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Improving Patient Care Via Development of a Protein-Based Diagnostic Test for Microbe-Specific Detection of Chronic Rhinosinusitis

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Abstract

Objectives—The hypothesis is that signature bacterial proteins can be identified in sinus secretions via high-throughput, proteomic based techniques. Non-typeable *Haemophilus influenzae* (NTHI) is the most common bacterial pathogen associated with sinusitis and will serve as a proof of principle pathogen for identifying biomarkers.

Study Design—*In vitro* and *in vivo* studies using proteomic based analysis of cultures of NTHI and a novel, experimental chinchilla polymicrobial sinusitis model.

Methods—Nano-liquid chromatography/tandem mass spectrometry (nano-LC-MS/MS) was performed to annotate the secretome from an NTHI biofilm. A model of NTHI induced sinusitis was developed in a chinchilla and NTHI proteins were detected in chinchilla secretions. A reference standard RT-PCR based assay was adapted to allow for sensitivity and specificity testing of the identified signature biomarkers in human patients.

Results—Outer membrane proteins P2 (OMP-P2) and P5 (OMP-P5) were identified as promising candidates for detection of NTHI biofilms and positively detected in nasopharyngeal secretions of chinchillas experimentally infected with NTHI. An RT-PCR based test for the presence of NTHI biofilms demonstrated 100% sensitivity and 100% specificity when tested against 8 unique strains commonly found in human bacterial rhinosinusitis.

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Supplementary Information

1. Laboratory Methods
2. Laboratory Methods Bibliography

Conclusions—Proteomic analysis was successful in identifying signature proteins for possible use as a biomarker for CRS. OMP-P2 and OMP-P5 were validated as promising candidates and were positively detected from nasopharyngeal secretions from chinchillas experimentally infected with NTHI. Collectively, these data support the use of OMP-P2 and OMP-P5 as biomarkers for a human clinical trial to develop a point of care medical diagnostic test to assist in the diagnosis and treatment of CRS.

Keywords

sinusitis; diagnostic; medical device; bacterial; proteomics

Introduction

The initial diagnosis of chronic rhinosinusitis (CRS) continues to remain challenging for primary care practitioners. This difficulty is directly linked to the lack of diagnostic markers for the different disease subtypes of CRS^{1,2,3}. However, diagnostic and therapeutic precision has increased from recent stratifications of CRS. One such subset of patients with CRS may have biofilm-induced CRS. A biofilm is defined as a sessile microbial community, which includes bacteria that exhibit an altered growth phenotype, gene expression, and protein expression⁴. Recent evidence suggests that a large percentage of CRS patients with and without polyposis are likely to have an associated pathogenic biofilms within their sinus cavities^{5,6,7,8,9}. Collectively, these studies suggest that the presence of pathogenic biofilms and/or the inability of host defenses to control biofilm development or to mediate its eradication may be associated with the development of CRS.

There is wide variation in the ability to detect biofilms based on the types of assays used^{10,11} and moreover, it is unclear if the mere presence of biofilms in CRS tissue implies pathogenesis¹². It is likely that the host innate and/or acquired immune response to these biofilms play a role in the chronicity of biofilm-associated inflammation¹³ and phenotypic differences in biofilms may play a role in the ability of bacteria to act as a commensal or pathogenic organism¹⁴. Therefore, whereas simple detection of biofilms in CRS is not likely to be sufficient, the development of clinically useful assays sensitive enough to distinguish among commensal versus pathogenic forms of bacterial colonization would add significantly to the arsenal of methods to detect, treat, and ultimately prevent CRS.

The central hypothesis to be tested in this research plan is that signature, trace bacterial proteins that distinguish between commensal and pathogenic phenotypes of bacteria can be identified in sinus secretions via high-throughput, proteomic based techniques¹⁵ and clinically useful biomarkers can be selected from these identified proteins to develop a low-cost diagnostic test for biofilm-associated CRS. Detection of specific signature proteins from bacterial biofilms associated with CRS may result in the identification of an objective biomarker to monitor success of therapies and ultimately lead to less morbidity and risk for complications compared to conventional empiric antibiotic and surgical therapy. In addition, transforming this paradigm would help achieve the goals of the U.S. Department Health and Human Services Healthy People 2010 objective 14–19¹⁶ which is “reducing the number of courses of antibiotics prescribed for the sole diagnosis of the common cold.”

Methods

Laboratory Methods (See Supplemental Information)

Procedural Methods

Initial investigation into spatial and temporal characteristics of NTHI biofilms: To initially investigate the temporal and spatial characteristics of NTHI secretomes, NTHI strain 86-028NP biofilms were cultured *in vitro* in chamber slides. Supernatants were analyzed via SDS-PAGE and silver staining to examine the stability and temporal characteristics of an early biofilm.

Annotation of non-typeable *Haemophilus influenzae* (NTHI) biofilm secretome: To determine the identity of the secretome, it was chosen to: 1) analyze the supernatants of a biofilm strain with a sequenced and annotated genome, 2) purify and separate proteins from the supernatants (as a surrogate for CRS secretions that we plan to recover in clinical trials), 3) perform high performance liquid chromatography separation of proteins, 4) utilize tandem mass spectrometry to identify the molecular weights of the complex mixture of peptides, 5) perform bioinformatic analysis of the complex mixture with NCBI and GenBank databases to identify the identity and abundance of the proteins compared to planktonic states (loosely associated, growing in fluid, and not attached to a surface) of the bacteria. A low passage number, clinical isolate of NTHI, strain 86-028NP, isolated in 1986 from the nasopharynx of a chronically infected child, was utilized¹⁷. This strain has been well characterized *in vitro* and in chinchilla models of otitis media¹⁸. Chromosomal DNA from this strain underwent sequencing and gene annotation using Basic Alignment Search Tool (BLAST), BLASTX, algorithm sequencing utilizing National Center for Biotechnology Information (NCBI) nucleotide and protein database searches against known and previously published strains of NTHI, and microarray analysis to annotate previously unknown genes. This annotation was published in the NCBI databases (GenBank Accession number CP000057).

Selection of initial candidate biomarkers: Proteins were individually graded from the most abundant protein to the 20th most abundant protein on their potential ability to fulfill the following criteria: 1) be visible early in a disease, prior to histopathological changes, 2) be sensitive and correlate with disease severity, 3) be non-invasively accessible, 4) be analytically stable, 5) bridge across species to allow for experimental animal modeling, 6) be associated with the disease via a known mechanism and not simply statistically associated with a disease, and, 7) be able to help pinpoint location of a disease and not simply be associated with tissue damage. Initial criteria allowed for between 1 and 10 proteins to be selected.

Development of a clinically relevant, chinchilla model of sinusitis: To perform validation testing of the selected candidate biomarkers, a clinically relevant, experimental model of NTHI-induced sinusitis was necessary to support human clinical trials for the development of a medical diagnostic device. Chinchillas have been the host of choice for experimental modeling of polymicrobial otitis media. Initial feasibility testing for the use of the chinchilla as a host for experimental sinusitis was performed by analysis of paranasal sinus anatomy

via micro-computed tomography. An initial cohort of 5 juvenile animals were inoculated with adenovirus serotype 1 followed by NTHI 86-028NP/pRSM2211, which is a low-passage number clinical isolate of NTHI containing a plasmid with strong promoter for outer membrane protein P2 driving expression of green fluorescent protein (GFP). This expression of GFP allowed for direct, continuous, non-invasive imaging of a bacterial infection within the chinchilla paranasal sinus cavities (Figure 1). A second cohort of 20 juvenile animals were inoculated with saline, adenovirus serotype 1 only, NTHI 86-028NP only, or adenovirus serotype 1 followed by NTHI 86-028NP, and underwent serial micro-computed tomography scanning. Mucosa was harvested following the experiment to examine for the presence of recoverable NTHI from upper respiratory mucosa.

Validation of selected biomarkers in multiple bacterial strains and chinchilla

secretions: After selection of the initial candidate biomarkers, testing across multiple clinical NTHI strains via Western blot testing with polyclonal antibodies was performed to confirm detection ability of the selected proteins. After successful *in vitro* testing with multiple clinical isolates, Western blot testing with polyclonal antibodies against nasopharyngeal secretions from chinchillas previously infected with NTHI strain 86-028NP was performed to confirm detection ability within an animal host and demonstrate increased likelihood of successful detection within humans.

Development of reference standard RT-PCR based test for detection of NTHI: In order to perform sensitivity and specificity testing in human clinical samples, a reference standard test to confirm presence or absence of NTHI was developed. A real-time, quantitative, RT-PCR based test was selected using published primers against the *hpd* gene encoding Protein D in NTHI. This assay was highly sensitive (98%) and specific (100%) for detection of NTHI within cerebrospinal fluid samples of patients suffering from meningitis in Mongolia¹⁹.

RESULTS

NTHI biofilms produce stable proteins consistently detectable in supernatants

Figure 2 demonstrates confocal imaging and silver staining of NTHI strain 86-028NP which reveals discrete bands of proteins seen to be consistently detectable in supernatants from NTHI biofilms from day 1 to day 10. Proteins bands initially chosen as potential biomarkers of interest were seen in regions with molecular weights between 100 and 160 kdaltons, and between 25 and 50 kdaltons.

Annotation of secretome via proteomic analysis of NTHI strain 86-028NP supernatant

Several hundred proteins and their relative abundance within NTHI secretomes were determined. Figure 3 lists the 13 most abundant proteins identified from the NTHI secretomes.

Selection of initial candidate biomarkers

Four proteins were selected as candidate biomarkers for NTHI biofilm associated sinusitis: High molecular weight adhesin 1(HMW1) – one of the known and most important non-

fimbriated adhesins for NTHI containing a distinct molecular weight that made it attractive for use as a biomarker, outer membrane protein P2 (OMP-P2) – the predominant NTHI outer membrane protein and an important porin for which antibody-mediated therapy has bacteriocidal efficacy, outer membrane protein P5 (OMP-P5)– an important adhesion protein for NTHI, and IgA-specific serine endopeptidase - an important virulence factor thought to protect NTHI against antibody-mediated clearance

Micro-computed tomography (Micro-CT) reveals chinchilla to be suitable candidate for experimental modeling of CRS

Micro-CT scans of chinchillas demonstrated the presence of sinus cavities comparable to human paranasal sinus anatomy; therefore chinchillas were deemed suitable for development of a model for sinusitis (Figure 4A). Contrast rhinography was performed demonstrating the ability to intranasally deliver an inoculum to the paranasal sinus cavities (Figure 4B).

Experimental modeling of sinusitis in the chinchilla

Fluorescent signal after inoculation with NTHI 86-028NP/pRSM2211 was detectable up to 18 days post-inoculation to allow for direct, non-invasive, continuous monitoring of a sinus infection (Figure 4). Evidence of characteristic signs of mucosal inflammation and secretion formation were detected in several animals inoculated with adenovirus + NTHI (Figure 5). NTHI was recovered from 100% of septal, nasopharyngeal, nasal turbinates, and ethmoid turbinate mucosa in animals treated with adenovirus + NTHI. Control animals (those receiving adenovirus only or saline only) demonstrated no CFU of NTHI. CFU recovered from adenovirus + NTHI treated animals demonstrated a significant increase in CFU compared to controls ($p < 0.03$), and a 500% increase in CFU counts from day 4 to day 10 (Figure 6).

Validation of selected biomarkers in multiple strains and chinchilla secretions

After selection of the 4 candidate biomarkers: high molecular weight adhesin 1, outer membrane protein P2, outer membrane protein P5, and IgA-specific serine endopeptidase, validation of these biomarkers was performed in multiple clinical isolates of NTHI and in chinchilla secretions after NTHI inoculation. Testing across multiple clinical strains revealed the lack of suitability for the use of high molecular weight adhesin 1 (Figure 7). Lack of specificity in testing, considerable genetic polymorphism, and a shared mosaic-like pattern with the IgA-specific serine endopeptidase with the Hap protein, an adhesive and penetrative protein, precluded the use of IgA-specific serine endopeptidase as a suitable biomarker^{20,21}. Western blot testing with the use of polyclonal antibodies against OMP-P2 and OMP-P5 revealed positive detection in all tested nasopharyngeal secretions of infected chinchillas and across all clinical isolates of NTHI tested (Figure 7,8).

Development of reference standard RT-PCR based test for detection of NTHI

The RT-PCR based test was 100% sensitive (5 of 5 clinical isolates of NTHI and 5 of 5 nasopharyngeal secretions following NTHI inoculation) and 100% specific (3 of 3 clinical isolates of *Moraxella catarrhalis* and 5 of 5 nasopharyngeal secretions prior to NTHI

inoculation) for accurate identification of NTHI species in nasopharyngeal secretions using clinical isolates of *Moraxella catarrhalis* as negative controls (Figure 9).

DISCUSSION

Because of the great need for a molecular biomarker for biofilm-associated CRS, this study endeavored to identify an ideal biomarker for this disease based on ideal criteria and progress through the stages of biomarker development with the goal of culminating with a clinically useful diagnostic test. While it is known that biofilm-associated CRS is likely caused by a myriad of pathogenic processes, the starting point chosen for this study was the most prevalent subtype of this disease, which is likely viral induced, bacterial biofilm associated CRS, and using with the most prevalent bacterial pathogen, which after the advent of the *Streptococcal pneumoniae* (Pneumovax™) vaccine, has become NTHI. NTHI also provided a distinct advantage as it was the first free-living microbe to have its entire genome sequenced and annotated²².

The initial validation of OMP-P2 and OMP-P5 holds great promise for the future development of a medical diagnostic that can change the paradigm of care for sinusitis. Specifically, it is planned in stage 2 of the development of this device to produce antibodies to OMP-P2 and OMP-P5 to be used in a lateral flow immunoassay that reports a change in color as an indicator of the presence of the identified diagnostic trace proteins in sinus secretions. The experimental model of sinusitis in the chinchilla will be used to determine the optimal antibody concentrations on a lateral flow immunoassay to provide the greatest sensitivity and specificity for the diagnostic test. A clinical trial has been opened with enrollment of over 30 patients of an estimated 300 patients to determine the optimal antibody concentrations to maximize the sensitivity and specificity of these biomarker proteins to diagnose NTHI-associated CRS. The reference-standard RT-PCR based test utilized in this study will be used to determine optimal antibody concentrations and detection limits for OMP-P2 and OMP-P5 to optimize diagnostic accuracy of the test. The final product of this diagnostic is planned to be a test strip that rapidly identifies the presence of trace proteins that serve as a reliable biomarker of NTHI-induced sinusitis. This test strip will be similar to test strips used for *streptococcal* colonization, as well as the common pregnancy detection tests. This diagnostic test will also enhance the development of an emerging market in respiratory medicine of molecularly targeted biologic medicines for specific diseases. For example, unique therapies against NTHI biofilms are currently in the state of development. If an accurate diagnosis of NTHI induced sinusitis could be ascertained, then a specific molecular-based therapy against NTHI (and particularly when this microbe is residing within a highly resistant-to-treatment-or-eradication biofilm community) could be utilized^{23,24} instead of the commonly used, empiric, broad-spectrum antibiotics. Empiric antibiotics often inadequately treat biofilm-based disease, destroy non-pathogenic bacteria required for health, and promote antibiotic-resistance in pathogenic bacteria.

While this device is being developed to non-invasively access nasal secretions draining from the multiple sinuses within one's nasal cavity, a large subset of sinusitis sufferers have completely obstructed ostia to their sinuses, particularly their maxillary and frontal sinuses.

The recent development and commercial application of balloon technology to provide in-office access to these sinus cavities^{25,26} represents an important breakthrough for sampling secretions from obstructed sinuses. It is envisioned that the protein-based molecular diagnostic will be engineered to pass through a balloon catheter and be able to directly sample sinus secretions from an obstructed sinus as well as sample nasopharyngeal secretions.

Finally, the development of these biomarkers for NTHI will serve as a platform technology and will drive development of diagnostics for other bacteria involved in CRS, such as *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, and others. The use of rapid, point-of-care testing for the diagnosis of sinusitis can change the paradigm for diagnosis to a personalized approach and revolutionize sinus care.

This study demonstrated the ability of proteomic techniques and bioinformatic analysis to characterize the secretome of NTHI, the most prevalent pathogen of CRS. Furthermore, two proteins found in abundant quantities in the NTHI biofilm secretome, outer membrane protein P2 and outer membrane protein P5 fulfilled initial criteria as potential early and sensitive biomarkers for this disease. In addition, a novel experimental model of sinusitis in the chinchilla that allows for direct, continuous, and live monitoring of infection was developed. Also, initial validation of the two candidate biomarkers was successfully completed across many human clinical isolates of NTHI and using nasopharyngeal secretions from chinchillas experimentally infected with NTHI. Finally, a reference standard RT-PCR based test was adapted to allow for sensitivity and specificity testing for these two biomarkers in a future human clinical study. Collectively, the successful identification and initial validation of candidate biomarkers for NTHI-associated CRS supports the development of a novel diagnostic test that could provide rapid, point-of-care testing for the objective detection of NTHI-associated CRS and serves as the platform for the development of additional biomarkers to other pathogens commonly associated with CRS.

Conclusions

The use of proteomic analysis and annotation of the secretome from a human clinical isolate of a non-typeable *Haemophilus influenzae* biofilm was successful in identifying signature proteins for possible use as a biomarker for CRS. OMP-P2 and OMP-P5 were validated as promising candidates and were positively detected from nasopharyngeal secretions from chinchillas experimentally infected with NTHI. Collectively, these data support the use of OMP-P2 and OMP-P5 as biomarkers for a human clinical trial to develop a point-of-care diagnostic test to assist in the prevention, diagnosis, and treatment of CRS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Functional imaging of NTHI-strain 86-028NP containing a GFP-fluorescent plasmid reporter construct

The pRSM2211 plasmid contains the strong promoter for the outer membrane protein P2 driving the expression of green fluorescent protein. Strain 86-028NP/pRSM2211 is a modification of a promoter trap vector and was created for its near constitutive expression to allow for direct, continuous, biofluorescent imaging of an experimental chinchilla infection with NTHI. Fluorescent signal was detectable up to 18 days post-inoculation to allow for continuous monitoring of a sinus infection.

Figure 2. Detection of proteins within the secretome of NTHI biofilms

(A). Confocal imaging of NTHI biofilms' growth over 10 days and stained with live-dead stain. Pronounced predominance of green (live) staining over red fluorescence (dead cells) demonstrates viability of bacteria resident within the biofilm.

(B). Silver staining of the secretome demonstrates multiple proteins that are readily detectable and stable in appearance throughout the first ten days of biofilm growth. Proteins of varying molecular weights between 25–160 kilodaltons were selected as potential candidate biomarkers (red arrows).

IDENTIFIED PROTEINS	Score	Mass (KDa)	Accession #
Outer membrane protein P2	1227	39.9	gi 68248747
HMW1A, high molecular weight adhesin 1	1205	154.5	gi 68250281
putative periplasmic chelated iron binding protein	1089	32.4	gi 301169065
IgA-specific serine endopeptidase	948	197.5	gi 68249575
Outer membrane protein P5	886	38.4	gi 68249712
galactose-1-phosphate uridylyltransferase	791	34.0	gi 145640927
HMWA	720	160.5	gi 68249817
phosphate ABC transporter phosphate-binding protein	703	36.6	gi 16273649
putative adhesin B precursor FimA	402	35.0	gi 3003012
HMW2A, high molecular weight adhesin 2	326	160.7	gi 68249817
HMWA	321	160.5	gi 5929966
Outer membrane protein P5; Precursor	283	37.7	gi 585614
Outer membrane protein P1	215	49.7	gi 9716607

Figure 3. Identification of the NTHI secretome via proteomic and bioinformatic analysis
 NTHI strain 86-028NP biofilm secretome was analyzed via high performance liquid chromatography and tandem mass spectrometry. Bioinformatic analysis was performed via BLAST searches using NCBI and GenBank databases which contained the sequenced genome for strain 86-028NP. Several hundred proteins were positively identified. The thirteen most abundant proteins found in biofilm supernatants (used as a surrogate for CRS patient fluids we intend to recover in clinical trials) are reported. From this list of several hundred proteins, four candidate biomarkers were selected based on biologic function and potential relevance to sinusitis. The four proteins selected were high molecular weight adhesin 1 (HMW1), outer membrane protein P2 (OMP-P2), outer membrane protein P5 (OMP-P5), and IgA-specific serine endopeptidase.

Figure 4. Micro-computed tomography of paranasal sinus cavities in the chinchilla

- (A). Axial, coronal, and sagittal reconstructions of chinchilla sinus anatomy in live animals showing similarity of paranasal sinus anatomy in this small mammal compared to humans. The ethmoidal sinuses of the chinchilla correspond to ethmoid sinus cavities in humans
- (B). Contrast rhinography demonstrating ability to topically deliver an inoculum of NTHI to the paranasal sinus cavity. Left ethmoid cavities received iodinated contrast intranasally and demonstrate hyperintense uptake compared to the right ethmoid cavities.

Figure 5. Micro-computed tomography evidence of experimental sinusitis

Chinchillas inoculated with adenovirus serotype 1 followed by NTHI 86-028NP display early evidence of secretion accumulation within their maxillary sinus cavities. Both images are axial sections of ct scans taken from two chinchillas at a time point corresponding to day 10 after adenoviral infection and day 3 after NTHI inoculation.

Figure 6. Colony forming units (CFU) recovered from chinchillas inoculated adenovirus and NTHI 86-028NP

100% of juvenile chinchillas inoculated with adenovirus followed by NTHI had recoverable CFU of NTHI at sacrifice. NTHI CFU increased on average by 500% from Day 4 to Day 10 post inoculation. Control animals (adenovirus only or saline only) did not demonstrate detectable NTHI CFU (data not shown). All values (both in Day 4 and Day 10) demonstrated significant increases in CFU over control animals; statistical testing via Student's T test; $p < 0.03$.

Figure 7. Validation of selected biomarkers across multiple clinical isolates of NTHI

After annotation of the NTHI biofilm secretome and selection of 4 candidate biomarkers: 1) high molecular weight adhesin, 2) IgA-specific serine endopeptidase, 3) outer membrane protein P2, and 4) outer membrane protein P5, western blot testing using polyclonal antibodies across multiple clinical isolates was performed. IgA-specific serine endopeptidase was found to share significant homology with the Hap protein, including mosaic-like patterns of gene homology, and no acceptable polyclonal antibody to this protein was identified; as a result, IgA-specific serine endopeptidase was excluded from further consideration. High molecular weight adhesin displayed incomplete sensitivity across multiple strains and was excluded from further consideration. OMP-P2 and OMP-P5 were readily detected in all clinical isolates of NTHI tested and chosen to be suitable biomarker candidates for further validation in secretions from chinchillas experimentally infected with NTHI.

Figure 8. Validation of outer membrane protein P2 and outer membrane protein P5 as biomarkers from chinchilla nasopharyngeal secretions inoculated with NTHI

Western blot testing using polyclonal antibodies to outer membrane protein P2 and outer membrane protein P5 confirmed the detection of both biomarkers with nasopharyngeal secretions, validating the feasibility of the use of these two proteins as potential biomarkers for the detection of NTHI-induced sinusitis.

Figure 9. Adaptation of reference standard, real-time, quantitative RT-PCR based test for detection of NTHI

Primers against the *hpd* gene encoding Protein D were utilized. The RT-PCR based test was 100% sensitive and specific for accurate identification of NTHI species in nasopharyngeal secretions using 5 clinical isolates of NTHI and 3 clinical isolates of *Moraxella catarrhalis* as negative controls.