UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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SIMILARIDADES GENOTÍPICAS E ANÁLISE DA PATOGENICIDADE DE *PREVOTELLA* SPP. ISOLADAS DO BIOFILME SUPRAGENGIVAL DE PACIENTES COM FIBROSE CÍSTICA.

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Trabalho de Conclusão de Curso apresentado à Comissão de Graduação da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgião-Dentista.

Orientador: Prof. Dr. Juliano Cavagni

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APRESENTAÇÃO

O presente estudo foi desenvolvido na Instituição *The Queen`s University of Belfast*, Reino Unido, por meio de uma bolsa de estudos ofertada pelo programa Ciências sem Fronteiras - CAPES. O mesmo é parte integrante de um projeto amplo com objetivo de avaliar o papel da infecção por bactérias anaeróbias na fibrose cística. Todas as análises a serem apresentadas neste trabalho de conclusão de curso foram realizadas no laboratório *CF & Airways Microbiology Research Group*, como parte do programa de graduação em Microbiologia, durante o período de Graduação Sanduíche na Universidade supracitada.

O trabalho foi concebido de acordo com o artigo 1º do regulamento geral do trabalho de conclusão de curso, sendo composto por uma introdução, um artigo científico a ser submetido a uma revista de circulação internacional e conclusões.

RESUMO

RANZAN, Nicolle. **Similaridades genotípicas e análise da patogenicidade de *Prevotella* spp. isoladas do biofilme supragengival de pacientes com fibrose cística.** 2014. 29 f. Trabalho de Conclusão de Curso (Graduação em Odontologia) - Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2014.

O objetivo do estudo foi o de investigar a expressão de fatores de virulência (beta-lactamase, cápsula e hemólise), determinar a suscetibilidade antimicrobiana e as características genotípicas de espécies de *Prevotella* retiradas da placa supragengival de pacientes com fibrose cística (FC). Para isso, o biofilme supragengival de sete adultos portadores de FC estável foi coletado e imediatamente levado a uma câmara de anaerobiose. As bactérias do gênero *Prevotella* foram selecionadas para o estudo baseado na sequência 16S rRNA, totalizando uma amostra de 16 diferentes espécies bacterianas de *Prevotella*. A produção da enzima beta-lactamase foi analisada por meio de uma solução de nitrocefina. A presença de cápsula foi analisada utilizando um microscópio e a capacidade de hemólise verificada utilizando-se ágar sangue. A suscetibilidade antimicrobiana da amostra foi testada para 12 diferentes antibióticos, utilizando-se E-test®. A análise genotípica inter e intra espécie foi realizada através da técnica de Eletroforese em Campo Pulsante. Como resultados, mais de 50% das espécies de *Prevotella* produziram a enzima beta-lactamase e hemólise e aproximadamente 40% apresentaram cápsula. A amostra testada apresentou resistência antimicrobiana para os antibióticos clindamicina, metronidazol, tetraciclina e tobramicina. Ainda, foram observadas características genotípicas similares em uma análise intra espécie da amostra de *Prevotella* analisada. Assim, conclui-se que espécies de *Prevotella* da placa dentária de pacientes com fibrose cística foram capazes de expressarem fatores de virulência, bem como apresentaram resistência antimicrobiana. Ainda, observou-se características genotípicas similares entre a amostra.

Palavras-chave: Fibrose Cística. Depósitos dentários. *Prevotella*.

ABSTRACT

RANZAN, Nicolle. **Genotypic similarities and potential pathogenicity of supragingival *Prevotella* strains isolated from cystic fibrosis patients**. 2014. 29 f. Final Paper (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2014.

The aims of the study were to investigate the production of virulence factors (beta-lactamase, haemolysis and capsule), to determine the antibiotic susceptibility and the genotypic characteristics of *Prevotella* species from dental plaque of cystic fibrosis (CF) patients. For this, supragingival biofilm of seven CF stable patients was collected and immediately placed into the anaerobic cabinet. A PCR reaction was carried out to amplify the 16S rRNA gene and *Prevotella* species were selected to the study, with a total of 16 different species of *Prevotella*. Nitrocefin solution was used to identify if *Prevotella* isolates were capable to produce β-lactamases. The presence of capsules was observed using a microscope and the haemolysis activity analysed using Supplemented Brucella Blood Agar (SBBA). E-tests® were used to test the antimicrobial susceptibility to 12 different antibiotics. Genotypic characteristics intra and inter-species were analysed using Pulsed Field Gel Electrophoresis (PFGE) method. As a result, more than 50% of *Prevotella* species from dental plaque of CF patients were capable to produce beta-lactamases and haemolysis and approximately 40% of them presented capsules. Furthermore, the isolates were resistant to the antibiotics clindamycin, metronidazole, tetracycline, and tobramycin. The study found closely related genotypic characteristics among *Prevotella* species, using PFGE method. In conclusion, *Prevotella* species from dental plaque of CF patients were capable to produce virulence factors and also presented antibiotic resistance. Additionally, some isolates were genetically similar.

Keywords: Cystic Fibrosis. Dental deposits. *Prevotella*.

SUMÁRIO

1 INTRODUÇÃO

Acometimentos pulmonares representam a maior causa de morbidade e mortalidade em pacientes com fibrose cística (FC) (1, 3) e esta é considerada a doença genética de maior caráter letal entre Caucasianos (2). A FC ocorre devido a uma mutação em uma proteína de membrana de células exócrinas (4, 5), fato que gera a produção de um muco espesso nas vias aéreas (6), causando uma infecção crônica e inflamação nos pulmões (6-8).

A doença pulmonar na FC tem uma origem polimicrobiana de infecção, com a presença de bactérias aeróbias e anaeróbias (6, 9). As bactérias comumente associadas a FC são *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus*, e membros do complexo *Burkholderia cepacia* (9-11). Recentemente, uma das bactérias anaeróbias predominantemente identificada no escarro pulmonar de pacientes com FC foi a *Prevotella* (12-14).

Alguns estudos sugerem que a cavidade bucal possivelmente seja um reservatório de patógenos associados à FC, incluindo espécies de *Prevotella* (13, 16-19). Um estudo que investigou a flora bacteriana normal da cavidade bucal mostrou que espécies de *Prevotella* são bactérias comuns presentes na língua, palato mole e duro, tonsilas, superfície dentária e placa subgengival (20). Sugeriu-se que a colonização bacteriana pulmonar de pacientes com FC está associada com patógenos de transição ou devido à flora orofaríngea (15). Ainda, associou-se a cavidade bucal como sendo um reservatório de bactérias respiratórias devido à relação anatômica entre os pulmões e a cavidade bucal (19).

Como a literatura associa a cavidade bucal como um reservatório de patógenos respiratórios, é razoável sugerir a associação e migração de espécies de *Prevotella* do biofilme bucal para o pulmão de pacientes com FC. Assim, os objetivos do presente estudo foram investigar os fatores de virulência, determinar a resistência antimicrobiana e características genotípicas de espécies de *Prevotella* retiradas da placa supragengival de pacientes com FC.

2 ARTIGO

Genotypic similarities and potential pathogenicity of supragingival *Prevotella* strains isolated from cystic fibrosis patients.

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INTRODUCTION

Lung disease is the foremost cause of morbidity and mortality among patients with cystic fibrosis (CF) (1, 3) and the most potentially lethal genetic disease among Caucasians (2). It is caused by a mutation in the CF transmembrane protein of exocrine cells (4, 5), which stimulates the secretion of thickened mucus on the airways (6), leading to persistent infection and inflammation in the lungs (6-8).

CF lung disease has a polymicrobial nature of infection, with the presence of both aerobic and anaerobic bacterial pathogens (6, 9). Different species of bacteria can be regularly found in CF pulmonary infections, such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus*, and members of the *Burkholderia cepacia* complex (9-11). Recently, one of the anaerobic bacteria predominantly identified in the sputum samples of CF patients is within the genus *Prevotella* (12-14).

There are some studies that proposed the oral cavity as a potential reservoir of CF pathogens, including *Prevotella* species (13, 16-19). A study investigating the normal bacterial flora of the oral cavity showed that *Prevotella* are a common microorganism of tongue, hard and soft palate, tonsils, tooth surface, and subgingival plaque (20). It has been suggested that bacterial communities that colonize the lung of CF patients are originated from either transient pathogens or due to oropharyngeal flora (15). Furthermore, it was proposed that due to the anatomical structure between the lungs and the oral cavity, it is possible that the latter be a reservoir of respiratory bacteria (19).

As the literature identifies the mouth as a reservoir of respiratory pathogens, it is reasonable to assume the association and migration of *Prevotella* from the oral biofilm to the lungs of CF patients. Thus, the aims of this study were to investigate the virulence factors that *Prevotella* species from supragingival plaque of CF patients might produce, to determine the antibiotic susceptibility phenotype and the genotypic characteristics of the isolates, since they might contribute to the pulmonary infection in CF patients.

METHODS

STUDY DESIGN

All the experiments were done *in vitro*, at the laboratory CF and Airways Microbiology Research Group, at The Queen`s University of Belfast, United Kingdom. The present study was approved by the Office of Research Ethics Committees in Northern Ireland (ORECNI).

PREVOTELLA IDENTIFICATION

Plaque samples were collected from UK CF patients (n=7) attending the Adult Cystic Fibrosis Centre at the Belfast City Hospital, Belfast, United Kingdom. A hospital professional dentist collected the dental plaque using a periodontal probe from a site/tooth that presented an accumulation of oral biofilm. After collection, the dental plaque was placed into a sterile eppendorf with 1ml of Phosphate Buffered Saline (PBS) buffer, which was put into an anaerobic pouch immediately and then into the anaerobic cabinet. A serial dilution was performed and 100µl of each dilution was plated out onto various agar (ABA, KVLB, SBBA). The same process was repeated after, but aerobically. After incubation of 5 days to allow an anaerobic growth, each colony that was different was picked off the agar plate and streaked onto a fresh agar plate. After pure growth was achieved, a loopful of each isolate was stored in 1ml blood and DNA extracted using a ZR Fecal MiniPrep kit. A PCR reaction was carried out with the DNA to amplify the 16S rRNA gene. This PCR product was purified and sent to a company for sequencing. After the determination of the DNA sequences, they were compared with online database sequences, using BLAST, to determine the *Prevotella* species used in this study (Table 1).

Table 1- *Prevotella* species isolated from dental plaque of adult CF patients that were used in this study. Shaded colours are representing 7 different patients on their first visit to the Adult CF Clinic.

Isolate	Genus	Species	From
B115-V1-S1-PLQ-P	*Prevotella*	*denticola*	CF stable patients attending the Adult CF Centre at Belfast City Hospital, Belfast, UK.
B115-V1-S1-PLQ-U	*Prevotella*	*intermedia*	
B116-V1-S1-PLQ-V	*Prevotella*	*maculosa*	
B116-V1-S1-PLQ-X	*Prevotella*	*nigrescens*	
B122-V1-S1-PLQ-R	*Prevotella*	*histicola*	
B122-V1-S1-PLQ-U	*Prevotella*	*histicola*	
B125-V1-S1-PLQ-R	*Prevotella*	*histicola*	
B125-V1-S1-PLQ-T	*Prevotella*	*salivae*	
B125-V1-S1-PLQ-W	*Prevotella*	*salivae*	
B126-V1-S1-PLQ-W	*Prevotella*	*denticola*	
B126-V1-S1-PLQ-Y	*Prevotella*	*intermedia*	
B132-V1-S1-PLQ-S	*Prevotella*	*nigrescens*	
B132-V1-S1-PLQ-T	*Prevotella*	*histicola*	
B132-V1-S1-PLQ-U	*Prevotella*	*histicola*	
B140-V1-S1-PLQ-O	*Prevotella*	*histicola*	
B140-V1-S1-PLQ-P	*Prevotella*	*histicola*	

CULTURE OF *PREVOTELLA* SPECIES

An anaerobic cabinet was used throughout all the laboratory experiments, with strict anaerobic conditions (10% Hydrogen, 10% Carbon Dioxide and 80% Nitrogen). After identification, all the *Prevotella* isolates used were previously stored in 1ml defibrinated horse blood (TCS Biosciences, United Kingdom) or Microbank beads and froze at -80°C. Supplemented Brucella Blood Agar (SBBA) plates were used for the culture of *Prevotella* isolates, under anaerobic conditions. The SBBA plates were previously incubated under anaerobic conditions for 48 hours. One plate was used as an aerobic control by incubating aerobically at 37^{o}C for 2 days and the other plate

was examined for anaerobic growth after incubation for 2-5 days. *Prevotella* isolates were considered pure if they were Gram-negative, strictly anaerobic and rod-shaped.

SCREENING FOR PRESENCE OF A CAPSULE

Colonies of the isolates were used to prepare bacterial broth, using a basal anaerobic media, and then the broth was incubated anaerobically for 48 hours. After bacterial growth in broth, capsule smear procedure was conducted using carbon fuchsin and eosin solution for staining the capsule. The smear was examined using bright-field microscopy (x100 objective lens). All the images of capsule smears were compared to a positive control strain (Bacteroides fragilis, NCTC 9343). A Premier® digital microscope eyepiece (Key Scientific Products, Texas, USA) was used to record images of capsule smears.

β-LACTAMASE ASSAY

After 60 minutes of suspension of bacterial colonies into nitrocefin solution, it was observed the colour change of the assay. If a colour change (yellow to red) was produced, then the isolate was positive for β-lactamase production. All the outcomes for β-lactamases production were compared to a control bacterial free sample, with just nitrocefin solution. The control sample had a stable yellow colour that did not change over 60 minutes.

HAEMOLYSIS ACTIVITY

Cultures of *Prevotella* were streaked onto an SBBA plate and then incubated at 37^{o}C, under anaerobic conditions for 5 days. Areas for haemolysis (clearing or degradation of the media) were examined and compared to a negative control plate with no bacterial growth. All results were read by a second expert investigator independently and all the results were agreed.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

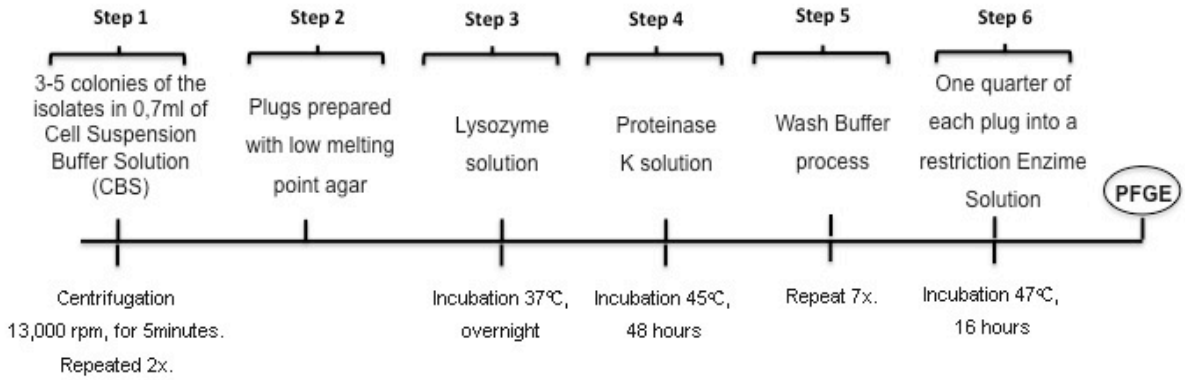
All the isolates were tested to 12 different antibiotics, using E-test®. Controls strains were used according to manufacture`s instructions and the Clinical and Laboratory Standards Institute (CLSI). The reagents and test procedure were considered satisfactory when minimum inhibitory concentration (MIC) values fell within the quality control ranges.

After the period of incubation required for each isolate, MIC was read directly from the strip scale where a symmetrical inhibition ellipse intersected the strip. All MIC values were read by a second expert investigator independently and all the results were agreed. *Prevotella* isolates were classified as susceptible, intermediate or resistant to the antibiotics.

PULSED FIELD GEL ELECTROPHORESIS

To start the Pulsed Field Gel Electrophoresis (PFGE) method, a few steps were previously conducted with the *Prevotella* isolates. These steps were described in Figure 1.

Figure 1 – Flowchart of 6 steps that were done previously of the PFGE method.



*Restriction Enzime used was *Xba I*.

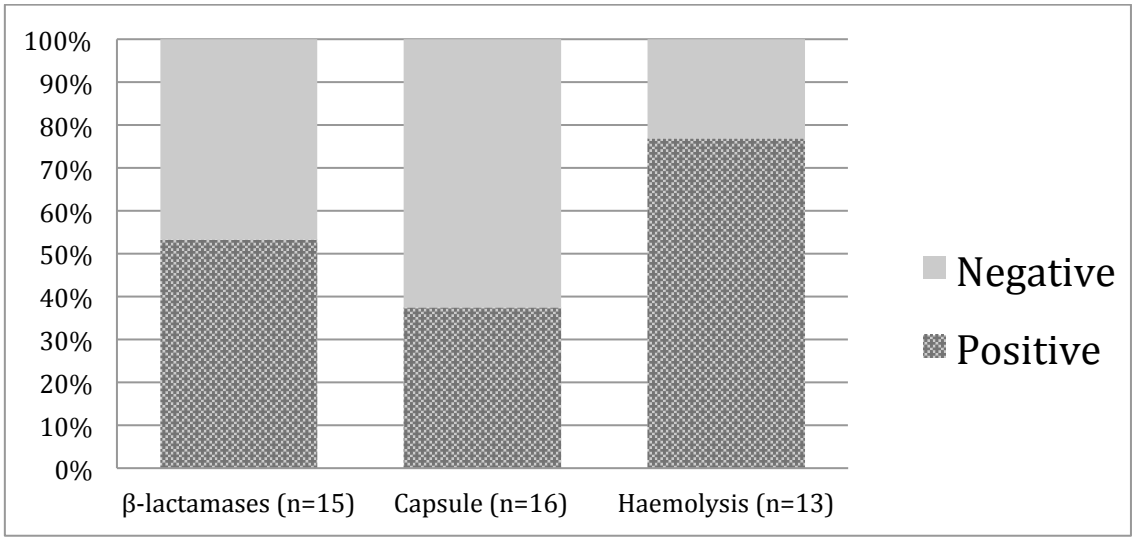
For the PFGE process, a gel was made of 1% of agarose. There were 15 wells in the gel to fill with one quarter of the plug that was previously incubated. However, the first, 8th and 15th wells were reserved for the size marker ladder

(lambda DNA fragment that sizes 50-1,000 kb). After 20 hours of running in the PFGE system, the gel was stained using ethidium bromide and de-stained in water. The visualization of the bands could be seen using UV transilluminator equipment, and the banding patterns analysed using a software named Gel Compar II V5.1.

RESULTS

The β-lactamase assay showed that 8/15 *Prevotella* isolates produced β-lactamase enzyme and that 6/16 *Prevotella* isolates presented capsule. Haemolysis screen procedure showed that from 13 *Prevotella* isolates analysed, 10 isolates produced haemolysis. All the results for the virulence factors are demonstrated in Figure 2.

Figure 2 - Percentages of isolates with capsule, β-lactamase production and haemolysis activity from *Prevotella* isolates.



The Minimum Inhibitory Concentration (MIC) range, MIC_{50}, MIC_{90} and the percentage of the isolates that were considered susceptible (S), intermediate (I) and resistant (R) to the 12 antibiotics tested are presented in Table 2. The isolates tested for the antibiotics amoxicillin, azithromycin, ceftazidime, doxycycline, and tobramycin were not classified as susceptible, intermediate or resistant, as there are no anaerobic breakpoints approved by the CLSI for these antibiotics.

Table 2 - Antimicrobial susceptibility testing of *Prevotella* isolates from dental plaque of CF patients.

Antimicrobial agent (number of isolates tested)	MIC (µg/ml)			% of isolates with indicated susceptibility		
	Range	**50%**	**90%**	**S**	**I**	**R**
Amoxicillin (11)	<0.016->256	0.75	>256	NA**	NA	NA
Amoxicillin/clavulanic acid (11)	<0.016-6	0.064	1.5	91	9	0
Azithromycin (11)	0.032->256	192	>256	NA	NA	NA
Ceftazidime (12)	0.50->256	3	>256	NA	NA	NA
Chloramphenicol (10)	0.50-8	2	4	100	0	0
Clindamycin (12)	<0.016->256	0.032	>256	50	0	50
Doxycycline (12)	0.047->256	6	16	NA	NA	NA
Meropenem (10)	0.023-0.38	0.094	0.25	100	0	0
Metronidazole (12)	<0.016->256	0.064	0.19	92	0	8
Piperacillin/tazobactam (11)	0.016-1.0	0.25	0.75	100	0	0
Tetracycline (11)	0.023->256	24	>256	27	0	73
Tobramycin (10)	>1024	>1024	>1024	NA	NA	NA

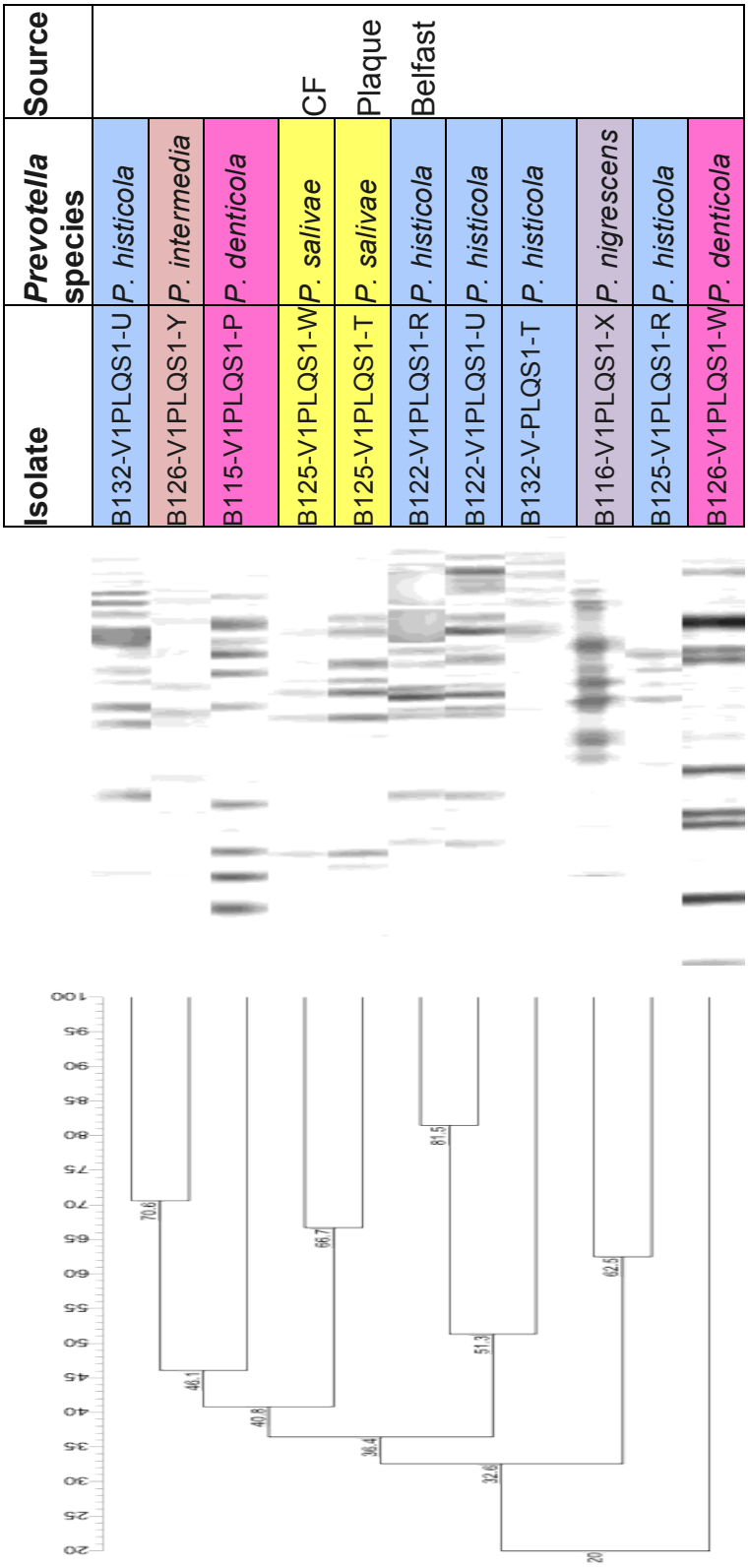
MIC_{50} and MIC_{90} (antibiotic concentration required to inhibit growth of 50% and of 90% of isolates, respectively); NA: no anaerobic breakpoints approved by the Clinical and Laboratory Standards Institute (CLSI).

All the isolates tested were susceptible to chloramphenicol (n=10), meropenem (n=10), and piperacillin/tazobactam (n=11). Moreover, MIC_{90} presented a susceptible range for the antibiotics amoxicillin/clavulanic acid, chloramphenicol, meropenem, metronidazole and piperacillin/tazobactam. However, MIC_{90} fell within a

resistant range to clindamycin and tetracycline. Some isolates were considered resistant to clindamycin (50%), metronidazole (8%) and tetracycline (73%). From the antimicrobials tested, only amoxicillin/clavulanic acid presented isolates with intermediate susceptibility (9%). Although there is no anaerobic breakpoint approved by CLSI to tobramycin, all the isolates (n=10) were resistant to this antibiotic, given that the growth occurred along the entire E-test® strip and no inhibition ellipse was visualized when tobramycin was tested.

PFGE typing results for 11 *Prevotella* isolates are described in the Figure 3. The cut-off value used on the dendrogram to describe if the isolates were genetically similar was 60%. Values under 60% demonstrated no genetic similarity among the isolates tested.

Figure 3 - Dendrogram constructed using DICE coefficient (optimization 0.49% and position tolerance 2.3%) indicating genetic relationships for 11 *Prevotella* isolates based on the banding patterns produced by PFGE.



A low degree of intra-species homology was observed when the isolates were from different patients. An example of that is a low degree of intra-species homology among three *P. histicola* (B122-V1-PLQ-S1-R, B122-V1-PLQ-S1-U and B132-V1-PLQ-S1-T), from two different patients. Similarly, *P. denticola* (B115-V1-PLQ-S1-P and B126-V1-PLQ-S1-W) from different patients also presented low degree of intra-species homology, with 20% of similarity. However, PFGE also demonstrated a relative high degree of intra-species similarity when a comparison between *Prevotella* isolates was done from the same patient. For example, *P. salivae* isolates (B125-V1-PLQ-S1-T and B125-V1-PLQ-S1-W) from the same patient showed 66.7% of similarity and both *P. histicola* isolates (B122-V1-PLQ-S1-R and B122-V1-PLQ-S1-U) from the same patient were considered highly similar by the PFGE method, with 81.5% of similarity.

A low degree of inter-species homology was also observed at the individual level among the majority of *Prevotella* species, e.g., *P. intermedia* (B126-V1-PLQ-S1-Y) and *P. denticola* (B126-V1-PLQ-S1-W), with 20% of similarity. However, *P. histicola* (B132-V1-PLQ-S1-U) and *P. intermedia* (B126-V1-PLQ-S1-Y) presented a relative high percentage of similarity as well as *P. nigrescens* (B116-V1-PLQ-S1-X) and *P. histicola* (B125-V1-PLQ-S1-R).

DISCUSSION

Studies have suggested the oral cavity as a possible source of pathogens to the lung of CF patients (13, 16-19). Given the fact that *Prevotella* species are common microorganisms in the oral cavity (20) and that they were found in high numbers in the sputum of CF patients (12), the present study had the aim to investigate *Prevotella* species from dental plaque of CF patients. Virulence factors, such as the presence of capsules, β-lactamase production, and haemolysis activity were observed in *Prevotella* species in the present study, in the approximately frequency of 40%, 55%, and 75%, respectively. It has been suggested that capsules protect microorganisms from phagocytosis by polymorphonuclear leukocytes and promote bacteria adhesion to host surfaces. Furthermore, capsules are considered the major pathogenicity factor of bacteria (21, 22). Haemolysis is characterized by

the ability of a bacterium to lyse erythrocytes and also possibly play a role in lysing other cells types, such as neutrophils (38). The results for the virulence factors presented are in accordance with other studies that also detected these virulence factors in *Prevotella* species (23-29).

A high number of isolates were capable of producing β-lactamases in the present study, which might result an important impact in the antibiotic treatment of CF lung disease, especially beta-lactam antibiotics, such as penicillin and cephalosporins. Accordingly, it was suggested that some bacteria that are capable of producing β-lactamases have the ability to release the free enzyme into the host environment and it might protect other penicillin-susceptible pathogens from the penicillin activity (30). Consequently, this fact suggests that penicillin and cephalosporin therapy may result in an unsuccessful treatment to patients with CF lung disease.

The antibiotics amoxicillin, azithromycin, amoxicillin/clavulanic acid, ceftazidime, chloramphenicol, clindamycin, doxycycline, meropenem, piperacillin/tazobactam tested in this study are largely used in the antibiotic treatment of CF lung infection and they demonstrate anaerobic activity against anaerobic bacteria. The antibiotics metronidazole and tetracycline are not used in CF lung treatment, but have putative anaerobic activity. In contrast, tobramycin has no anaerobic activity, but it is routinely used in the treatment of CF lung infection, especially against *Pseudomonas aeruginosa* infection, which is a common pathogen in CF (31). Despite of the fact that tobramycin does not present an anaerobic breakpoint approved by CLSI, the present study considered that all the isolates were resistant to this antibiotic, given that it was possible to observe *Prevotella* growth in all the E-tests® tested.

The present study showed that some of the *Prevotella* isolates tested were susceptible to the antibiotics chloramphenicol, meropenem and piperacillin/tazobactam. In addition, 50% of the isolates tested were resistant to clindamycin, which is used for the CF lung disease treatment. Some isolates also presented resistance to the antibiotics metronidazole and tetracycline, however they are not commonly used on the CF treatment. Interestingly, Tunney and colleagues (12) also found resistant strains to clindamycin and metronidazole. Additionally, in the

same study, they showed that more than 50% of *Prevotella* species tested were susceptible to meropenem and more than 90% were susceptible to piperacillin/tazobactam, in accordance with the present study. Worlitzsch *et al.* (13) also observed that *Prevotella* isolates were susceptible to the antibiotics piperacillin/tazobactam and meropenem tested, but only 3 strains were tested in the study. These findings show that some results for antimicrobial susceptibility testing in the present study are in accordance with previous studies.

Prevotella also was previously reported as containing antibiotic resistance genes that can be transmitted to others bacterial populations by horizontal gene transfer (32). This fact is a concern because it demonstrates a bacterial strategy to spread antibiotic resistance among different bacterial populations. Thus, it is important to consider the antibiotic treatment that is going to be used during the treatment of CF patients and also use an antibiotic that is capable to target *Prevotella* species.

The PFGE method was described in the literature as a technique to overcome the limitations of the conventional (steady-field) agarose gel electrophoresis, due to the fact that PFGE allows the separation of large DNA fragments (1 kb up to 1,000 kb), while the conventional method just separates DNA fragments of 0.5 kb to 25 kb (33, 34). Furthermore, PFGE was previously used to compare DNA segmentation patterns of *Prevotella* species (35). A 60% of similarity was the cut-off value used in this present study to classify if the *Prevotella* species were genetic related. In fact, interpretations in the PFGE method are not very well established for *Prevotella* species. However, a 60% cut-off value was also used in a previous study using the PFGE method (36) .

Tenover and colleagues (37) described a method for the interpretation of the PFGE results. They classified the PFGE results as indistinguishable, closely related, possibly related and different, when there were 0, 2-3, 4-6 and ≥ 7 fragment differences compared to an outbreak, respectively. For example, using a 60% cutoff value, both *P. salivae* (B125-V1-PLQ-S1-W and B125-V1-PLQ-S1-T) were classed as genetically similar by the PFGE method and using the classification of Tenover *et al.* (37) they can be classed as closely related, given that 3 different bands are clearly possible to be seen when comparing these two isolates. Moreover, a limitation that

was observed in the present study was that different species of *Prevotella* were classed as genetically similar by the PFGE method, e.g., the isolates *P. histicola* (B132-V1-PLQ-S1-U) and *P. intermedia* (B126-V1-PLQ-S1-Y) from different patients presented more than 70% of similarity on the PFGE analysis. One possible reason for this outcome is that the dendrogram generated by the PFGE is more accurate as more bacterial isolates are presented in the test. In the present study just 11 from 16 isolates were tested, because some of the isolates didn`t present enough growth to perform the PFGE test. Therefore, despite of the limitations of the study regarding the PFGE method, the majority of different species of *Prevotella* showed no genetic similarity and a high degree of intra-species homology was observed, as it was expected.

Thus, it is possible to conclude that *Prevotella* isolates from dental plaque of CF patients were capable to produce virulence factors and presented antibiotic resistance for some antibiotics, including clindamycin and tobramycin, which are commonly used on the treatment of CF. Besides that, it was possible to observe that same species of *Prevotella* from the same patient tend to be genetically similar when compared to same species from different patients. These results altogether suggest that if *Prevotella* from the dental plaque can migrate to the airways of CF patients, an important attention to *Prevotella* is needed, as they might contribute to the pathogenicity of the CF disease, by the production of virulence factors and antimicrobial resistance.

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3 CONCLUSÃO

Os resultados deste trabalho demonstram que espécies de *Prevotella* do biofilme supragengival de pacientes com FC apresentaram fatores de virulência importantes, como também resistência antimicrobiana, especialmente à clindamicina, à tetraciclina e à tobramicina. Ainda, os isolados de *Prevotella* apresentaram similaridades genotípicas em uma análise intra-espécie e de um mesmo paciente. Assim, é de suma importância atentar para bactérias do gênero *Prevotella* da cavidade bucal de pacientes com fibrose cística, pois em caso de migração (translocação) para o pulmão, poderiam contribuir com o processo infeccioso crônico e com a patogenicidade de doença nesses pacientes.

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APÊNDICE A – CONTROL STRAINS USED FOR THE ANTIBIOTIC SUSCEPTIBILITY TESTING.

Table 1 - E-tests® used for the antibiotic susceptibility testing of *Prevotella* isolates and their appropriate control strains used.

Antibiotic tested	**Quality Control Strain**	**Quality Control Range (MIC - µg/ml)**
Amoxicillin (AC)	*Streptococcus pneumoniae* – ATCC 49619	0.032 - 0.125
Azithromycin (AZ)	*Staphylococcus aureus* – ATCC 29213	0.5 - 2
Amoxicillin/clavulanic acid (XL)	*Bacteroides fragilis* – ATCC 25285	0.25 - 1
Ceftazidime (TZ)	*Pseudomonas aeruginosa* – ATCC 27853	1 – 4
Chloramphenicol (CL)	*Bacteroides fragilis* – ATCC 25285	2 – 8
Clindamycin (CM)	*Bacteroides fragilis* – ATCC 25285	0.5 - 2
Doxycycline (DC)	*Staphylococcus aureus* – ATCC 29213	0.064 – 0.25
Meropenem (MP)	*Bacteroides fragilis* – ATCC 25285	0.064 – 0.25
Metronidazole (MZ)	*Bacteroides fragilis* – ATCC 25285	0.25 - 1
Piperacillin/Tazobactam (PTC)	*Bacteroides fragilis* – ATCC 25285	0.125 – 0.5
Tobramycin (TC)	*Bacteroides fragilis* – ATCC 25285	0.125 – 0.5
Tetracycline (TM)	*Pseudomonas aeruginosa* – ATCC 27853	0.25 - 1

APÊNDICE B – PFGE EQUIPMENTS AND MATERIALS

Figure 1 - Reagents, equipment and software used during the development of PFGE.

	Source	
Reagent	**Company**	**Location**
Brij® 58	Sigma	Dorset, United Kingdom
EtBr	Sigma	Dorset, United Kingdom
Lambda Ladder PFG Marker	New England Biolabs	Herts, United Kingdom
LMP – SeaKem® Gold	Lonza	Slough, United Kingdom
Lysozyme	Sigma	Dorset, United Kingdom
NaCl	Sigma	Dorset, United Kingdom
Na Citrate	BDH	Lutterworth, United Kingdom
Proteinase K	Sigma	Dorset, United Kingdom
Sodium deoxycholate	Sigma	Dorset, United Kingdom
Sodium lauryl sarcosine	Sigma	Dorset, United Kingdom
Sucrose	BDH	Lutterworth, United Kingdom
TBE	Bio-Rad	Hertfordshire, United Kingdom
UltraPure™ Agarose	Invitrogen	Paisley, United Kingdom
UltraPure™ EDTA (0.5M, pH8.0)	Invitrogen	Paisley, United Kingdom
UltraPure™ Tris HCL(1M,pH8.0	Invitrogen	Paisley, United Kingdom
X*ba* I	Invitrogen	Paisley, United Kingdom
Equipament & Software		
Anaerobic Cabinet – MG500 Workstation	Don Whitley Scientific	Shipley, United Kingdom
PFGE System – CHEF-DR® II	Bio-Rad	Hertfordshire, United Kingdom
PFGE System – CHEF-DR® III	Bio-Rad	Hertfordshire, United Kingdom
UV transilluminator – Gel Doc™	Bio-Rad	Hertfordshire, United Kingdom