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Haemophilus influenzae in children with cystic fibrosis: Antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation

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ABSTRACT

Haemophilus influenzae commonly infects the respiratory tract of patients with cystic fibrosis (CF), early in childhood. In this investigation, 79 *H. influenzae* isolates were recovered from the respiratory secretions of 64 CF patients (median age: 5 years) included in a 5-year follow-up study. Fifteen of the 64 patients contributed two or more *H. influenzae* isolates overtime. Serotyping, antibiotic susceptibility testing, genotyping, detection of both *hmwA* and *hia* adhesin genes and hypermutable strains was carried out. Biofilm formation ability was investigated. Most strains (72/79, 91.2%) were nonencapsulated or nontypeable (NTHi). Resistance to ampicillin (13.9%) and imipenem (17.7%) was the most detected. Few isolates (2.5%) exhibited the hypermutable phenotype. The NTHi strains showed 55 different genotypes, but 19 clusters of closely related strains were identified. Nine clusters included strains that cross-colonised several patients over a long-time period (mean: 3.7 years). Most patients with sequential isolates harboured strains genetically unrelated, but persistent colonisation with the same clone was observed in 37.5% of patients. Over 45% of NTHi strains contained *hmwA*-related sequences, 26.3%, *hia*, 8.3% both *hmwA* and *hia*, while 19.4% lacked both. A significant association was found between occurrence of an adhesive gene (irrespective of which) and both persistence ($P < 0.0001$) and long-term cross-colonisation ($P < 0.0001$). Mean biofilm level formed by the persistent strains was found significantly increased compared to non-persistent ones ($P < 0.0001$). *Hia*-positive strains produced significantly more biofilm than *hmwA*-carrying strains ($P < 0.01$). Although a high turnover of NTHi strains in FC patients was observed, distinct clones with increased capacity of persistence or cross-colonisation occurred.

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Introduction

Cystic fibrosis (CF) is a human recessive hereditary disorder caused by mutations in the CF transmembrane conductance regulator gene that regulates transport of electrolytes across the epithelial cell membranes (Tomashefski et al., 1993). Mutations in this gene disrupt electrolyte secretion, leading to a hyper-osmolar viscous mucus and, in the end, to an impairment of the mucociliary clearance function.

As a consequence, the respiratory tract of the CF patients is colonised by pathogenic micro-organisms early in childhood and, in the vast majority of cases, chronic infections are established (Tomashefski et al., 1993; Foweraker, 2009). Recurrent acute respiratory infections together with an aggressive host inflammatory response are thought to play a key role in the irreversible airway damage for which some patients ultimately die (Lyczak et al., 2002). A variety of bacterial pathogens including *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and the *Burkholderia cepacia* complex contributes to morbidity, but the prevalence of the respiratory infections due to the different species change overtime (Harrison, 2007). In infancy, colonisation of the respiratory tract is often initiated by *S. aureus* and *H. influenzae* that can cause acute respiratory infections sustained by either each individual bacterial species or both species (co-infections) (Harrison, 2007; Pettigrew et al., 2008).

It has previously been reported that, among the different *H. influenzae* serotypes, nonencapsulated *H. influenzae* (NTHi) is mostly associated with chronic lung infections and acute exacerbations in CF patients (Murphy et al., 2009a,b). The exposure to multiple antibiotic treatments begins very early in the life of children suffering of CF disease, leading to the possible selection of multiple

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resistant strains, that are difficult to eradicate despite antibiotic therapy. Moreover, NTHi possesses several adherence factors including the HMW1/HMW2 and the *Hia* proteins that may play a role in both successful bacterial colonisation of the human respiratory tract and persistence within it (St. Geme et al., 1993, 1998; Barenkamp and St. Geme, 1996). Since *H. influenzae* has recently been found capable to form biofilms on both middle ear mucosa and airway epithelia, such capability may be a contributing factor to bacterial persistence and disease pathogenesis (Hall-Stoodley et al., 2006; Hall-Stoodley and Stoodley, 2009; Starner et al., 2006).

Whether "*H. influenzae* persistence" is due to permanent colonisation with the same *H. influenzae* clone or subsequent colonisation with different *H. influenzae* clones, is currently the subject of several investigations (Moller et al., 1995; Román et al., 2004; Sá-Leão et al., 2008).

In this study, 79 *H. influenzae* strains isolated from 300 CF pediatric patients, who were included in a 5-year follow-up study, were phenotypically and genotypically characterized in order to (i) study the dynamics of colonisation in each patient (persistence of the same strain vs colonisation with multiple strains) or among different patients (possible presence of cross-colonising clones); (ii) investigate whether *H. influenzae* persistence is associated with either antibiotic resistance or presence of specific adhesins or capability to form biofilm or the sum of these factors.

Materials and methods

Patients and bacterial strains

Three hundred patients (median age 15.4 years), with a history of CF, who were attending the "Bambino Gesù" pediatric hospital in Rome, were included in a 5-year follow-up study (September 2004–September 2009). All patients were screened for *H. influenzae* and other pathogens at three month intervals (once every three months) and at any exacerbation occurrence.

A total of 79 *H. influenzae* strains were recovered from the respiratory secretions (bronchoscopic samples, expectorate sputum and oropharyngeal swabs) of 64 out of 300 patients. Fifteen of the 64 patients contributed two or more *H. influenzae* isolates overtime. At the time of collection of clinical samples positive for *H. influenzae*, patients were visited either at the outpatient department (54 patients) or were admitted to the hospital (10 patients). *H. influenzae* strains were selectively cultured on chocolate blood agar containing Iso-Vitalex and bacitracin (Becton Dickinson, Sparks, USA) and were identified based on the requirement for hemin (X factor) and NAD (V factor) on identification (ID) QUAD Plates (Becton Dickinson). Identification was further confirmed by using the VITEK 2 System (Biomerieux, Marcy l'Etoile-France).

As a control group for the hypermutability study, a sample of randomly selected *H. influenzae* isolates (8 from cerebrospinal fluid and 8 from blood) from non-CF patients was included. This sample was chosen from our collection of *H. influenzae* strains isolated from patients with invasive disease detected through the National Surveillance of Invasive Bacterial Disease (Giufrè et al., 2011). Serotyping was determined by the PCR capsular genotyping, following procedures previously reported (Falla et al., 1994).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, cefotaxime, cefixime, imipenem, azithromycin and chloramphenicol were determined by *E*-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations and using *Haemophilus* Test Medium (HTM). The interpretative criteria were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (http://www.eucast.org/clinical_breakpoints/), except for azithromycin, for which the interpretative criteria were adjusted according to the *E*-test protocol for CO_2 incubation (susceptibility breakpoint $\leq$0.250 μg/ml). *H. influenzae* ATCC 49247 was used as control strain. beta-Lactamase activity was detected by the chromogenic cephalosporin test with nitrocefin as the substrate.

Pulsed-field gel electrophoresis (PFGE)

Genetic relationships among the 79 *H. influenzae* isolates were assessed by PFGE after digestion of genomic DNA with SmaI (Roche Diagnostics, Mannheim, Germany) following procedures previously described (Cerquetti et al., 2000), with the only exception of *H. influenzae* type e isolates, for which digestion of plugs was carried out using Apa I (Roche diagnostics) (Cerquetti et al., 2003). Similarity analysis was performed with Dice's coefficient and clustering was carried out by means of the unweighted pair group mean association (UPGMA), with GelCompar II v6.0 (Applied Maths, Sint-Martens-Latem, Belgium). Isolates showing a coefficient of similarity $\geq$80% were considered to belong to the same clonal group or cluster.

Detection of hmw1A, hmw2A and hia genes

The presence of *hmw1A*, *hmw2A* and *hia* adhesion-encoding genes was detected by PCR and confirmed by dot blot hybridization technique. Briefly, PCR analysis was carried out by using primers and following PCR conditions as previously described (Giufrè et al., 2006; Cardines et al., 2007). For dot blot analysis, genomic DNA was spotted onto a nylon membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) and hybridized with *hmw1A*–, *hmw2A*– and/or *hia*-specific probes generated by PCR amplifications of the corresponding genes in prototype strains NTHi12 and NTHi 11, respectively (St. Geme et al., 1998). Positive samples were detected using the ECL system (Amersham Biosciences).

Determination of mutation frequencies

To determine the mutation frequencies, 10 bacterial colonies were re-suspended in 20 ml of Brain Heart Infusion (BHI) broth added with HTM Supplement (Oxoid Ltd., Basingstoke Hampshire, United Kingdom) and grown at 37 °C overnight. Bacterial cells were then collected at 4000 rpm for 10 min and re-suspended in 1 ml of BHI broth. A 100-μl sample from this suspension, as well as samples from successive dilutions, was plated onto HTM with and without rifampin (10 μg/ml). After 48 h of incubation at 37 °C with CO_2, the number of colonies grown on rifampin-containing medium was counted and the mutation frequencies were determined as relative proportions of the total counts of viable organisms plated. A strain was considered to be hypermutable when the mutation frequency was at least 50 times higher than the average mutation frequency found for *H. influenzae* strains isolated from invasive disease. For each hypermutable strain, the experiment was repeated in triplicate and results were indicated as mean value.

Biofilm formation assay

The 79 *H. influenzae* strains were evaluated in sixtuplicate and repeated on two different occasions, for biofilm formation in 96-well culture microplates. Briefly, 200 μl of a standardized inoculum ($2–5 \times 10^6$ CFU/ml) prepared in BHI broth with HTM Supplement (Oxoid Ltd.) was dispensed to each well of a sterile flat-bottom polystyrene 96-well microtiter tissue culture plate (Iwaki, Bibby srl; Milan, Italy) and incubated at 37 °C with 5% CO_2 for 48 h. Quantification of biofilm biomass was then performed by crystal violet assay and expressed as optical density measured at 492 nm

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