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Production of extended-spectrum β-lactamases and the potential indirect pathogenic role of *Prevotella* isolates from the cystic fibrosis respiratory microbiota

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ABSTRACT

Extended-spectrum β-lactamase (ESBL) production and the prevalence of the β-lactamase-encoding gene bla_{TEM} were determined in *Prevotella* isolates ($n = 50$) cultured from the respiratory tract of adults and young people with cystic fibrosis (CF). Time-kill studies were used to investigate the concept of passive antibiotic resistance and to ascertain whether a β-lactamase-positive *Prevotella* isolate can protect a recognised CF pathogen from the action of ceftazidime in vitro. The results indicated that approximately three-quarters (38/50; 76%) of *Prevotella* isolates produced ESBLs. Isolates positive for ESBL production had higher minimum inhibitory concentrations (MICs) of β-lactam antibiotics compared with isolates negative for production of ESBLs ($P < 0.001$). The bla_{TEM} gene was detected more frequently in CF *Prevotella* isolates from paediatric patients compared with isolates from adults ($P = 0.002$), with sequence analysis demonstrating that 21/22 (95%) partial bla_{TEM} genes detected were identical to $bla_{TEM-116}$. Furthermore, a β-lactamase-positive *Prevotella* isolate protected *Pseudomonas aeruginosa* from the antimicrobial effects of ceftazidime ($P = 0.03$). *Prevotella* isolated from the CF respiratory microbiota produce ESBLs and may influence the pathogenesis of chronic lung infection via indirect methods, including shielding recognised pathogens from the action of ceftazidime.

1. Introduction

Prevotella spp. are dominant obligate anaerobic bacteria belonging to the oral and respiratory microbiota. They have been shown to occur in healthy individuals and in people with chronic pulmonary infection including cystic fibrosis (CF), chronic obstructive pulmonary disease and bronchiectasis [1–8]. This opportunistic pathogen has the potential to produce virulence factors that inhibit the action of antibiotics, facilitate immune evasion and contribute to tissue degradation [9,10]. Nevertheless, the pathogenesis of *Prevotella* infection in chronic polymicrobial lung diseases is not understood.

Pulmonary infection in CF is managed using a range of antimicrobial agents, including β-lactam antibiotics, which target pathogens such as *Pseudomonas aeruginosa* [11]. Although *Prevotella* spp. are not currently targeted by antibiotic treatment of chronic lung infections, we recently detected resistance to penicillin and cephalosporin antibiotics in CF isolates from this genus, with reduced susceptibility associated with β-lactamase production [9]. Extended-spectrum β-lactamases (ESBLs), classed in functional subgroup 2be, including those encoded by a TEM-type β-lactamase gene (bla_{TEM}), are known to confer resistance to such β-lactam antibiotics [12]. ESBL-producing bacteria have also been associated with clinical failure of cephalosporin antibiotics

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and increased mortality in hospital-acquired infections [13,14]. These data suggest that it may be clinically important to determine whether *Prevotella* spp. specifically produce ESBLs, which is currently unknown. Furthermore, in polymicrobial infections, β-lactamase-producing bacteria may protect β-lactam-susceptible members of the community from antimicrobial agents [15]. Therefore, β-lactamase production by *Prevotella* spp. may be an indirect method by which this genus contributes to disease pathogenesis in CF by enabling recognised pathogens to persist in the presence of β-lactam antibiotics.

The objective of this study was to investigate the production of ESBLs by clinical *Prevotella* isolates ($n = 50$) from the respiratory microbiota of adults and young people with CF and to establish the prevalence of bla_{TEM} among these isolates. We also investigated the concept of passive resistance and hypothesised that a β-lactamase-producing *Prevotella* isolate could protect a *P. aeruginosa* isolate (from CF sputum) from the action of ceftazidime, an antibiotic commonly used in the treatment of CF pulmonary exacerbations.

2. Materials and methods

2.1. *Clinical* Prevotella *isolates*

Fifty *Prevotella* isolates from people with CF living in Northern Ireland were included in this study to investigate ESBL production. The CF isolates were cultured from 14 adult (≥18 years) CF patients attending the adult CF clinic, Belfast, UK (sputum, $n = 25$) and 11 paediatric (6–17 years) CF patients attending the paediatric CF clinic, Belfast, UK (sputum, $n = 4$; cough swab, $n = 21$). The isolates originated from single bacterial colonies with different morphotypes from each clinical sample and those identified as *Prevotella* spp. were subsequently used in this study. Isolates were identified via 16S rRNA sequencing (Fig. A1, Appendix). Current prescription of long-term antibiotics (flucloxacillin, azithromycin, tobramycin and colistin) was documented for each CF patient (Table A1, Appendix).

2.2. *Extended-spectrum β-lactamase production by* Prevotella

Prevotella isolates ($n = 50$) were tested for production of ESBLs under strict anaerobic conditions using the combined disk method according to the manufacturer's instructions. Briefly, each isolate was inoculated onto supplemented Brucella blood agar (SBBA) with disks (Neo-Sensitabs™; Rosco Diagnostica, Taastrup, Denmark) containing the indicator cephalosporins (ceftazidime 30 μg and cefotaxime 30 μg) as well as ceftazidime + clavulanic acid (30 μg + 10 μg) and cefotaxime + clavulanic acid (30 μg + 10 μg). Isolates were classed as ESBL-positive or ESBL-negative according to Clinical and Laboratory Standard Institute (CLSI) guidelines [16] as follows: isolates were identified as ESBL-positive if the diameter of the inhibition zone was increased by ≥5 mm when the tested indicator cephalosporin was combined with clavulanic acid for at least one of the combinations; and isolates were identified as ESBL-negative if the difference between the zone diameters was <5 mm for both combinations. *Klebsiella pneumoniae* ATCC 700603 was used as a quality control strain.

2.3. *Susceptibility of* Prevotella *isolates to β-lactam antibiotics*

Amoxicillin, ceftazidime and amoxicillin/clavulanic acid (AMC) were selected for in vitro susceptibility testing as these β-lactams are among the antibiotics recommended for the treatment of CF respiratory infection according to clinical guidelines [17]. Under strict anaerobic conditions, *Prevotella* isolates ($n = 50$) were inoculated onto SBBA and the minimum inhibitory concentrations (MICs) determined by Etest (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. *Streptococcus pneumoniae* ATCC 49619 (amoxicillin), *P. aeruginosa* ATCC 27853 (ceftazidime) and *Bacteroides fragilis* ATCC 25285 (AMC) were used as quality control strains. Where anaerobic breakpoints are available, isolates were categorised as susceptible, intermediate-resistant or resistant as defined by CLSI guidelines [18].

2.4. *PCR amplification of* bla$_{TEM}$ *and DNA sequencing*

DNA extraction was performed as previously described [19]. An alignment of 109 different $bla_{TEM\text{-}type}$ nucleotide sequences (861 bp, downloaded from BLAST) was used to identify consensus regions for primer annealing. Primers (bla_{TEM}-Forward, 5′-CCG AAG AAC GTT TTC CAA TG-3′; and bla_{TEM}-Reverse, 5′-GAA GCT AGA GTA AGT AGT TCG-3′) had 100% coverage with the reference sequences and were purchased from Eurofins MWG Operon (Wolverhampton, UK). The PCR screening assay was performed using My*Taq*™ Red Mix (Bioline, London, UK). The final reaction mixture (50 μL) contained 0.2 μM of each forward and reverse primer and 2 μL of DNA template. *Escherichia coli* NCTC 11560 ($bla_{TEM\text{-}1}$) was used as a positive control. PCR was performed using an Applied Biosystems® Veriti® Thermal Cycler (Thermo Fisher Scientific, Paisley, UK) with the following parameters: initial denaturation at 94 °C for 5 min; 33 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min; and a final extension at 72 °C for 10 min. Amplicons (424 bp) were analysed as previously described [19]. PCR products positive for bla_{TEM} were purified using a QIAquick® PCR Purification Kit (QIAGEN, Manchester, UK) in accordance with the manufacturer's instructions. Both strands of DNA were sequenced by Eurofins MWG Operon using the forward and reverse primers, and a consensus DNA sequence was determined with CLUSTALW software. The deduced nucleotide sequences were compared with sequences deposited in GenBank.

2.5. *Passive resistance*

A β-lactamase-positive *Prevotella* isolate (ceftazidime MIC > 256 mg/L) and a β-lactamase-negative *P. aeruginosa* isolate (ceftazidime MIC = 0.5 mg/L) (cultured from CF sputum and identified using 16S rRNA sequencing) were selected to investigate the concept of passive resistance [15].

Time-kill studies were carried out under strict anaerobic conditions according to CLSI guidelines [20]. Assays (*Prevotella* monoculture, *P. aeruginosa* monoculture and co-culture) were performed using basal anaerobic medium containing 1% w/v potassium nitrate (details of this medium are provided in the Appendix) and a cephalosporin antibiotic (ceftazidime; AAH Pharmaceuticals, Belfast, UK) at a pre-determined concentration of 64× (32 mg/L) the *P. aeruginosa* MIC; this concentration has also been detected in CF sputum [21]. The initial inoculum of each bacterium was prepared to ca. 5×10^5 CFU/mL. No drug assays were included as controls. Killing activity was assessed at 0, 2, 4, 6, 24 and 48 h and was repeated on three different occasions with colonies enumerated on SBBA (under anaerobic conditions) and on Mueller–Hinton agar (under aerobic conditions) for the *Prevotella* and *P. aeruginosa* isolates, respectively. Drug carry-over was minimised by carrying out serial dilutions (10^{-1} to 10^{-6}) in sterile saline.

A protective effect (antagonism) by *Prevotella* was defined as a ≥2 $\log_{10}$ increase in viable count of *P. aeruginosa* at 48 h compared with that of the *P. aeruginosa* isolate alone [22]. The limit of detection was 2×10^2 CFU/mL. To investigate whether survival of *P. aeruginosa* in the presence of *Prevotella* was secondary to the development of ceftazidime resistance, susceptibility testing (Etest) was performed using individual colonies ($n = 10$) isolated from the co-culture.