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Increased Biofilm Formation by Nontypeable *Haemophilus influenzae* Isolates from Patients with Invasive Disease or Otitis Media versus Strains Recovered from Cases of Respiratory Infections

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Biofilm formation by nontypeable (NT) *Haemophilus influenzae* remains a controversial topic. Nevertheless, biofilm-like structures have been observed in the middle-ear mucosa of experimental chinchilla models of otitis media (OM). To date, there have been no studies of biofilm formation in large collections of clinical isolates. This study aimed to investigate the initial adhesion to a solid surface and biofilm formation by NT *H. influenzae* by comparing isolates from healthy carriers, those with noninvasive respiratory disease, and those with invasive respiratory disease. We used 352 isolates from patients with nonbacteremic community-acquired pneumonia (NB-CAP), chronic obstructive pulmonary disease (COPD), OM, and invasive disease and a group of healthy colonized children. We then determined the speed of initial adhesion to a solid surface by the BioFilm ring test and quantified biofilm formation by crystal violet staining. Isolates from different clinical sources displayed high levels of biofilm formation on a static solid support after growth for 24 h. We observed clear differences in initial attachment and biofilm formation depending on the pathology associated with NT *H. influenzae* isolation, with significantly increased biofilm formation for NT *H. influenzae* isolates collected from patients with invasive disease and OM compared with NT *H. influenzae* isolates from patients with NB-CAP or COPD and healthy colonized subjects. In all cases, biofilm structures were detached by proteinase K treatment, suggesting an important role for proteins in the initial adhesion and static biofilm formation measured by crystal violet staining.

Nontypeable (NT) *Haemophilus influenzae* is an opportunistic pathogen which is highly adapted to colonize the human upper respiratory tract and which can subsequently progress to cause mucosal infections in children and adults (1–3). This Gram-negative unencapsulated microorganism is responsible for causing upper respiratory tract infections (otitis media, sinusitis, and conjunctivitis), community-acquired pneumonia (CAP), and acute exacerbations of lower respiratory tract infections in adults with chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) and is increasingly present in invasive disease (1, 2, 4).

The pathogenesis of many human infections, including chronic and recurrent respiratory infections, has been associated with biofilm communities; these biofilms represent a protective mechanism that enhances bacterial resistance to clearance (5–7). This mechanism has been observed in CF-related pulmonary infections, mainly involving *Pseudomonas aeruginosa* (8–10). However, recent data have also revealed the presence of NT *H. influenzae* in biofilm communities in the lower and upper airways, and physical evidence has been shown in experimental models of otitis media (OM) with the detection of biofilm-like structures in the middle-ear mucosa of chinchillas (11, 12). Moreover, these biofilms could be important in early lung injury and could facilitate colonization and infection by *P. aeruginosa* (1, 7). Despite these observations, biofilm formation by NT *H. influenzae* remains a controversial topic, because NT *H. influenzae* lacks a specific polysaccharide associated with the extracellular matrix (13).

To date, biofilm formation by NT *H. influenzae* has been studied in only a limited number of strains, and a repertoire of genes and bacterial surface structures have been implicated in biofilm formation and maturation. These include type IV pili (PilA) overexpression (14), the presence of fimbriae (13), quorum sensing (15), the presence of outer membrane proteins (OMPs) P2 and P5 (16), and the presence of phosphorylcholine (PCho) and sialic acid in the lipooligosaccharide (LOS) molecule (17, 18). Despite the previously shown role of PCho in biofilm growth (19), a longitudinal study on NT *H. influenzae* isolates from patients with chronic respiratory disease found no clear correlation between biofilm growth and the presence of PCho in the LOS molecule (20). We previously showed the absence of a clear correlation between *in vitro* biofilm formation and the presence of PCho in the LOS of NT *H. influenzae* using a collection of 111 clinical isolates from different clinical sources (21). Despite the limitation imposed by the number of isolates, our previous study suggested that isolates from the middle ear fluid of children with OM formed

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denser biofilm structures than isolates from patients with either COPD or nonbacteremic community-acquired pneumonia (NB-CAP). Given that no association had previously been observed between the sample source and biofilm formation for NT *H. influenzae*, our study suggested the need for further investigation. Therefore, to provide a more comprehensive analysis of the differences in biofilm formation among different clinical sources, we significantly expanded our collection to 352 isolates and included NT *H. influenzae* isolates obtained from patients with invasive disease. This study assessed the initial bacterial adhesion to and biofilm formation on a solid surface by these isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We analyzed 352 NT *H. influenzae* strains from five different patient groups. These included the following: (i) 92 isolates from sputum samples from patients with NB-CAP; (ii) 60 isolates from sputum samples from patients with COPD; (iii) 29 isolates from the middle ear fluid of children with OM; (iv) 54 isolates from the blood, cerebrospinal fluid, and pleural fluid of patients with invasive disease; and (v) 117 oropharyngeal isolates from healthy children in day care centers.

Isolates from NB-CAP (22), COPD (23), and invasive disease were obtained from the Hospital de Bellvitge, Barcelona, Spain. Informed consent was not required, as this process formed part of the normal microbiological routine; patient confidentiality was always protected.

OM isolates were obtained from the University Medical Center, St. Radboud, Nijmegen, The Netherlands (24), and approved by the Committee on Research Involving Human Subjects of the Radboud University Medical Centre, Nijmegen (CMO 2007/239, international trial registry number NCT00847756).

Isolates from healthy children were obtained in a point prevalence study conducted in day care centers and schools in Oviedo, Spain, and approved by the Ethics Committee of the Hospital Universitario Central in Asturias, Spain (25).

All NT *H. influenzae* isolates were identified according to standard microbiological procedures (26). Additionally, all isolates were identified by mass spectrometry (matrix-assisted laser desorption ionization [MALDI] Biotyper, version 3.0; Bruker) according to the manufacturer's instructions and preserved in frozen stocks at −80°C. *H. influenzae* and *Haemophilus haemolyticus* were differentiated by detection of the *lgtC*, *fucK*, and *iga* genes, as previously described (22). Capsular serotype was determined by PCR using primers and conditions previously described (27); only nontypeable strains were considered for this study. Isolates were cultivated on brain heart infusion (BHI; BD) supplemented with 10 μg/ml hemin (Sigma-Aldrich) and 10 μg/ml NAD (Merck) (sBHI). Growth was performed at 37°C in a 5% CO_2 atmosphere.

Molecular genotyping. Molecular typing was performed on bacterial suspensions by pulsed-field gel electrophoresis (PFGE) as previously described (22). Genomic DNA embedded in agarose plugs was digested with SmaI, and the fragments were separated using a CHEF-DRIII apparatus (Bio-Rad). The PFGE band patterns were analyzed using Fingerprinting II software 3.0 (Bio-Rad). Similarity of PFGE banding patterns was estimated with the Dice coefficient (1% optimization and tolerance), and isolates that were ≥85% similar were considered genetically related.

Biofilm formation. The static biofilm formation assay was performed on 96-well plates with crystal violet staining, as previously described (21). Before staining, the optical density at 600 nm (OD_{600}) was determined to assess bacterial growth. We obtained biofilm values by calculating the mean absorbance from at least three independent tests and comparing it with the absorbance of negative controls (sBHI). Isolates were defrosted and used without additional passages for each repetition. The cutoff for biofilm formation was three times the value of the negative control. Strong biofilm formation was defined as three times the value of this cutoff, and any value between was considered indicative of moderate biofilm formation.

Bacterial adhesion assay. The speed of initial bacterial adhesion was evaluated by the BioFilm ring test (Biofilm Control, St Beauzire, France). Biofilm and adhesion assays were performed in parallel to reduce differences in strain behavior. The adhesion assay was performed on modified 96-well polystyrene plates obtained from Biofilm Control, as described by Chavant et al. (28). Briefly, bacterial suspensions were mixed with magnetic beads, incubated for 2 or 4 h at 37°C, and placed on a magnetic block. Free beads migrated to the center of the well and formed a spot, while bead migration was blocked in the presence of adherent bacteria. We used the BioFilm Control software to obtain the biofilm index (BFI); values of >7 corresponded to a total lack of bacterial adherence, while values of <5 were associated with different degrees of bacterial adherence.

Classification regarding adhesion and biofilm. The BioFilm ring test method was used to determine the speed of initial bacterial adhesion to a surface, independently of bacterial biofilm formation after 24 h growth. This is because faster and slower adhesion could be associated with different bacterial adhesion mechanisms. Regarding the association between initial adhesion and biofilm formation, four groups can be identified and defined as follows: B^+Ad^+, biofilm formation at 24 h with a fast initial adhesion to the surface; B^+Ad^-, biofilm formation at 24 h with a slow initial adhesion to the surface; B^-Ad^+, no biofilm formation at 24 h with a fast initial adhesion to the surface; B^-Ad^-, No biofilm formation at 24 h with a slow initial adhesion to the surface.

Biofilm detachment assays. Biofilms were developed in 96-well plates for 24 h as described. Biofilms attached to the bottom of the plate were washed with water and separately treated with 10 mM sodium metaperiodate or with 100 μg/ml proteinase K, as previously described (29). After treatment for 2 h at 37°C, biofilms were washed and then stained with crystal violet. The detachment assay was performed on a selection of 150 strains (52% of the biofilm-forming isolates) distributed among clinical sources and with different intensities of biofilm formation.

The effect of proteinase K on bacterial viability was assessed on five randomly selected isolates. Bacterial cultures were treated with 100 μg/ml proteinase K at 37°C for 2 h. After serial dilutions were performed, treated and untreated cultures were plated to determine bacterial viability by calculating the number of CFU/ml.

Statistical analysis. Statistical analysis were performed using the GraphPad Prism 5 software. Differences were evaluated using the Fisher exact test or the chi-squared test with Yates' correction. A *P* value less than 0.05 was considered statistically significant. Means ± standard errors of the means of at least three independent replicates are depicted. One-way analysis of variance with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis.

RESULTS

Molecular typing associated with adhesion and biofilm formation. Molecular typing was performed on all the studied isolates, and their clonal relationship was compared among the independent clinical sources (Table 1). (i) Ninety-two NB-CAP isolates from 92 adult patients were separated into 48 genotypes. (ii) Sixty COPD isolates from 60 adult patients were separated into 57 genotypes. (iii) One hundred seventeen carrier isolates from 117 children were separated into 85 genotypes. (iv) Twenty-nine OM isolates from 29 children were separated into 27 genotypes. (v) Fifty-four invasive isolates from 54 adult patients were separated into 47 genotypes.

Genotypically identical isolates obtained from unrelated patients displayed phenotypic differences. Thus, the initial adhesion to the surface and biofilm formation patterns were not always maintained; in fact, only half of the genotypes from unrelated patients had the same initial adhesion and biofilm formation profiles.

TABLE 1 Genotype distribution within the five groups of NT *H. influenzae* isolates considered in this study

Source of NT *H. influenzae*[a]	No. of isolates	No. of genotypes		No. of clusters with:						
		Total	Unique	2 isolates	3 isolates	4 isolates	5 isolates	6 isolates	7 isolates	8 isolates
NB-CAP	92	48	27	10	7	1	1	0	1	1
COPD	60	57	54	3	0	0	0	0	0	0
Carriers	117	85	67	13	1	1	1	2	0	0
OM	29	27	25	2	0	0	0	0	0	0
Invasive disease	54	47	40	7	0	0	0	0	0	0

[a] OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

Relationship between NT *H. influenzae* adhesion and bacterial origin. The speed of initial adhesion to a solid surface was determined by the BioFilm ring test after 2 and 4 h static growth at 37°C, where NT *H. influenzae* strains that adhere were defined by a BFI less than 5 (Fig. 1), being inversely proportional to the number of bacteria adhered to the surface. The number of NT *H. influenzae* isolates that adhered after 2 h was low for all the bacterial groups tested (range, 3% to 14%). Isolates collected from patients with invasive disease and from pediatric patients with OM showed a significantly higher adhesion than isolates from carriers and those from patients with COPD and NB-CAP. OM isolates also showed a significantly higher adhesion rate than isolates from invasive disease (Fig. 1A). After 4 h growth, the percentage of adherent NT *H. influenzae* isolates was variable among the groups of isolates (NB-CAP, 35%; invasive disease, 52%; healthy carriers, 56%; COPD, 58%; OM, 83%). NB-CAP isolates showed significantly less adhesion than those from patients with invasive disease, COPD, and OM and from healthy children (Fig. 1B). In addition, following the trend observed after 2 h growth, the OM isolates showed a significantly higher adhesion than isolates from the other sources (NB-CAP and COPD strains, invasive strains, and isolates from healthy children).

Relationship between NT *H. influenzae* biofilm formation and bacterial origin. Most NT *H. influenzae* isolates from the five clinical sources showed a clear ability to form biofilms (i.e., the OD_{570} was more than three times that of the negative control) on a static solid support after 24 h growth (range, 67% to 100%); however, the percentage of strongly biofilm-forming isolates (the OD_{570} was more than three times the cutoff) was variable among the groups (range, 18% to 63%). As in the case of initial adhesion, differences in the intensity of the biofilm formed were observed between the five isolate groups, with no significant differences in the stationary-phase culture (Fig. 2A). Isolates collected from patients with invasive disease and OM formed denser biofilms, as measured by crystal violet staining, while isolates from NB-CAP patients exhibited a lower capacity for biofilm formation (Fig. 2B) than all the other groups studied. Although the lower biofilm formation observed for NB-CAP isolates was not statistically significant compared to that of isolates from COPD patients and healthy carriers, the number of isolates that did not form biofilms (i.e., that were biofilm negative [B^-], defined as having an OD_{570} less than three times that of the negative control) was significantly higher (Fig. 3). Conversely, isolates from patients with COPD and from healthy children showed similar levels of biofilm formation.

Correlation between NT *H. influenzae* adhesion and biofilm formation. The relationship between initial adhesion to a solid surface and biofilm formation varied between isolates. Four independent groups were identified based on the amount of biofilm formed and the speed of the initial adhesion to the surface (Fig. 3): B^+Ad^+, B^+Ad^-, B^-Ad^+, and B^-Ad^-.

Most OM isolates (>80%) showed a fast initial adhesion to the surface, which translated into strong biofilm formation after 24 h of growth, while invasive isolates presented a high level of biofilm formation independently of the speed of the initial adhesion to the surface (Fig. 3). NB-CAP isolates were mostly associated with slow adhesion, although some (>40%) were able to form biofilms after 24 h of growth. Isolates from COPD patients and healthy carriers had similar patterns of adhesion and biofilm formation. As shown

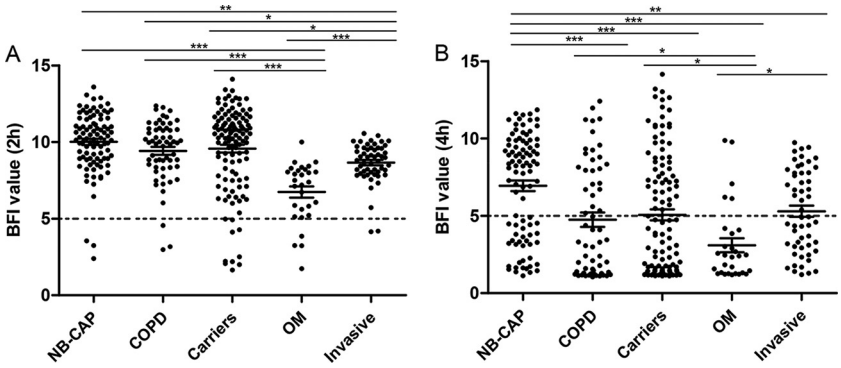

FIG 1 Initial adhesion to a solid surface determined by the BioFilm ring test after 2 h (A) and 4 h (B) of static growth at 37°C. The biofilm formation index (BFI) was adjusted by the test software and is inversely proportional to the number of adherent bacteria. Dotted lines represent the cutoff for adhesion (BFI = 5), with values of <5 representing high levels of adhesion to the surface. Means ± standard errors of the means for at least three independent replicates are presented. One-way analysis of variance with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Abbreviations: OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

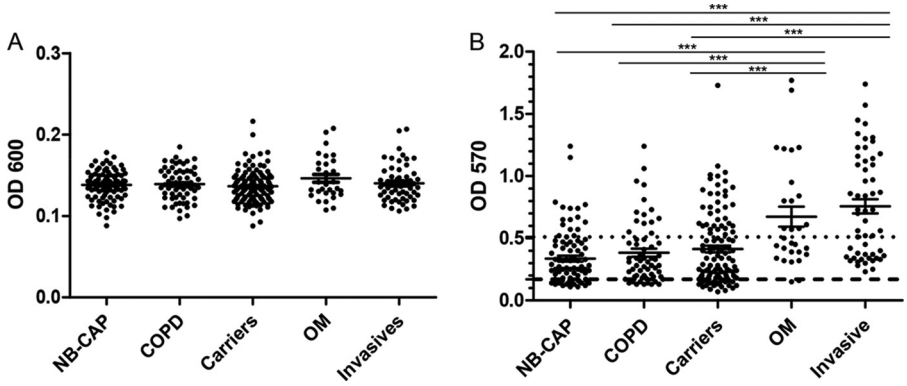

FIG 2 Stationary-phase cultures and biofilm formation determined for 352 NT *H. influenzae* isolates from patients with NB-CAP, COPD, OM, and invasive disease and from healthy carriers. (A) Stationary-phase cultures measured by optical density at 600 nm (OD_{600}); (B) biofilm formation measured by crystal violet light absorbance at 570 nm (OD_{570}). Means ± standard errors of the means of at least three independent replicates are presented. One-way analysis of variance with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Abbreviations: OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease. The dashed line shows the OD_{570} that is more than three times that of the negative control; the dotted line shows the OD_{570} that is more than three times the biofilm breakpoint.

in Fig. 4, no significant relationship was observed between initial adhesion and biofilm formation for individual NT *H. influenzae* isolates in any of the five groups studied.

Biofilm detachment. To compare the nature of the biofilm structures formed by the clinical isolates, a biofilm detachment assay was performed on 150 strains from the five sources. We also considered the four categories established (B^+Ad^+, B^+Ad^-, B^-Ad^+, and B^-Ad^-) to determine differences in biofilm composition. The biofilm structures of all the studied isolates were sensitive to proteinase K and were highly resistant to sodium metaperiodate. These findings were independent of the amount of biofilm formed, the speed of adhesion to the solid surface, and the isolate origin, indicating that initial attachment and biofilm formation depends on the presence of proteins but not on sugar components in the extracellular matrix (Fig. 5). Proteinase K treatment did not affect viability.

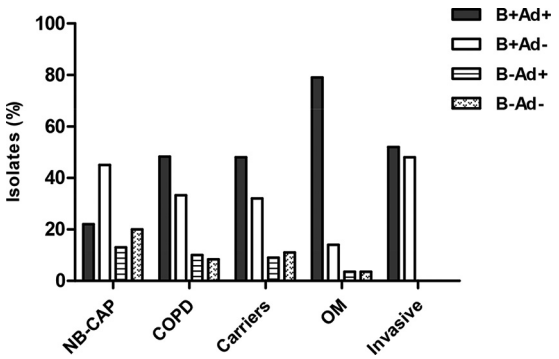

FIG 3 Distribution within the NT *H. influenzae* clinical sources of four independent groups regarding the amount of biofilm formed and the speed of initial adhesion to the surface. OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease. B^+Ad^+, biofilm formation with a fast initial adhesion to the surface; B^+Ad^-, biofilm formation with a slow initial adhesion to the surface; B^-Ad^+, no biofilm formation after 24 h with a fast initial adhesion to the surface; B^-Ad^-, no biofilm formation after 24 h with a slow initial adhesion to the surface.

DISCUSSION

Biofilm formation in chronic and recurrent infections is a persistence mechanism used by a wide range of microorganisms (10, 30, 31). Biofilm structures are common, both in nature and in clinical settings, and protect bacteria from bactericidal agents, bacteriophages, or host clearance mechanisms (30).

Despite the controversy over the inability to identify a specific polysaccharide link to the extracellular matrix, biofilm formation by NT *H. influenzae* has been widely described (11–13, 32). Additionally, there is evidence that bacterial adhesion to human epithelial cells in the respiratory epithelium leads to microcolony and biofilm formation (2). For this reason, a comprehensive analysis of the capacity of NT *H. influenzae* isolates from different clinical sources to form biofilms will contribute further insights into their involvement in bacterial infection.

Bacterial adhesion. Previous studies used crystal violet staining after 2 h growth to determine the initial adhesion to solid surfaces (33, 34). This staining is an established method for quantifying biofilm formation; however, in common with other approaches that involve washing the surface where bacteria adhere, this is a controversial technique for assessing initial adherence (33, 35). In fact, initial adhesion is a reversible process based on physicochemical interactions (36) and, for this reason, repeated washes can remove bacteria from the surface (33, 35, 37). Consequently, we used the BioFilm ring test, a system based on the immobilization of beads by adherent sessile bacteria (28) which allows quantification of initial attachment while avoiding the washing steps. Furthermore, it has been shown to be suitable for the study of adhesion with *Campylobacter* spp. (38).

To date, no studies have shown the initial surface adhesion of NT *H. influenzae*. After 4 h growth, NT *H. influenzae* isolates from OM patients presented higher adherence levels than the other isolates, while NB-CAP isolates showed the lowest level of adhesion. It has been stated that bacterial isolates from different niches can exhibit differences in adhesion patterns (34, 39). However, why NT *H. influenzae* OM isolates should adhere faster than the other isolates remains unresolved.

Biofilm formation. A biofilm starts to develop after bacteria

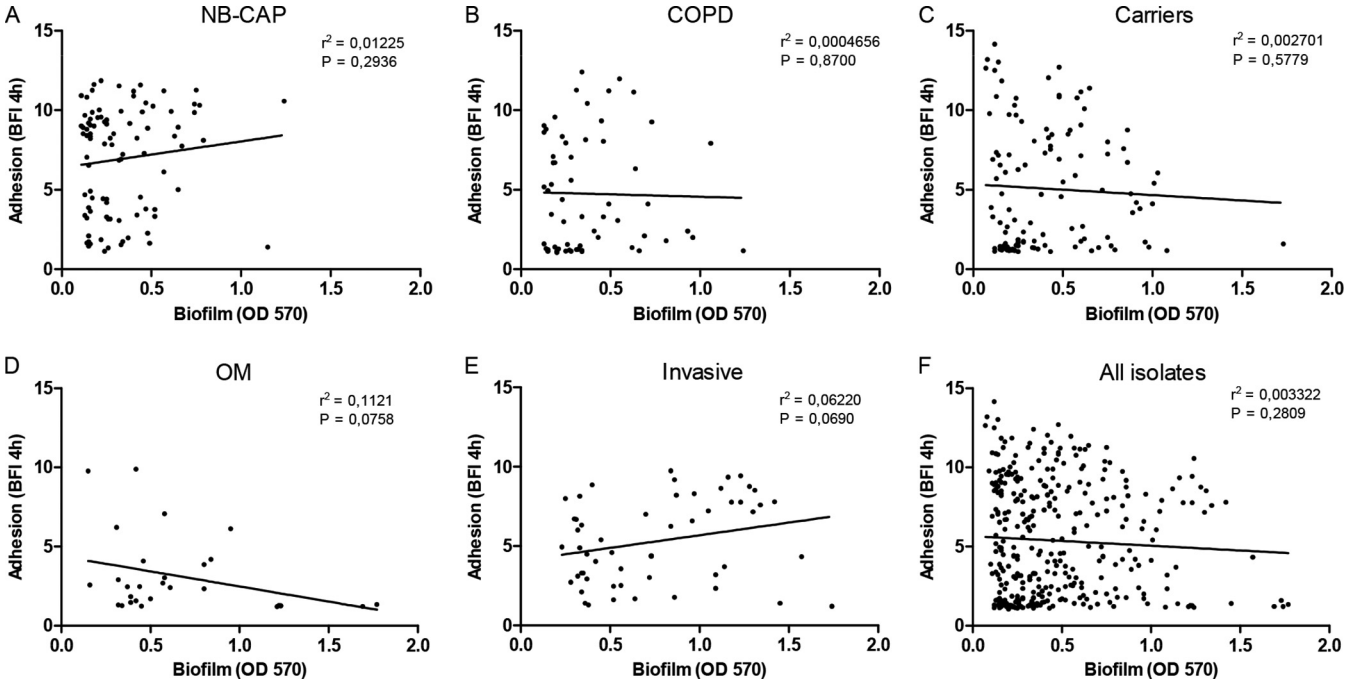

FIG 4 Correlation between initial adhesion to a solid surface by the BioFilm ring test after 4 h growth and levels of biofilm formation measured by crystal violet light absorbance at 570 nm (OD_{570}) in 352 NT *H. influenzae* isolates from different sources. (A) NB-CAP patients; (B) COPD patients; (C) healthy children; (D) children with OM; (E) patients with invasive disease; (F) all groups combined. Abbreviations: OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

have irreversibly attached to the surface (30, 36). After 24 h growth, the biofilm was more resistant to washing, and crystal violet staining was selected for quantification. Biofilm formation was significantly stronger for isolates from OM and invasive disease, supporting our previous findings on the increased ability of OM isolates to form biofilm (21). OM isolates were obtained from The Netherlands, and therefore we cannot completely exclude the possibility of differences due to geographic variation. However, studying 15 COPD and 15 OM isolates, Murphy and Kirkham found no relationship between biofilm formation and the clinical source of the sample (5). Their findings might have been related to the limited number of isolates included in the study.

Interestingly, invasive isolates displayed the highest level of biofilm formation. Previous studies on *Streptococcus pneumoniae* showed that biofilm-producing isolates had an enhanced ability to attach to host cells and a reduced ability to cause invasive disease (40). However, other authors have linked *in vitro* biofilm formation to spread through tissue barriers (41, 42) and the adhesion mediated by PilA with meningococcal sepsis (43), and studies on *H. influenzae* type b suggested that fimbrial structures contribute to bacterial spread into the circulation and secondary infection sites (42).

Biofilm detachment. Requirement of a sugar moiety or a protein-based interaction with the surface was determined by treatment with sodium metaperiodate (which cleaves sugar components) and proteinase K (for protein degradation) (29). Biofilm formation by all tested NT *H. influenzae* isolates was sensitive to proteinase K, suggesting that proteins play an important role in adhesion and biofilm formation. Izano et al. demonstrated rapid biofilm detachment in eight NT *H. influenzae* isolates after adding proteinase K and suggested that adhesins existed within the biofilm structure (44). Our study significantly expands this observation based on a large collection of NT *H. influenzae* isolates from different sources. Conversely, treatment with metaperiodate did not affect biofilm, adding to the controversy regarding the role of polysaccharides in NT *H. influenzae* biofilms (32).

Correlation between adhesion and biofilm formation. Bacterial isolates from different areas can exhibit differences in adhesion to solid surfaces (34, 39). We showed that, although adhesion is the first step in biofilm formation, there is no relationship between speed of initial adhesion and biofilm formation. However, given that the adhesion process is due to physicochemical interactions between cellular components and the solid surface (36, 45, 46), differences in the initial surface adhesion could reflect variability in adhesive proteins (since no role was found for extracellular polysaccharide). Further experiments to determine the proteins associated with each type of disease could bring further insights into the mechanisms used to cause infection.

Correlation between adhesion/biofilm and clinical infection. Acute infections are often caused by planktonic bacteria, while biofilm-producing bacteria are mostly associated with chronic infection and colonization (10, 30). Our results partially support this notion, because NB-CAP isolates exhibited slower adhesion and lower biofilm formation than isolates from either patients with chronic infections (COPD and OM) or healthy children. However, isolates from invasive disease showed the highest levels of biofilm formation. A previous study showed that *Acinetobacter baumannii* isolates from blood and from a single meningitis sample formed biofilm, while those isolated from respiratory tract infections were mostly unable to form biofilm (47). This difference could result from the fact that invasive isolates must cross tissue barriers before causing infection.

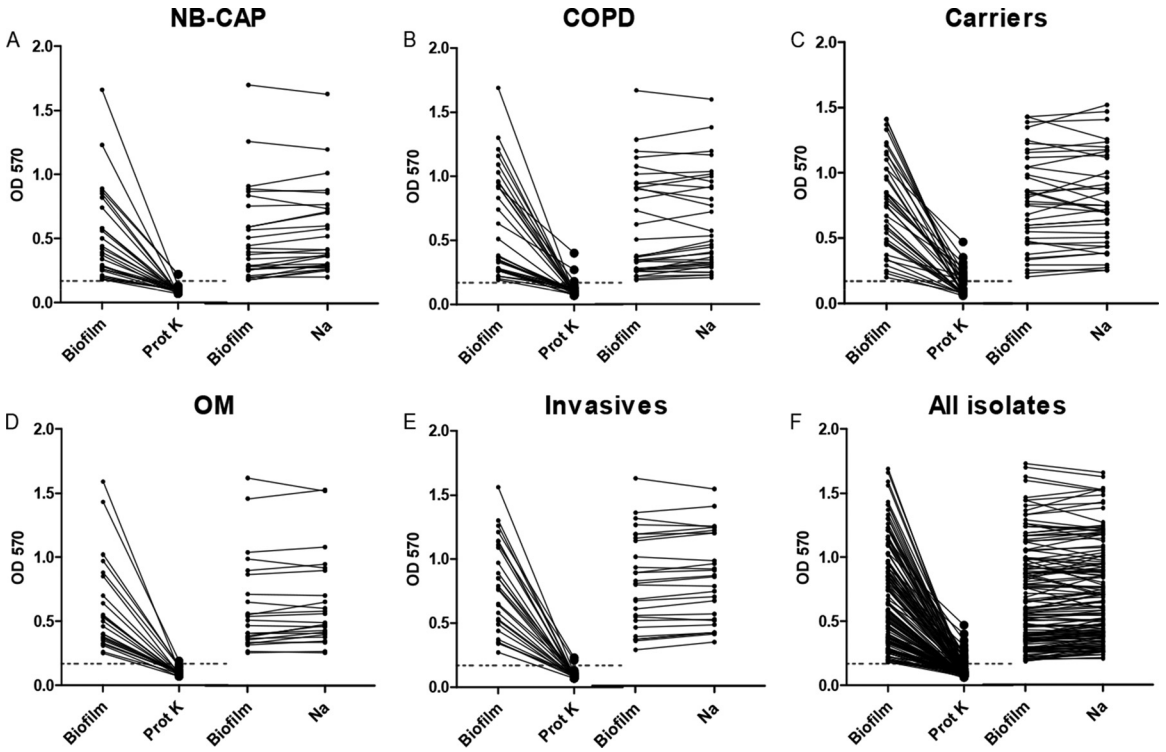

FIG 5 Effect of proteinase K and sodium metaperiodate treatment on 24-h biofilm structures measured by crystal violet light absorbance at 570 nm (OD_{570}) in 150 NT *H. influenzae* isolates from different sources. (A) NB-CAP patients; (B) COPD patients; (C) healthy children; (D) children with OM; (E) patients with invasive disease; (F) all groups combined.

NT *H. influenzae* binds to a variety of receptors in the respiratory tract (16). The OMPs P2 and P5, lipoproteins such as OapA, and proteinaceous adhesins have been attributed early roles in colonization (13, 48). Colonization by *H. influenzae* can result in epithelial damage and eventually reach the circulatory system (49), a process that could depend on the proteins expressed. Previous studies have shown that OMPs are likely to be expressed differently in colonizing bacteria than in invasive isolates (50). Therefore, studies on adhesion to eukaryotic cell lines and the identification of proteins involved in this adhesion would shed light on the differences among groups observed in this study.

Molecular typing associated with adhesion and biofilm formation. PFGE-based genotypically identical isolates from different episodes in the same patient maintained the adherence behavior and biofilm formation (data not shown), but this was not the case for the genotypically identical isolates from unrelated patients whose samples were used in this study. Thus, closely related isolates from different individuals may undergo modifications in the environment or within the host that can alter their ability to adhere and form biofilms. Host-pathogen interactions, including pathology, antimicrobial therapy, and inflammatory responses of different degrees, could be responsible for the variability in adhesion patterns. Bakker et al. suggested that isolates from different sources exhibit modifications in their adhesion patterns, not only because of the environment but because bacterial outer components have adapted through selective pressure over time (39). We acknowledge the limitation imposed by PFGE-based genotyping on bacteria obtained from single colonies and cannot exclude an association between the observed phenotypic differences and nonsynonymous polymorphisms in closely related isolates.

In conclusion, our results suggest differences in biofilm formation depending on the type of disease caused by NT *H. influenzae*. Specifically, there was a clear increase in biofilm-forming ability among isolates from OM and invasive disease. We also found that biofilm stability was dependent on protein interaction; this may represent a novel therapeutic target for disrupting established biofilms *in vivo*.

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We have no conflicts of interest to declare.

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