

Infectious exacerbations of chronic obstructive pulmonary disease associated with respiratory viruses and non-typeable *Haemophilus influenzae*

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Received 3 January 2003; received in revised form 7 February 2003; accepted 3 March 2003

First published online 3 April 2003

Abstract

Infectious exacerbations of chronic obstructive pulmonary disease (COPD) have been reported to occur with both viral and bacterial pathogens. In this study, 35 exacerbations associated with the isolation of non-typeable *Haemophilus influenzae* from sputum were identified as part of a prospective longitudinal study. Samples from these patients were subjected to immunoassays to identify a new immune response to the homologous isolate of non-typeable *H. influenzae* to more accurately assess a bacterial etiology. These patients also were studied carefully for evidence of viral infection using viral culture, serology and polymerase chain reaction-based assays. Sixteen of 35 exacerbations (45.7%) were associated with evidence of acute viral infection and 11 of the 35 exacerbations (31.4%) were associated with the development of new serum IgG to homologous non-typeable *H. influenzae*. Overall, evidence of infection with a respiratory virus or non-typeable *H. influenzae* was seen in 24 of 35 exacerbations (68.6%). No association between viral infection and immune response to non-typeable *H. influenzae* was observed, although a trend toward an immune response to non-typeable *H. influenzae* and absence of viral infection was seen. The results show that exacerbations in adults with COPD were associated with infection caused by virus alone, non-typeable *H. influenzae* alone, or virus and non-typeable *H. influenzae* simultaneously.

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Keywords: Viral respiratory tract infection; Bacterial respiratory tract infection; Chronic obstructive pulmonary disease; Enzyme-linked immunosorbent assay; Exacerbation; *Haemophilus influenzae*

1. Introduction

The course of chronic obstructive pulmonary disease (COPD) is characterized by periodic exacerbations of the disease. These exacerbations are associated with substantial morbidity, lead to approximately 2 million hospital

admissions annually, cause respiratory failure requiring mechanical ventilation in some patients, and are an important cause of death in COPD [1]. Exacerbations are caused by a wide range of factors, including viral infection, bacterial infection, allergy, fluid overload, environmental factors and others [1,2]. In order to develop successful strategies to better treat and prevent exacerbations, it will be important to elucidate more precisely the etiology of exacerbations. Unfortunately, the etiology of exacerbations is difficult to determine with certainty in individual patients.

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Determining the infectious etiology of exacerbations is particularly challenging in view of the limitations of routine laboratory tests. Viral pathogens have been identified by viral cultures, analysis of clinical samples by the polymerase chain reaction (PCR) and by serological tests. Since these assays are not routinely performed, the diagnosis of a viral infection is generally a presumptive, clinical diagnosis by the practicing physician. Several studies which utilized assays to identify viral pathogens have established that viral infection is associated with a significant proportion of exacerbations of COPD [3–7]. One study demonstrated that respiratory tract viral infections were present in 23% of hospitalizations in adults with COPD and in 45% of those admitted during winter months [3].

Non-typeable *Haemophilus influenzae* is the most common bacterium implicated as a cause of exacerbations of COPD [1]. A goal of the present study is to assess the immune response to non-typeable *H. influenzae* isolated from the sputum of patients experiencing exacerbations. Several studies have used this approach [8]. However, one must be cautious in interpreting these studies because recent work has established that the immune response to non-typeable *H. influenzae* in COPD is directed at strain-specific antigens [9–14]. Therefore, in order to detect a human immune response to non-typeable *H. influenzae*, it is critical that the homologous infecting strain be used as the source of antigen in the immunoassays.

While several studies have assayed for the presence of viral infection in exacerbations and several studies have looked carefully for evidence of infection caused by non-typeable *H. influenzae*, no studies have used rigorous methods to look for both in the same set of patients. In the present study, 35 exacerbations during which non-typeable *H. influenzae* was isolated from the sputum, were identified in a cohort of adults with COPD followed prospectively at Baylor College of Medicine. The goal of the present study was to rigorously assess these 35 exacerbations for the presence of viral infection and for infection by non-typeable *H. influenzae* as evidenced by the presence of the organism in sputum and a new immune response to the homologous isolate.

2. Materials and methods

2.1. Study population

Samples were collected from subjects receiving care at Ben Taub General Hospital of the Harris County Hospital District and enrolled in a longitudinal study of chronic bronchitis. To be eligible for the longitudinal study, a subject had to have chronic bronchitis as defined by the American Thoracic Society [15]. Subjects with known asthma, bronchiectasis, malignancy or immunocompromising conditions were excluded. Subjects also

had to agree to be followed monthly for evaluation and to be seen when signs or symptoms of a respiratory illness developed.

Study samples were selected from subjects who had an exacerbation, had non-typeable *H. influenzae* isolated from a sputum sample collected during the illness, had pre- and post-exacerbation sera available for analysis, and had no other illness in the interval covered by the paired sera. An exacerbation was defined as upper and/or lower respiratory symptoms, and one of the following symptoms: increased sputum production, cough or dyspnea.

2.2. Specimens

Sputum specimens were collected and transported on ice to the laboratory within 4 h of collection for bacteriologic studies as previously described [16]. Nasal washes and throat swab specimens were collected for virologic studies, placed in veal infusion broth, and transported on ice to the laboratory within 4 h of collection. Serum for antibody studies was collected at each visit, at the time of an exacerbation, and 2–4 weeks later, and it was stored at -20°C until used in serologic assays.

2.3. Virologic studies

Viral cultures and virus identification were performed as described previously [3,17,18] with the following modifications. Primary rhesus monkey kidney cells (BioWhittaker, Walkersville, MD, USA) were used in place of Madin-Darby canine kidney (MDCK) cells for some samples, and two human lung embryonic lung fibroblast lines (WI-38, MRC-5) were used for some samples. All samples were inoculated onto human epidermoid cells (Hep-2) and monkey kidney (LLC-MK2) cells.

Serologic studies for antibody levels to respiratory viruses were performed as described previously [3]. These included microneutralization tests for influenza A and B viruses, parainfluenza viruses types 1–3, respiratory syncytial virus, and coronavirus type 229E [3,19], hemagglutination inhibition assays for influenza A and B viruses, and an enzyme-linked immunosorbent assay (ELISA) for coronavirus type OC43 [20].

Reverse transcription PCR assays were performed using virus-specific primers and probes for influenza A and B viruses, picornaviruses, coronavirus type OC43, parainfluenza virus types 1 and 3, and respiratory syncytial virus as previously described [21–25]. For parainfluenza virus type 2, a region of the F gene [26] was amplified using the following virus-specific primers: upstream primer, 5'-CATGTACTATACTGATGGTGG-3'; downstream primer, 5'-GTTAGTAACTTAAATAGGGTAAC-3'. Parainfluenza virus type 2-specific amplimers were identified using a digoxigenin-labeled oligoprobe: 5'-AATGGAACATGCAACATCACC-3'.

2.4. Bacterial strains and growth conditions

Isolates of non-typeable *H. influenzae* were grown on chocolate agar overnight at 37°C under 5% CO₂. For ELISA, strains were grown in a shaking incubator to mid-exponential phase (optical density at 600 nm (OD₆₀₀) ~0.2) in brain heart infusion broth containing hemin and nicotinamide adenine dinucleotide (NAD) both at 10 µg ml⁻¹. Bacteria that did not reliably grow to mid-exponential phase by this method (total of 10) were grown in chocolate broth (15 g proteose peptone No. 3, 1 g corn starch, 4 g potassium phosphate dibasic, 1 g potassium phosphate monobasic, 5 g sodium chloride, 10 mg hemin and with 10 µg ml⁻¹ NAD in 800 ml distilled water).

2.5. Typing of non-typeable *H. influenzae*

Isolates of non-typeable *H. influenzae* were subjected to typing by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole bacterial cell lysates, as previously described [27,28]. This method is based largely on the mobility of outer membrane proteins in SDS–PAGE [29]. Isolates were grown on chocolate agar overnight. A loopful of bacteria was suspended in 0.1 ml of phosphate-buffered saline (PBS). An equal volume of 2× sample buffer (0.1 M Tris, 2% SDS, 10% β-mercaptoethanol, 40% glycerol and 0.05% bromophenol blue) was added, and the mixture was heated in a boiling water bath for 15 min. A 20-µl aliquot was subjected to SDS–PAGE on 11% gels. Gels were stained with Coomassie blue.

2.6. ELISA

The level of IgG in human serum to whole cell preparations of homologous isolates of non-typeable *H. influenzae* was determined by ELISA. Wells of a 96-well microtiter plates (Immulon 4; Dynatech) were coated overnight at 4°C with 0.1 ml of mid-exponential phase bacteria suspended in PBS to an OD₆₀₀ of ~0.20. Wells were washed twice with PBS containing 0.05% Tween 20 (PBS-Tween). Unbound sites on plastic were blocked with 3% non-fat dry milk in PBS-Tween for 1 h at room temperature. Wells were washed three times with PBS-Tween. Serum was diluted in 1% non-fat dry milk in PBS-Tween, 100 µl was added to wells in multiple dilutions (ranging from 1:500 to 1:64 000), and incubated at 37°C for 2 h. After the wells were washed three times with PBS-Tween, horseradish peroxidase-conjugated rabbit anti-human F(ab')₂ IgG (DAKO, Carpinteria, CA, USA), diluted 1:3000 in 3% goat serum, was added to the wells and incubated for 1 h at room temperature. After washing wells, substrate and color developer (0.1 mg 3,3',5,5'-tetramethyl-benzidine-dimethyl sulfoxide–0.2% hydrogen peroxide per ml in 0.1 M sodium acetate adjusted to a pH 4.5 with citric acid) were

added to the wells and incubated for 15 min at room temperature before the reactions were stopped by addition of 4 N H₂SO₄. The OD₄₅₀ was read.

Paired samples of pre- and post-exacerbation sera were always run together. The percentage change in OD between the pre-exacerbation serum and the post-exacerbation serum for each dilution was calculated using the following formula: (OD post-exacerbation serum–OD pre-exacerbation serum) × 100/OD pre-exacerbation serum. To determine the percentage change which represents a significant increase in the antibody response from a pre-exacerbation to a post-exacerbation sample, ELISA was performed on 15 paired serum samples, collected 2 months apart from adults with COPD followed with monthly sputum cultures and who did not grow non-typeable *H. influenzae* in the sputum. These paired sera were tested against isolates of non-typeable *H. influenzae*. The percentage change in OD was calculated as described above.

3. Results

3.1. Exacerbations

Thirty-five of 152 exacerbations were studied. Reasons for exclusion from study included the failure to obtain a sputum sample during the illness (*n* = 13), failure to isolate non-typeable *H. influenzae* from sputum samples collected (*n* = 75), lack of availability of either a pre-exacerbation or a convalescent serum for the illness (*n* = 14), lack of availability of at least one strain of non-typeable *H. influenzae* from the illness sample (*n* = 8), and illness not selected for study (*n* = 7). The 35 exacerbations occurred in 24 subjects during which non-typeable *H. influenzae* was isolated from sputum. Table 1 shows the baseline clinical characteristics of the patients. Pre-exacerbation sera were collected a median of 24 days (range 5–104 days) before the onset of the illness, and post-exacerbation sera were collected a median

Table 1
Baseline characteristics of 24 patients with COPD

Characteristic	Value
Age at enrollment (mean ± S.D.)	55.5 ± 8.1 years
Age at first exacerbation	56.4 ± 8.0 years
Sex	
Male	10
Female	14
Race	
White	17
Black	5
Hispanic	2
Smoking status	
Former smoker	9
Current smoker	15
Pack years of smoking (mean ± S.D.)	63.8 ± 34.3 pack years
FEV ₁ ^a (mean ± S.D.)	1.3 ± 0.6 l
FEV ₁ % of predicted (mean ± S.D.)	50.3 ± 23.5%

^aFEV₁: forced expiratory volume in 1 s.

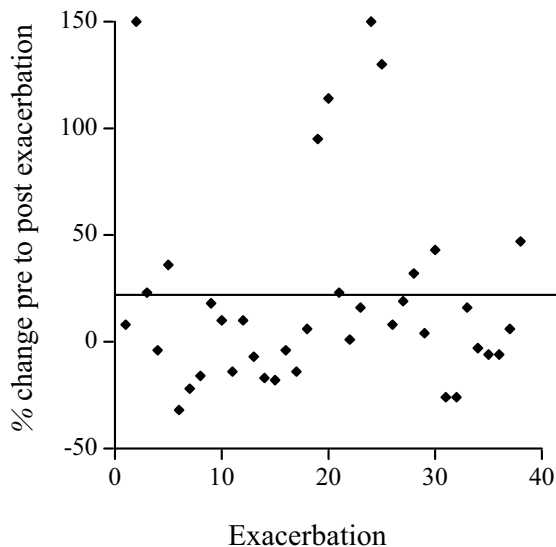


Fig. 1. Results of whole cell ELISAs measuring percentage of change in serum IgG levels between pre- and post-exacerbation serum samples, using homologous isolates of *H. influenzae*. The horizontal line at 22.1% shows the cutoff for significant change. Thirty-eight values are shown for 35 exacerbations; in three exacerbations, two isolates of *H. influenzae* were isolated simultaneously and each was tested individually with paired pre- and post-exacerbation sera.

of 39 days (range 24–83 days) after illness onset. The median time from illness onset to clinic visit was 2 days (range 0–15 days).

3.2. Typing of isolates of non-typeable *H. influenzae*

Multiple colonies of non-typeable *H. influenzae* were picked from original culture plates and grown individually when possible. Of 35 exacerbations, two or more colonies (range 2–19 colonies) were recovered from the original culture plate in 28. An average of 4.3 colonies per sample was recovered with 134 isolates in total. In seven exacerbations, a single colony was recovered. Molecular typing by SDS–PAGE was performed to determine the frequency with which single or multiple isolates were present in the sputum simultaneously. Of the 28 samples with multiple colonies, 25 had a single strain of non-typeable *H. influenzae*. Three sputum samples contained isolates with two banding patterns in SDS–PAGE that differed in multiple bands from one another, indicating that these two sputum samples had two genetically different strains of non-typeable *H. influenzae* simultaneously.

3.3. Serum IgG response to homologous non-typeable *H. influenzae*

ELISAs were performed to measure serum IgG to the homologous isolate in pre-exacerbation and post-exacerbation sera of 35 exacerbations during which non-typeable *H. influenzae* was isolated from sputum. Paired pre- and post-exacerbation sera were always tested in the same as-

say. Varying dilutions of each set of paired samples were tested and the OD was plotted against dilution. The percentage change between paired pre- and post-exacerbation sera was measured at a dilution of serum which demonstrated a linear relationship between OD and dilution (1:500 to 1:16 000).

To determine the percentage change which represents a significant increase in the antibody response from a pre-exacerbation to a post-exacerbation sample, ELISA was performed on 15 paired serum samples, collected 2 months apart from adults with COPD who did not grow non-typeable *H. influenzae* from monthly sputum samples. These paired control sera demonstrated a $0.43 \pm 8.58\%$ (mean \pm S.D.) change when tested with heterologous isolates of non-typeable *H. influenzae*. A change in OD of 22.1% represented the upper limit of the 99% confidence interval for the controls. Therefore, any change in OD from pre- to post-exacerbation serum samples of 22.1% or more was regarded as a significant change.

Fig. 1 shows the distribution of results of percent change in serum IgG to homologous isolates following 35 exacerbations. A significant increase in serum IgG was observed in 11 of 35 (31.4%) exacerbations. The range of increase in OD among the 11 samples was 22.5–201.9%. This represents a 1.22–3.01-fold increase in serum IgG to homologous strains of non-typeable *H. influenzae* in the 11 exacerbations demonstrating a significant response.

Three sputum samples contained two different isolates of non-typeable *H. influenzae* simultaneously during exacerbations. Each isolate was tested individually in ELISA with homologous pre- and post-exacerbation sera. None of these three patients had a statistically significant IgG response to either of the homologous sputum isolates.

3.4. Virological studies

Eighteen respiratory viral infections were identified in 16 of the 35 (46.7%) exacerbations (Table 2). Rhinovirus was cultured from four subjects, parainfluenza type 3 from three subjects, and influenza A/H3N2 from one subject. Serologic evidence of infection with influenza A virus was seen in four subjects and parainfluenza type 3 virus in two subjects and coronavirus OC43 in one subject. RT-PCR assays identified three additional infections (two picornavirus and one parainfluenza type 3 virus). Dual infections were identified in two subjects (one rhinovirus

Table 2

Viral etiology of documented respiratory tract infection during 35 exacerbations in which non-typeable *H. influenzae* was present in sputum culture

Viral infection	Number of infections
Parainfluenza type 3	6
Picornavirus	6
Influenza A/H3N2	5
Coronavirus OC43	1
Total	18

Table 3
Summary of virological studies and immune response to non-typeable *H. influenzae* in 35 exacerbations

	+ Evidence of viral infection	No evidence of viral infection	Totals
+ Immune response to non-typeable <i>H. influenzae</i>	3	8	11
No immune response to non-typeable <i>H. influenzae</i>	13	11	24
Totals	16	19	35

$P=0.167$, Fisher's exact test (two-tailed).

plus influenza A virus and one influenza A virus plus coronavirus OC43). Persons with infection identified by culture or PCR were more likely to be seen within the first 2 days of illness onset than those with infection documented by serology (7/9 vs. 1/6, $P=0.04$, Fisher's exact test). The average time from illness onset to evaluation was no longer in persons for whom no respiratory virus infection was documented compared to that for those with a documented respiratory viral infection (3.8 vs. 4.9 days, respectively).

3.5. Correlation of virological studies and immune response to non-typeable *H. influenzae*

Table 3 shows a summary of the results of virological studies and ELISAs to non-typeable *H. influenzae* in the 35 exacerbations. All possible combinations were observed, including evidence of simultaneous viral and bacterial infection, absence of both viral and bacterial infection and evidence of each individually. Three exacerbations (8.6%) showed evidence of both viral infection and also a new serum IgG response to non-typeable *H. influenzae*, suggesting co-infection. Overall, evidence of infection with a respiratory virus or non-typeable *H. influenzae* was seen in 24 of 35 exacerbations (68.6%). The data were analyzed to test the hypothesis that when evidence of viral infection was absent, an immune response to non-typeable *H. influenzae* was more likely. An immune response to non-typeable *H. influenzae* occurred more frequently in the absence of viral infection but did not reach statistical significance ($P=0.167$).

4. Discussion

An important limitation in developing strategies to prevent exacerbations of COPD is the relative lack of accurate information regarding the etiology of exacerbations in individual patients. Because specialized techniques are required to establish a diagnosis of viral infection, the diagnosis of viral respiratory tract infection by clinicians is generally made on clinical grounds. To complicate matters further, the isolation of a bacterial pathogen from the sputum of an adult with COPD during an exacerbation does not prove etiology because colonization by bacteria occurs in the absence of an exacerbation. Therefore, assays beyond sputum cultures are necessary to more accurately predict bacterial etiology.

In the present study, 35 exacerbations associated with the isolation from sputum of non-typeable *H. influenzae* were identified as part of a prospective study. These patients were studied carefully for evidence of viral infection using viral culture, serology and PCR-based assays. In addition, samples from these patients were subjected to immunoassays to identify a new immune response to the homologous isolate of non-typeable *H. influenzae* to more accurately assess a bacterial etiology. This careful evaluation allows a far more accurate assessment of the etiology of exacerbations. Previous studies have used similar methods, but no studies thus far have evaluated the same set of patients with exacerbations of COPD for both viral and bacterial infection in this way [3,6,7,30].

Sixteen of the 35 exacerbations (45.7%) were associated with evidence of acute viral infection. This number is similar to that observed in previous studies of COPD patients with exacerbations, including those whose sputum cultures were negative for non-typeable *H. influenzae* [3,5,31]. The most frequent viruses were parainfluenza type 3 and picornaviruses. This pattern is similar to that observed in previous studies [3,31].

The demonstration of a new immune response to non-typeable *H. influenzae* isolated from the sputum at the time of the exacerbation provides evidence that the organism caused the exacerbation. Eleven of the 35 exacerbations (31.4%) were associated with the development of new serum IgG to the homologous isolate detected in whole cell ELISA. Although the demonstration of new serum IgG to non-typeable *H. influenzae* provides supportive evidence that the organism in the sputum was etiologic, no gold standard exists for unequivocally identifying the organism as the cause of an individual exacerbation. Some patients who experience exacerbations due to bacteria may develop an immune response which is not detected by using whole cell ELISA with serum. For example, a mucosal immune response can occur independent of a systemic immune response [32,33]. Indeed, in a recent study, analysis of systemic and mucosal antibody responses to *Moraxella catarrhalis* isolated during exacerbations of COPD revealed instances of the development of mucosal IgA responses in the absence of serum IgG responses to the homologous isolate [34]. Similarly, cell-mediated immune responses to antigens of non-typeable *H. influenzae* may occur in adults with COPD independent of antibody responses [35]. Therefore, the absence of a new serum IgG response does not exclude the possibility that the exacerbation was caused by non-typeable *H. influenzae*. Further-

more, it is possible that colonization with an isolate of non-typeable *H. influenzae* may induce an immune response, even in the absence of a clinical exacerbation.

In spite of these limitations of measuring new serum IgG to non-typeable *H. influenzae*, several elements of the design of the present study should be emphasized. First, the homologous isolate was used as the antigen in ELISA for all assays. A serious limitation of a large number of published studies on the immune response to non-typeable *H. influenzae* is the use of laboratory strains or heterologous isolates to detect immune responses [8]. The antibody response following infection with non-typeable *H. influenzae* is predominantly directed at strain-specific epitopes [9–14]. Therefore it is important to use homologous isolates to measure immune responses to non-typeable *H. influenzae* as was done in this study. Second, adult human serum contains ‘background’ antibodies to many antigens of non-typeable *H. influenzae*. These antibodies are cross reactive with antigens on other bacteria and do not bind to the surface of non-typeable *H. influenzae*; thus, the antibodies are unlikely to mediate protective immune responses. The present study utilized whole bacterial cells in ELISA to increase the likelihood that antibodies which bind to the intact bacterial cell were detected.

A third important element of the present study is the availability of samples as a result of the prospective study design. Serum from prior to the onset of symptoms was used as the pre-exacerbation serum. Such samples are preferable to ‘acute’ serum collected at the time of the exacerbation since partial antibody responses may already be developing by the time the patient is evaluated. Furthermore, assays of pre- and post-exacerbation serum allowed us to detect exclusively new antibody responses in spite of the presence of varying titers of background antibodies.

Finally, multiple strains of non-typeable *H. influenzae* can colonize the human respiratory tract simultaneously [28,36]. In the present study, multiple colonies from the original sputum culture plates were subcultured separately and subjected to molecular typing to assess the possibility that multiple strains were present. In three of the 35 exacerbations, multiple strains were present. Whole cell ELISAs were performed with both of the isolates individually in these cases.

We hypothesized that patients who showed evidence of viral infection would be less likely to develop an immune response to non-typeable *H. influenzae* than those patients who lacked evidence of viral infection. While a trend in this direction was observed, the data did not support this hypothesis. This study showed that exacerbations may be caused by virus alone, non-typeable *H. influenzae* alone, or virus and non-typeable *H. influenzae* simultaneously. In the case of evidence of simultaneous infection by virus and non-typeable *H. influenzae*, several interpretations are possible. First, the exacerbation may be caused by virus and *H. influenzae* is a colonizer that induces an immune response. Second, a viral infection may be the initial

inciting event in the exacerbation and secondary infection with non-typeable *H. influenzae* occurs. This scenario has been proposed in several respiratory tract infections including otitis media, sinusitis and exacerbations of COPD. Third, a virus may be causing upper respiratory tract infection while the lower respiratory tract symptoms characteristic of an exacerbation are caused by non-typeable *H. influenzae*. It is not possible to draw a conclusion regarding these possibilities based on the data in the present study.

In summary, rigorous methods to identify evidence of viral infection were applied to samples from patients experiencing exacerbations of COPD from whom non-typeable *H. influenzae* was isolated from sputum. A total of 45.7% of these exacerbations were associated with evidence of viral infection and approximately one third were associated with the development of serum IgG responses to the homologous isolate of non-typeable *H. influenzae*; overall, two thirds of exacerbations were associated with a viral infection or a rise in serum IgG to the homologous isolate of non-typeable *H. influenzae*. The data indicate that exacerbations may be caused by virus alone, non-typeable *H. influenzae* alone, or virus and non-typeable *H. influenzae* simultaneously. These results highlight the challenge facing practicing physicians who treat patients with exacerbations of COPD and emphasize the need for better methods to accurately identify the etiology of exacerbations of COPD.

Acknowledgements

The study was supported by Public Health Service contract N01 AI 65298 and Grant AI 19641 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health and the Department of Veterans Affairs.

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