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Journal of Infection (2014) 68, 125—130

www.elsevierhealth.com/journals/jinf

Detection of viral and bacterial pathogens in acute respiratory infections

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Accepted 31 October 2013
Available online 7 November 2013

KEYWORDS
Upper respiratory infections;
Bacteria;
Common cold;
Inflammation

Abstract *Objectives:* The role of bacteria in acute respiratory illnesses (ARI) of adults and interactions with viral infections is incompletely understood. This study tested the hypothesis that bacterial co-infection during ARI adds to airway inflammation and illness severity.
Methods: Two groups of 97 specimens each were randomly selected from multiplex-PCR identified virus-positive and virus-negative nasal specimens obtained from adults with new onset ARI, and 40 control specimens were collected from healthy adults. All specimens were analyzed for *Haemophilus influenzae*(*HI*), *Moraxella catarrhalis*(*MC*) and *Streptococcus pneumoniae*(*SP*) by quantitative-PCR. General linear models tested for relationships between respiratory pathogens, biomarkers (nasal wash neutrophils and CXCL8), and ARI-severity.
Results: Nasal specimens from adults with ARIs were more likely to contain bacteria (37% overall; *HI* = 28%, *MC* = 14%, *SP* = 7%) compared to specimens from healthy adults (5% overall; *HI* = 0%, *MC* = 2.5%, *SP* = 2.5%; $p < 0.001$). Among ARI specimens, bacteria were more likely to be detected among virus-negative specimens compared to virus-positive specimens (46% vs. 27%; $p = 0.0046$). The presence of bacteria was significantly associated with increased CXCL8 and neutrophils, but not increased symptoms.
Conclusion: Pathogenic bacteria were more often detected in virus-negative ARI, and also associated with increased inflammatory biomarkers. These findings suggest the possibility that bacteria may augment virus-induced ARI and contribute to airway inflammation.
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http://dx.doi.org/10.1016/j.jinf.2013.10.013

Introduction

Viruses are the major cause of acute respiratory infections (ARI) in both adults and children.[1] ARI, including both influenza and the common cold, is a worldwide problem that accounts for significant loss of productivity and financial burden on the healthcare system.[2–4] Although various experimental[5] and epidemiological[6,7] studies have identified viruses as the pathogens for most ARI, a significant number of ARI episodes have unknown etiologies despite improved diagnostic procedures.[8–10]

It has been suggested that bacterial pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* contribute to ARI, however; results from studies have been inconclusive.[11,12] Detection of bacteria is increased in symptomatic children[13,14] and adults[11] compared to healthy controls.[15,16] In a study involving 507 ARI sufferers[11] Heald et al. (1993) reported 56% positive bacteria cultures from nasopharyngeal secretions of adults with ARI illness, but found no bacteria in healthy controls. However, Winther et al. (1984) found no difference in nasal bacterial between healthy and ARI ill conditions.[12] Even so, antibiotics are often prescribed for uncomplicated ARI,[17] and widespread inappropriate use of antibiotics contributes to the emergence of antibiotic resistance and therefore to increased health care costs.[18]

There is evidence that virus-induced inflammation contributes to respiratory symptoms. During the course of viral illnesses, there are significant correlations between interleukin-8 (CXCL8)[7] levels and neutrophil counts[19] in nasal secretions and cold symptom severity. There is some evidence that detection of bacterial pathogens during ARI may be associated with increased inflammatory biomarkers.[11]

Given these findings, we hypothesized that during viral ARI, detection of specific bacterial pathogens would be associated with increased levels of inflammatory biomarkers and greater measures of severity of illness. A secondary goal was to examine nasal secretions for pathogenic bacteria in ARI adult sufferers with and without detectable viruses. The rationale for this stratified analysis is to determine if bacterial co-infection would lead to greater ARI illness severity compared to viral only or no pathogen detection. Stratification enabled us to determine whether symptoms were greater for "bacteria plus virus" vs. "virus alone", and also to determine whether symptoms were greater for "bacteria alone" compared to "no pathogen detected".

As a control group, we also evaluated the frequency of the same bacteria in nasal wash specimens from healthy adults. Finally, we assessed the relationship between these respiratory pathogens, inflammatory biomarkers and self-reported severity of illness.

Design and methods

Study populations

The study protocol was approved by the University of Wisconsin–Madison Institutional Review Board. The ARI specimens were obtained from a subset of participants in the NIH-sponsored randomized clinical trial, the "Physician, Echinacea, Placebo (PEP)" study.[20] A total of 712 nasal wash specimens were obtained from adults at the beginning of an ARI and were tested for viral nucleic acid by multiplex PCR multiplex.[21] Of these, 395 were found to be positive for virus and 317 found to be negative. For this study, 97 specimens per group were randomly selected (www.randomizer.org) from each of 2 groups: those with detectable respiratory viruses and those without detectable respiratory viruses. This sample size was selected based on 2-sided testing, with $\alpha = 0.05$, power = 80%, and hypothesized 20% difference in bacterial detection rates (effect size). The PEP trial spanned from January 2004 to August 2008 and enrolled 719 participants of whom 713 completed the study (one participant had missing viral nucleic acid result).[22] The study rationale and methods have been described previously.[23]

Briefly, the pill arm of the PEP trial examined placebo and Echinacea. Participants were eligible if they acknowledged having a cold, had $\geq$2 points on the Jackson symptom scale[24] and included $\geq$1 of the following symptoms within 36 h of enrollment: nasal discharge, obstruction, sneezing or sore-throat. Reasons for exclusion included active symptoms of allergy and asthma observed at enrollment, or use of antibiotics or other excluded medications.

Additional specimens were obtained from adults ($n = 40$) with no evidence of cold symptoms.

Outcome assessments

Global ARI severity was calculated using area-under-the-curve trapezoidal approximations with duration on the *x*-axis and symptom scores on the *y*-axis. Duration of illness was defined as time from symptom onset until the participant responded with "No" to the question "Do you think you still have a cold?" Symptom scores were self-reported on the Wisconsin Upper Respiratory Symptom Survey (WURSS-21).[25] The WURSS-21 consists of 10 symptom and 9-quality of life items used in severity estimations. Two remaining items assessing global severity "(How sick do you feel today?") and daily change of illness ("Compared to yesterday, I think my cold is...") were assessed separately. PEP findings showed no significant between-group differences in severity and duration of illness between treatment groups.

Nasal wash specimens were also analyzed for interleukin-8 (CXCL8) and neutrophils as described previously.[23,26]

Pathogen detection

Viral pathogens from nasal secretions collected on day-1 were identified using multiplex PCR (Respiratory MultiCode-PLx Assay, EraGen Biosciences, Madison WI). This assay detects all common respiratory viruses including; rhinovirus, coronavirus, influenza, respiratory syncytial virus, parainfluenza virus, adenovirus, bocavirus, metapneumovirus, and enterovirus.[21]

Nasal wash specimens were analyzed for specific bacterial pathogens. DNA was extracted from 300 μl of nasal wash specimens (BiOstic Bacteremia DNA isolation kit, MO

BIO Laboratories, Carlsbad, CA). To prevent potential degradation of the bacteria DNA, 0.1 mM of EDTA was added to the samples. Quantitative PCR was performed for *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*. The detection range for each bacterium was from 10 to 1 million colony-forming units (CFU), and the standard curve consisted of DNA extracted from bacteria assayed using the McFarland technique. Multiplex q-PCR employing P6 and copB genes were used for the detection of *H. influenzae*[27] and for *M. catarrhalis*[28] respectively, and PCR for lytA[29] was conducted separately for detection of *S. pneumoniae* (Table 1). All primers and probes were purchased from Applied Biosystems, Foster City, CA.

Statistical analysis

Differences between bacterial prevalence were tested using chi-square (χ^2) test. The general linear model (GLM) was used to examine the relationship between pathogens, CXCL8 and neutrophils and severity of illness on day-1. Box—Cox transformations were used to normalize the highly skewed distributions of the biomarkers CXCL8 and neutrophil count. Square-root transformation was employed to normalize the distribution of WURSS severity scores on day-1 (LAMBDA = 0.36), while natural log (LN) transformations were used to normalize distributions of the biomarkers (Lambda: CXCL8 = 0.04; neutrophils = 0.02). Covariates included age, gender and season of the year (spring, summer, fall and winter). NCSS® 2007 statistical program was used for analyses.[30]

Results

Study subjects and virology

Nasal wash specimens were obtained from a total of 40 healthy adults and 194 participants during acute colds (Table 2). The average age, income and proportion with $\geq$ college education were higher among the healthy group compared to the ARI group.

The samples from symptomatic participants were collected during fall (35%), winter (39%) spring (17%) and summer (9%) seasons, during the years 2004—2008, while samples from healthy adults were collected in August—September 2012. Among the virus positive specimens, the most common viral agents were rhinovirus (72%) and coronavirus (10%). Ninety-five percent of this group contained only one type of virus while the remaining 5% contained two different viruses.

Bacterial detection during illness vs. health

The frequency of bacterial detection was significantly greater among participants with ARI illness (37%; 71 out of 194 samples) compared to healthy participants (5%; 2 out of 40 samples, $p < 0.001$). Among the 194 nasal specimens from symptomatic adults, bacteria were detected significantly more often from the virus-negative samples compared to the virus-positive (47% vs. 27%, $p = 0.0046$).

Overall, in subjects with ARI regardless of viral etiology, *H. influenzae* was identified in 22% ($n = 43$) of the nasal specimens, *M. catarrhalis* in 14% ($n = 27$) and S. pneumoniae in 7% ($n = 13$).

Bacterial detection in viral vs. non-viral ARI

Bacterial detection varied depending on the presence of virus (Fig. 1); virus-negative specimens were more likely to have bacterial pathogens detected (46% overall, including *H. influenzae* 29%, *M. catarrhalis* 22% and *S. pneumoniae* 9%) compared to virus-positive nasal specimens (27% overall, including *H. influenzae* 19%, *M. catarrhalis* 6% and *S. pneumoniae* 4%).

ARI severity of illness

Within the ARI group, the severity of illness (mean; 95% confidence interval) was similar with bacteria only (6.5; 5—7.1), combination of bacteria and virus (6.5; 5.7—7.2), virus only (6.3; 5.8—6.7), or no pathogen (6.1; 5.5—6.6). Adjusting for age, gender and season of year did not significantly alter these trends (Table 3). In addition, patterns of pathogen detection were not associated with differences in individual rhinitis symptoms (results not shown).

CXCL8 and neutrophil biomarkers

Detection of a bacterial pathogen was associated with higher levels of CXCL8 and greater numbers of neutrophils (Table 3). The combination of virus and bacteria had the highest levels (mean; 95% CI) of CXCL8 (6.4, 5.8—6.9) and neutrophil counts

Table 1 Primers and probes for *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*.

	Haemophilus influenzae[27]	*Moraxella catarrhalis*[28]	*Streptococcus pneumoniae*[29]
Forward primer	Hi P6 F 5′-CCA GCT GCT AAA GTA TTA GTA GAA G-3′ (302-326)	Mc copB F 5′-GTG AGT GCC GCT TTT ACA ACC-3′ (50-70)	LytA F 5′-ACG CAA TCT AGC AGA TGA AGC A-3′
Reverse primer	Hi P6 R 5′-TTC ACC GTA AGA TAC TGT GCC-3′ (477-457)	Mc copB R 5′-TGT ATC GCC TGC CAA GAC AA-3′ (121-102)	LytA R 5′-TCG TGC GTT TTA ATT CCA GCT-3′
Probe	Hi P6 VIC 5′-CAG ATG CAG TTG AAG GTT ATT TAG- MGB-‘3	Mc copB NED 5′-TGC TTT TGC AGC TGT TAG CCA GCC TAA-3′-MGB (73-99)	LytA TaqMan probe FAM FAM-5′-TGC CGA AAA CGC TTG ATA CAG GGA G-3′ -MGB

Table 2 Demographic characteristics of study population.

	Participants with ARI		Healthy participants (n = 40)
	No-detectable virus group (n = 97)	Detectable virus group (n = 97)	
Age, mean (years) (SD)	35 (14)	34 (14)	50 (12)
Female n (%)	61 (63)%	63 (65%)	28 (70%)
Non-smokers' n (%)	66 (68%)	63 (65%)	25 (63%)
Race, white n (%)	81 (84%)	91 (94%)	37 (93%)
College graduate or higher, n (%)	43 (44%)	50 (52%)	30 (75%)
Income > $50,000, n (%)	35 (36%)	48 (49%)	22 (55%)

(3.8, 3.2—4.4); and were significantly different compared with viral only specimens (each $p = 0.01$).

Discussion

Bacterial pathogens cause respiratory illnesses such as sinusitis and pneumonia, but their role in the common cold is controversial. To test the hypothesis that bacterial co-infection during ARI adds to airway inflammation and the severity of illness, we examined the frequency of pathogenic bacteria in nasal wash specimens from healthy adults and adults with ARI. We also included further exploratory analysis based on the presence or absence of detectable respiratory viruses. Overall, bacteria were detected more often during illnesses. Within the ARI group, bacterial detection was significantly greater among samples without detectable viruses compared to samples with viruses. Furthermore, airway inflammation, but not illness severity, was increased with combined presence of bacterial and viral pathogens. These findings suggest that bacterial pathogens may contribute to a subset of ARI and that bacterial pathogens may augment airway inflammation during viral ARI.

Previous studies of bacterial pathogen detection during ARI have reported varied results.[11,12,31,32] In support of our results, Han et al. detected increased bacterial pathogens from nasal swabs obtained from symptomatic participants.[32] Similar findings have also been reported in a study of nasopharyngeal wall aspirates from adults with upper respiratory tract infection.[11] Other studies of experimentally[33] or naturally[12] acquired ARI have not found illness-related changes in bacterial frequency during ARI. None of these studies stratified based on the presence of viral pathogens.

When pathogens were identified among the group with ARI, the most frequent finding was detection of a virus alone (n = 71), consistent with the accepted concept that viruses are the most common cause of common colds. In the virus-negative samples, increased detection of bacteria suggests the possibility that bacteria contribute to illness pathogenesis. This concept is also supported by results of double-blinded randomized controlled trials of antibiotic treatment of ARI, in which beneficial effects were demonstrated in the subgroup of participants with detectable bacteria pathogens.[33,34]

In our analysis, samples that were positive for both bacteria and virus were associated with increased inflammatory changes in nasal secretions, as indicated by greater amounts of CXCL8 and neutrophils. Despite the increase in inflammation associated with detection of viruses plus bacteria, severity of illness was not increased compared to illnesses associated only with a virus. In most studies of ARI, markers of neutrophilic inflammation are positively correlated with symptoms during respiratory illnesses.[7,11] The lack of correlation between biomarkers and symptoms in our study may be due to the relatively low range in illness severity. In the PEP clinical protocol from which these subjects were selected, the participants needed to have a threshold level of symptoms to meet eligibility criteria. Most illnesses were mild to moderate in severity, and exclusion of asymptomatic infections and the limited

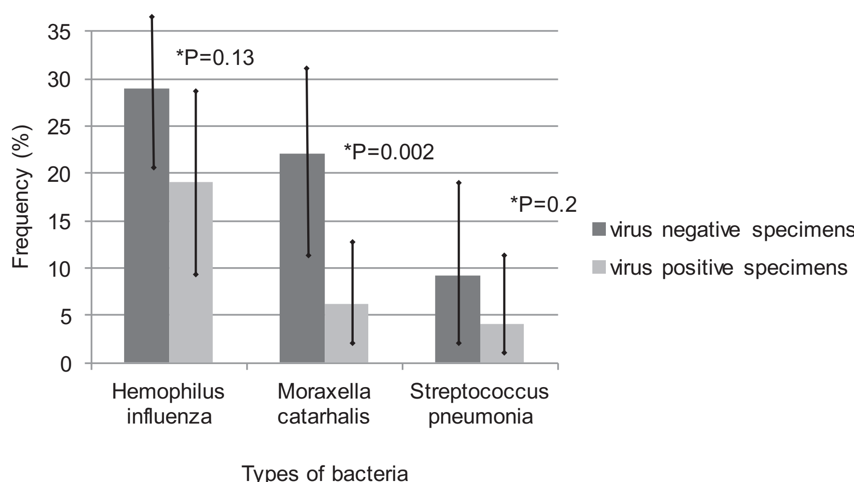

Figure 1 Type of bacteria detected during acute respiratory infections. *χ^2 test of bacteria estimates.

Table 3 Relationship between pathogen detection and illness severity, inflammatory markers during acute respiratory infections.

Outcomes	No pathogen (n = 52)	Virus only (n = 71)	Bacteria only (n = 45)	Virus and bacteria (n = 26)
Mean severity of illness[a] (95% CI)	37 (30, 44)	40 (34, 45)	43 (36, 50)	43 (32, 52)
Mean CXCL8 pg/ml[b] (95% CI)	203 (134, 309)	235[c] (179, 309)	303 (177, 521)	591[c] (320, 1092)
Mean neutrophil counts[b] (95% CI)	10 (6, 14)	15[c] (10, 21)	20 (11, 35)	45[c] (23, 92)

[a] Square root means.
[b] Geometric means.
[c] Two-sided p-value <0.05 comparing virus only to combined virus and bacteria.

number of more severe infections may have obscured relationships between etiology, inflammation, and symptoms of illness.

Limitations of this study include a moderate sample size, a homogenous population (predominantly Caucasians) and the likelihood that the course of illness could differ with types of pathogens. Strengths of the study design include the use of comprehensive viral diagnostics, as well as PCR-based detection of bacterial pathogens. The latter provides a sensitive and culture-independent diagnostic technique for bacterial detection.

In conclusion, we found higher levels of pathogenic bacteria in nasal wash specimens obtained from symptomatic ARI participants compared to healthy adults, and among symptomatic adults without detectable virus compared to those with virus. During viral illnesses, the presence of bacteria was associated with increased inflammatory markers. These findings suggest the possibility that bacterial pathogens are causal in a subset of ARI, and prospective studies are thus warranted to better define the temporal presence of viral and bacterial pathogens and relationship to inflammation and illness.

Funding support

"Physician, Echinacea, Placebo (PEP)" [1-R01-AT-1428] and Meditation or Exercise for Preventing Acute Respiratory Infection (MEPARI-2) [1 R01 AT006970-01] including the K24 Midcareer Investigator Award [K24AT006543] were funded by the National Center for Complementary and Alternative Medicine. Bruce Barrett was the Principal Investigator on both studies.

Chidi Obasi is supported by: 1) National Research Service Award from the Health Resources and Services Administration to the University of Wisconsin Department of Family Medicine (T32HP10010); and 2) Clinical and Translational Sciences Award (CTSA) program of the National Center for Research Resources (NCRR), National Institutes of Health (NIH) (Grant 1UL1RR025011). Laboratory costs for the current study were supported by the U.W. Department of Family Medicine small grant program.

Authors' contributions

All authors contributed substantially to the development of this manuscript, approved the final version of this report, and report no conflicts of interest pertaining to this work.

We are thankful to Mindy Smith, MD MS- Associate Professor affiliated with Family Medicine, Michigan State University USA; and Nnenna O.A. Kalu, MBBS- Family Medicine Resident with University of Uyo Teaching Hospital, Akwa Ibom State Nigeria, for their careful review of the manuscript.

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