736)).

DISCUSSION

With increasing resistance to some common antibiotics, enterococcal infections pose a very big threat as a nosocomial pathogen. *E. faecalis* was found to cause 90% of the enterococcal infections in humans, and it was frequently found in obturated root canals exhibiting symptoms of chronic apical periodontitis, especially in post dental monocultures [19-23]. In a previous study, by Salah et al. there was no *E. faecalis* found in healthy patients, whereas isolates collected from different dental diseased patients carried virulence genes, *ace* (100%), *efaA* (100%), and *cylA* (25%).

In our study, distributed virulence factors were observed. The presence of *eep* in 48% of the isolates indicates that bacterial pheromone secretion is necessary for inducing conjugation, and confirming its role in different dental conditions. It was also reported that *eep* also involved in biofilm formation [24] and it also provides lysozyme resistance to the host. Collagen-binding protein, *ace* gene was found in 95% (n=40) of the isolates indicating its ability to bind with dental collagen, used for dressing oral wounds. Out of 40 *ace* positive isolates, 14 of them were found to be lacking both *fsr,* and *gelE,* explaining its importance in collagen adherence because gelatinase activity was found to inhibit the Ace expression in previous studies [7]. *Asa1* and *asa373* were found in 65% and 33%, respectively, and all the *eep* positive isolates were found to have the aggregation substance (*asa*) gene that may be due

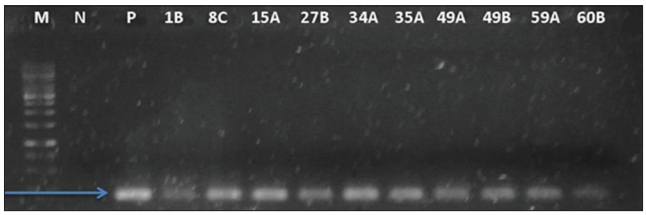

Fig. 1: Amplified products of 16s rRNA for identification of *Enterococcus faecalis* (M: Marker, N: Negative control, P: Positive control, Lane: 4-13 test isolates)

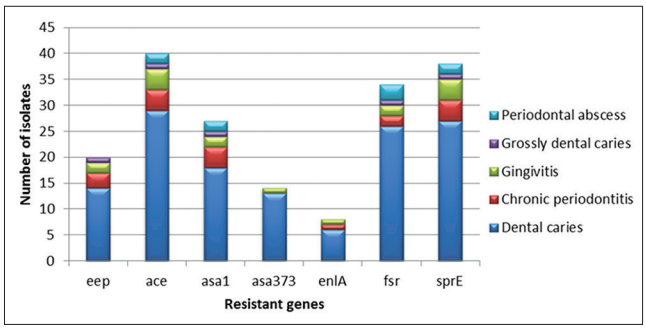

Fig. 2: Comparison of virulence determinants distributed among *Enterococcus faecalis* isolated from various clinical conditions

to positive effects of pheromone secretion on enterococci aggregation formation [5]. Enterolysin gene *enlA* was found only in 8 isolates (19%). The presence of *fsr* gene in 81% (n=34) of the isolates and its downstream genes *sprE* in 90.4% (n=38) of the isolates, and *gelE* (in another study) gene in 62% (n=26) of the isolates indicated that 98% of the isolates carried either of these three genes irrespective of its presence, and interestingly *fsr* was always found in combination with either *sprE* or *gelE*. 52% of the isolates carried all the three genes, and only one isolate was identified to lack all the three genes.

Isolates included in this study were identified as contain at least one gene and as many as six, with three or four being a common pattern. The high prevalence was found with the *ace* gene, and the most observed patterns were *eep+ace+asa1+fsr+sprE*. Out of seven genes detected, it was also identified that 17%, 24%, and 21% of the isolates carried a combination of 6, 5, or 4 genes. Most observed combination in dental caries was *eep+ace+asa1+fsr+sprE*, chronic periodontitis was *ace+asa1+fsr+sprE*, gingivitis was *eep+ace+asa1+sprE*, grossly dental caries *eep+ace+fsr+sprE,* and periodontal abscess *ace+fsr+sprE*. The role of *E. faecalis* in nosocomial infections such as urinary tract infection (UTI) and surgical site infection (SSI) are severe, and a study reported the presence of *E. faecalis* in 87.5% of UTI and SSI

patients [25]. So, the presence of resistant-virulent *E. faecalis* in a hospital environment is a very serious concern. Our data showed that virulence factors were noted to be distributed among all dental isolates regardless of its clinical condition. Both dental caries and gingivitis conditions had the highest prevalence of virulence factors. Gene silencing may also interfere with our data because the presence of virulence gene does not confer its activity but the presence of virulence gene was observed to be the cause for numerous resistant isolates in clinical settings.

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