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Bacteria in the nose of young adults during wellness and rhinovirus colds - detection by culture and microarray methods in 100 nasal lavage specimens

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Abstract

Background—Patients with viral respiratory infections/viral rhinitis/common colds are often treated with antibiotic although there is little information on whether or how bacterial microbiota in the nose and nasopharynx might influence the course of viral illnesses.

Methods—To initiate investigation of possible interaction between viral respiratory illness and microbiota of the nose/nasopharynx, we utilized microarray technology to examine 100 nasal lavage fluid samples (NLF) for bacterial species and recorded the bacterial titer of culturable bacteria. Rhinovirus illnesses were induced by self-inoculation using the “finger to nose or eye natural transmission route” in ten otherwise healthy young adults. NLF samples were collected during wellness and at specific time points following experimental rhinovirus inoculation.

Results—The rhinovirus infection rate was 70%. There were no consistent changes in the prevalence of different bacterial species determined by microarray and bacterial titer by culture methods during rhinovirus infection. The bacterial profile in NLF samples showed high variability between volunteers but low variability in multiple NLF's obtained before and following infection from the same volunteer. *S. epidermidis*/coagulase negative *staphylococcus* (CNS) were identified in all ten subjects. One or more bacterial sinus/otitis pathogens were identified by microarray in six of the ten volunteers. The microarray identified a few bacteria not included in traditional bacterial cultures.

Conclusion—Our pilot study showed that each of ten volunteers had a unique bacterial profile in the nose by microarray analysis and that bacterial load did not change during experimental rhinovirus colds. Larger scale studies are warranted.

Keywords

Bacteria; rhinovirus; nasopharynx; nasal lavage fluid; microarray

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Introduction

The symptom complex of viral respiratory infections (VRI's, common colds) and bacterial complications (bronchitis, otitis media and sinusitis) are self-limiting, but they cause billions of dollars in healthcare costs and millions of days of school and work missed in the US every year. Rhinovirus is the major cause of VRI's and young preschool age children contract as many as six rhinovirus infections per year [1, 2]. In the United States, the majority of antibiotics are used for VRI's, otitis, sinusitis and bronchitis. However, it remains unclear whether commensal bacteria may become pathogenic during the viral infection. Many pathogenic bacteria including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* reside in the nasopharynx in 60% -70% of healthy adults and children [3-7], but these bacteria may exacerbate symptoms during viral infections [8]. Further understanding of possible viral-bacterial interactions during VRI's are warranted to provide evidence for more appropriate use of antibiotics in colds.

Studies of nasopharyngeal bacteria during VRI's have been done without knowledge of the timing of the viral infection. Traditionally, bacterial identification was based on culture methods, but new technologies allow detection of bacterial genomes by microarray and sequencing (e.g. 454 pyrosequencing) [9]. In the present study, we utilized a new clinical model to study bacteria during colds by evaluating patients with experimentally induced rhinovirus colds and the application of a novel method of bacterial detection using microarray technology to determine the bacteria in the nasal cavity of otherwise healthy adults prior to and during rhinovirus infections. Experimental cold inoculation of volunteers with rhinovirus allows examination of changes in the commensal and pathogenic bacterial flora in the upper airways at precise time points during rhinovirus infections.

Methods

Participants

Volunteers between the ages of 18 and 65 were recruited by advertisement during October-November 2010. Subjects were eligible if they had a screening serum neutralizing antibody of 1:4 or less to the challenge rhinovirus type 39. Subjects were not eligible if they had a history of chronic sinus disease, history of sinus surgery, allergic rhinitis or if they had received topical or oral antibiotics within the four weeks prior to study participation or had upper respiratory tract symptoms during the two weeks prior to rhinovirus challenge. The study was approved by the Institutional Review Board for Health Sciences Research at the University of Virginia. Informed consents were obtained from all participants prior to enrollment.

Rhinovirus self- inoculation

Rhinovirus immunotype 39 was used as the challenge inoculum. This inoculum pool has been safety tested and approved for use by the FDA (IND12934). All subjects were exposed to a total of 100-300 TCID₅₀ of rhinovirus in 250 μ l by self-inoculation. Following hand washing, the volunteer used the tip of the pointer finger on the dominant hand to dip in the inoculum in a sterile Petri dish. The subject then inoculated her/himself by touching the medial cantus and conjunctiva of one eye or the septum in the nasal vestibulum of one side of the nose. The procedure was repeated once after a 5-15 minute interval.

Specimen collection

Nasal lavage fluid (NLF) was obtained by installation of 5mL of 0.9% saline into each nasal cavity while the subject had the head tilted backwards, repeating the "k-k-k" sound multiple times to close the soft palate to minimize fluid running into the throat. After a few seconds,

the subject moved the head forward and 5-8 mL of nasal lavage fluid was recovered into a waxed paper cup. Ten nasal washes were obtained from each volunteer: three during the week prior to HRV inoculation, one on each of five days immediately following inoculation, and on days 10 and 21 following inoculation (Figure 1). An aliquot of 1mL of each NLF samples was placed in tubes containing an equal volume of viral collection broth, kept on ice, and transported to the laboratory within one hour for rhinovirus isolation in tissue cultures. The remaining NLF was transferred to 2mL cryo-tubes and stored frozen at -80°C .

Assessment of Rhinovirus infection

The presence of rhinovirus in nasal and nasopharyngeal mucus was examined in NLF during each of five days following inoculation. 0.2 mL of NLF from each subject was inoculated into two fibroblast monolayer tubes (embryonic lung fibroblast cell/MRC-5 or WI 38) and incubated at 33°C in roller drums for 14 days. Tubes were examined every other day for rhinovirus cytopathic effect. One isolate from each subject was confirmed as the challenge virus by neutralization testing with antibody to rhinovirus 39. Subjects with a rhinovirus isolated from the nasal lavage specimens were considered to be infected. The serum antibody response to the challenge rhinovirus type 39 was examined on sera obtained prior to inoculation and three weeks following inoculation by standard methods [10]. Subjects with a 4-fold increase in antibody titer to the challenge virus were considered to be infected.

Assessment of illness

The severity of symptoms was recorded each morning by the study nurse when the subjects returned to the study site, prior to any study procedures. Upper respiratory tract symptoms/nasal symptoms including sneezing, runny nose, nasal obstruction and sore throat, and non-nasal symptoms including malaise, chilliness, cough and headache were recorded at baseline and each morning following rhinovirus inoculation by asking the subject to judge the severity of their symptoms on a scale from 0 to 4. The scale indicated 0 as absent (symptom not noticeable); 1 as mild (symptom by itself causes no limitation of usual activities); 2 as moderate (symptom by itself causes some limitation of usual activities); 3 as severe (symptom by itself causes severe limitation or inability to carry out usual activities); and 4 as very severe (symptom by itself leaves you unable to carry out usual activities). The score for each cold symptom present before the virus challenge was subtracted from each daily score for that symptom. The daily symptom scores were obtained at day zero and for 5 days following virus inoculation. The diagnosis of a cold illness (modified Jackson cold [11]) required a total symptom score of ≥ 6 for 5 days and either the presence of rhinorrhea on 3 or more days or the subjective impression of having a cold.

Assessment of bacterial load by culture

Nasal lavage fluid was transported to the laboratory on ice and cultured within one hour after collection. One tenth of 1 mL of NLF was inoculated onto both a blood and chocolate agar plate and incubated at 35°C for 48 hours. Number of bacterial colonies was examined at 24 and 48 hours. If there were less than 200 colonies, they were counted; 25-75 colonies was recorded as $10^{2.5}$ colonies per mL; 200 colonies was recorded as $10^{3.5}$ per mL; confluent growth on plates was recorded as $10^{4.0}$, and more than 200 but less than confluent was recorded as $10^{3.5}$ colonies per mL of NLF.

DNA extraction from nasal lavage fluid samples

From each NLF sample, 1.5 mL of NLF was centrifuged at 5000 rpm for 10 minutes, and the remainder of the sample was stored at -80°C . After centrifugation, 1mL of supernatant was discarded and 20 μL Proteinase K, at 20mg/mL (Roche Proteinase K, recombinant, PCR grade), was added to the 0.5 mL of sample and incubated at 60°C with 300 rpm agitation for

2 hours. Samples were then heated to 95°C to block Proteinase K activity. Extraction with a starting volume of 520µL and an elution volume of 60µL were done with Nordiag Arrow VIRAL NA® (CE/IVD) kit and Viral NA v1.0 program (NorDiag, Norway). Seven NLF samples did not have sufficient extracted DNA and were re-extracted using the remainder of the stored supernatant and a different extracting method suitable for smaller volumes (Nuclisens® easyMAG®, BioMerieux, Inc. France). DNA was quantified using 1µL of extracted DNA by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Concentrations and purity measures (A260/280) were recorded.

DNA Amplification and hybridization to the microarray

Broad range bacterial PCR was carried out according to the instructions of the Prove-it™ Bone and Joint assay (Mobidiag, Finland, <http://www.mobidiag.com>) which includes primers for bacteria tested (Table 2). Amplicons were hybridized onto the Prove-it™ Bone and Joint Strip Array using a modified version of the hybridization assay [12, 13]. The modified version of the assay used 10µL PCR product instead of using the standard volume of 3µL PCR product for hybridization, though all other steps were followed as stated in the protocol.

Microarray scanning and analysis

Microarray images were scanned using the Prove-it™ StripArray Reader and analyzed by Prove-it™ Advisor software using Mobidiag default parameters [12]. Analysis by Prove-it™ Advisor software includes simultaneous identification of bacterial targets and evaluation of the control probes included in the array. Results were detected by signals from the hybridization of PCR products with target specific oligonucleotides on the array. The software interpreted the array image using built-in rules and parameters which are specific for the assay type.

Results

Ten eligible subjects with serum neutralizing antibodies <4 were enrolled. Four were females (mean age 18 ½ years) and six were males (mean age 20 years). None of the subjects withdrew from the study or had serious adverse events.

Rhinovirus infection and illness rates

Six subjects were inoculated into the nose and four subjects into one eye. Overall, 70% of rhinovirus challenged subjects became infected. Four of six subjects inoculated into the nose got infected; three of the four inoculated into one eye got infected (Table 1). Four subjects shed rhinovirus into nasal secretion sometime during days 1-5 after inoculation, and six subjects had a 4-fold rise in serum neutralizing antibodies to the challenge virus. Three of the seven HRV infected subjects had clinical illness based on the modified Jackson colds method. The severity of illness in the rhinovirus infected volunteers appeared similar regardless of the nasal or eye inoculation method. One of three subjects who did not get infected with the challenge rhinovirus had clinical illness based on the modified Jackson method.

Bacterial culture

The titer of bacteria in NLF from each subject was surprisingly stable, varying only one log in 10 different samples, whereas the inter-individual variation was up to 4 fold. There were no apparent changes in the culturable bacterial load during rhinovirus infection compared to during wellness (Figure 2a-2c). Two of four HRV infected subjects (volunteer #'s 4 and 7) had a high bacterial load (>10³ cfu/mL) prior to inoculation and it remained high; the two

other subjects (volunteer #'s 1 and 5) dropped the bacterial load by one log from day 3 to 5 following rhinovirus inoculation. Three subjects (volunteer #'s 3, 6 and 8) had a low bacterial titer ($<10^3$ cfu/mL) throughout the study. The bacterial titer was not different in three symptomatic rhinovirus infected subjects (Figure 2a) compared to four rhinovirus infected subjects without symptoms (Figure 2b).

Microarray

Amplification of bacterial DNA and hybridization to the microarray was successful in all 100 NLF samples from ten volunteers. Prove-it™ Advisor software identified 13 bacterial species out of the bacterial species tested (Table 2). Samples analyzed for quality assurance were identical with original bacterial results. *S. epidermidis*/Coagulase negative *Staphylococcus* (CNS) was present in all volunteers, and *mecA* was present sporadically in samples from all ten volunteers. One or more of the potential otitis or sinus pathogens (*H. influenzae*, *S. pneumoniae* and *M. catarrhalis*) were detected in at least one NLF sample from 60% of volunteers. *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* were present in 40%, 30% and 30% of subjects, respectively (Figure 3). *H. influenzae* was present in 90% of samples from one volunteer (Table 3c, Vol #9). In each of the ten individuals, the prevalence of different bacterial species was similar across the ten samples obtained prior to and following rhinovirus inoculation (Table 3a-3c). A few bacterial species, such as *S. marcescens*, *E. aerogenes* and *K. kingae*, are not usually assayed with traditional culturing technique but were detected in the NLF samples by microarray (Table 3a-3c).

Discussion

This study examined the bacteria present in the nasal cavity of ten young healthy adults prior to and during rhinovirus infection. Rhinovirus infection was successfully induced using self-inoculation with inoculum applied with the finger into either the nose or eye. Inoculation with larger volume (½ mL) dropped into each nasal cavity was avoided in order to decrease the risk of spreading bacteria in the nasal cavity at time of inoculation.

An astonishing number of studies in the past have evaluated bacteria in the upper airways during colds by culturing bacteria on agar plates. Overgrowth of some bacteria may make less abundant pathogenic bacteria difficult to identify [14]. In this study, we have used microarray technology based on isolation of bacterial DNA to identify bacteria in the NLF samples. This approach allows for rapid detection of clinically relevant pathogens at the species level and the resistance marker *mecA* [12]. Bacterial species that are known to either be common in the upper airways or to have a pathogenic potential were included in the platform. Not surprisingly, the most common species found in this study was *Staphylococcus epidermidis*/CNS with at least one of these bacteria detected in 99 NLF samples out of the 100 total. The bacterial profile by microarray showed that samples from the same participant had a low variability, whereas inter-individual variability was high. The detection rate of bacterial sinusitis and otitis pathogens identified in nasal lavage fluid by microarray was similar to the detection rate by selective agar culture methods [3].

Nasal lavages were chosen as the sampling method (rather than swabbing specific areas) in order to obtain bacteria from the entire the nose/nasopharynx. The nasal wash sampling method has been shown to detect more potential pathogens than swabbing the nasopharynx in healthy adults [15]. The microarray did identify a few bacterial species that are not included in traditional culture methods. The importance of those bacterial species remains to be explored with a larger number of subjects.

During wellness, the nasopharynx and nasal vestibulum are colonized with bacteria, whereas the nasal turbinates and especially the osteomeatal complex are not colonized with

pathogenic bacteria [6, 7]. In this study, the bacterial profile in the NFL by microarray did not show any major changes following rhinovirus infection. This finding is in agreement with a prior study in adults with natural colds [16]. The overall bacterial titer by culture on blood agar plates also remained within one log difference in the ten nasal lavage samples obtained from each volunteer. Following rhinovirus inoculation into the eye, the virus enters the nasal cavity via the lacrimal duct and is transported back to the nasopharynx where it initially can be detected in the majority of subjects. Later, the rhinovirus can also be detected on the nasal turbinates, suggesting that the rhinovirus spread [17]. Similarly, potential pathogenic bacteria from the nasopharynx have been detected in the osteomeatal complex of the nasal cavity during natural colds but not during wellness [6]. Bacteria transient in the nose are removed by the mucociliary clearance mechanism during wellness but during colds the mucociliary clearance function is decreased for 3-4 weeks [18-20]. A longer transit time of the bacteria in the nose may increase the risk of further displacement into the paranasal sinuses and/or the middle ear cavity by nose-blowing [21-23]. Kaiser, et al. showed a benefit of antibiotic treatment in the subgroup of patients with early stage common colds with pathogenic bacteria in the nose/nasopharynx by culture method [24]. It would be of great interest to know whether displacement of potentially pathogenic bacterial to the nose during colds would increase the overall morbidity of colds and/or affect the severity of nasal symptoms. If so, treatment of colds should attempt to decrease spread of virus and bacteria into the nasal cavity by a combination of several different approaches such as 1) an antiviral and antibacterial nasal spray 2) decrease of mucus production and 3) enhancement of nasopharyngeal clearance by sniffing rather than nose blowing.

In conclusion, we present an experimental clinical model to study possible effects of bacteria during viral respiratory tract infection. We successfully infected healthy adults with rhinovirus by self-inoculation into the eye or nose and demonstrated that microarray is an excellent way to detect more bacterial species than traditional bacterial culture methods. We used a commercially available microarray platform for rapid detection of clinically relevant bacteria. In the future, a microarray platform designed using 16S rDNA sequence data or RNASeq data results of a cross-section of samples from normal children and adults and patients with upper respiratory diseases should be used. In this pilot study, each of 10 volunteers had a unique bacterial profile as evidenced by microarray and the bacterial load did not change during viral infection. Larger scale studies are needed to assess definitively whether bacterial load or species change during rhinovirus colds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Study timeline including when information or samples were collected from participants.

*NLF samples were obtained prior to Rhinovirus inoculation on this day.

Figure 2.

Bacterial titer by semi-quantitative cultures in nasal wash samples in HRV inoculated volunteers. Shaded region indicates nasal wash samples obtained during the first five days following HRV 39. a) Rhinovirus infected volunteers with illness; b) Rhinovirus infected volunteers without illness; c) Not rhinovirus infected volunteers

Figure 3.

Prevalence of bacteria identified by microarray in 100 samples from 10 volunteers inoculated with HRV39. Blue bars represent the percentage of **samples** (out of 100) which the species/gene was detected. The orange bars represent the percentage of **subjects** (out of 10) in whom the species/gene was detected

Table 1

Infection and Illness in Volunteers following self-inoculation of HRV 39

Subject	1	6	7	8	2	3	4	5	9	10
Route of inoculation	Eye	Eye	Eye	Eye	Nose	Nose	Nose	Nose	Nose	Nose
HRV infection	yes	no	yes	yes	no	yes	yes	yes	no	yes
Clinical illness	yes	no	yes	no	no	no	no	yes	yes	no
Total symptoms score	7	0	29	6	3	4	4	20	12	0

Table 2List of Species covered on the Mobidiag Prove-it™ Bone and Joint Assay (<http://www.mobidiag.com>)

Gram+ Bacteria	Gram – Bacteria
Coagulase negative <i>staphylococcus</i> *	<i>Haemophilus influenzae</i>
<i>Staphylococcus aureus</i>	<i>Moraxella catarrhalis</i>
<i>Staphylococcus epidermidis</i>	<i>Acinetobacter baumannii</i>
<i>Streptococcus agalactiae</i>	<i>Bacteriodes fragilis</i> group**
<i>Streptococcus dysgalactiae</i> subspecies <i>equisimilis</i>	<i>Enterobacteriaceae</i> ***
<i>Streptococcus pneumoniae</i>	<i>Enterobacter aerogenes</i>
<i>Streptococcus pyogenes</i>	<i>Enterobacter cloacae</i>
<i>Clostridium perfringens</i>	<i>Escherichia coli</i>
<i>Enterococcus faecalis</i>	<i>Fusobacterium necrophorum</i>
<i>Enterococcus faecium</i>	<i>Kingella kingae</i>
<i>Listeria monocytogenes</i>	<i>Klebsiella oxytoca</i>
	<i>Klebsiella pneumoniae</i>
*Detects <i>S. haemolyticus</i> , <i>S. hominis</i> , <i>S. lugdunensis</i> , <i>S. saprophyticus</i> , <i>S. warneri</i> , <i>S. xylosum</i>	<i>Neisseria meningitidis</i>
	<i>Neisseria</i> species non-meningitidis****
	<i>Proteus mirabilis</i>
<i>mecA</i> meticillin resistance marker	<i>Proteus vulgaris</i>
	<i>Pseudomonas aeruginosa</i>
	<i>Salmonella enterica</i> subspecies <i>enterica</i> *****
	<i>Serratia marcescens</i>
	<i>Stenotrophomonas maltophilia</i>
	<i>Campylobacter jejuni/coli</i>
	**Detects <i>B. fragilis</i> , <i>B. vulgatus</i> , <i>B. thetaiotaomicron</i>
	***Detects <i>C. amalonaticus</i> , <i>C. braakii</i> , <i>C. freundii</i> , <i>C. koseri</i> , <i>E. hormaechei</i> , <i>E. sakazakii</i> , <i>K. intermedia</i> , <i>M. morganii</i> , <i>P. agglomerans</i> , <i>P. rettgeri</i> , <i>P. stuartii</i> , <i>Y. enterocolitica</i> , <i>Y. pseudotuberculosis</i>
	****Detects <i>N. gonorrhoeae</i> , <i>N. subflava</i> , <i>N. sicca</i> , <i>N. cinerea</i> , <i>N. elongata</i> subspecies <i>nitroreducens</i> , <i>N. flavescens</i> , <i>N. lactamica</i> .
	*****Detects the following serovars: <i>Enteritidis</i> , <i>Oranienburg</i> , <i>Othmarschen</i> , <i>Panama</i> , <i>Paratyphi</i> , <i>Stanley</i> , <i>Typhimurium</i> , <i>Virchow</i> , group A, B, C, D.
	*****Detects the following serovars: <i>Enteritidis</i> , <i>Oranienburg</i> , <i>Othmarschen</i> , <i>Panama</i> , <i>Paratyphi</i> , <i>Stanley</i> , <i>Typhimurium</i> , <i>Virchow</i> , group A, B, C, D.

Table 3

Bacteria detected by microarray in nasal lavage fluid samples obtained during wellness (well) and on days following rhinovirus challenge (days 1-21). *S. epidermidis*/CNS was positive in 99 samples/all volunteers and *mecA* was detected in all volunteers sporadically and therefore are not included in the table. a) Rhinovirus infected volunteers with illness; b) Rhinovirus infected volunteers without illness; c) Not rhinovirus infected volunteers

a											
Volunteer 1	Well	Well	Well	Day1	Day2	Day3	Day4	Day5	Day10	Day21	
<i>S. aureus</i>				pos							
<i>S. pneumoniae</i>			pos		pos				pos		
<i>H. influenzae</i>			pos		pos	pos	pos				
<i>Enterobacteriaceae</i> family			pos	pos	pos						
<i>Neisseria</i> sp.					pos			pos			
Volunteer 5											
<i>Neisseria</i> sp.						pos	pos	pos			
Volunteer 7											
<i>Neisseria</i> sp.				pos	pos	pos			pos	pos	
b											
Volunteer 3	well	well	well	Day1	Day2	Day3	Day4	Day5	Day10	Day21	
No pathogens											
Volunteer 4											
<i>M. catarrhalis</i>		pos	pos			pos	pos	pos	pos	pos	
<i>Neisseria</i> sp.									pos		
Volunteer 8											
<i>S. aureus</i>						pos			pos	pos	
<i>S. pneumoniae</i>					pos		pos				
<i>H. influenzae</i>							pos				
<i>Neisseria</i> sp.					pos			pos			
Volunteer 10											
<i>Streptococcus</i> sp.						pos					
<i>H. influenzae</i>	pos	pos	pos							pos	
<i>M. catarrhalis</i>										pos	

[illegible]

* had symptomatic illness not associated with rhinovirus infection with challenge virus.