Pathogenic bacteria and viruses in induced sputum or pharyngeal secretions of adults with stable asthma

T H Harju, M Leinonen, J Nokso-Koivisto, T Korhonen, R Räty, Q He, T Hovi, J Mertsola, A Bloigu, P Rytilä, P Saikku

Background: Respiratory infections are well known triggers of asthma exacerbations, but their role in stable adult asthma remains unclear.

Methods: 103 asthmatics and 30 control subjects were enrolled in the study. Sputum was induced by inhalation of 3% NaCl solution. Oropharyngeal swab specimens were obtained from the posterior wall of the oropharynx. Respiratory specimens were analysed by RT-PCR for rhinovirus, enterovirus and respiratory syncytial virus and by PCR for adenovirus, Chlamydia pneumoniae, Mycoplasma pneumoniae and Bordetella pertussis.

Results: Sputum samples from two of the 30 healthy controls (6.7%), five of 53 patients with mild asthma (9.4%), and eight of 50 with moderate asthma (16.0%) were positive for rhinovirus. Rhinovirus positive asthmatic subjects had more asthma symptoms and lower forced expiratory volume in 1 second (FEV1) (79% predicted) than rhinovirus negative cases (93.5% predicted; p = 0.020). Chlamydia pneumoniae PCR was positive in 11 healthy controls (36.6%), 11 mild asthmatics (20.8%), and 11 moderate asthmatics (22%), and PCR positive asthmatics had lower FEV1/FVC than negative cases (78.2% v 80.8%, p = 0.023). Bordetella pertussis PCR was positive in 30 cases: five healthy controls (16.7%), 15 mild asthmatics (28.3%), and 10 moderate asthmatics (20%). Bordetella pertussis positive individuals had lower FEV1/FVC (77.1% v 80.7%, p = 0.012) and more asthma symptoms than B pertussis negative cases.

Conclusions: Rhinovirus, C pneumoniae, and B pertussis are found in the sputum or pharyngeal swab specimens of asthmatic subjects without concurrent symptoms of infection or asthma exacerbation, as well as in some healthy controls. Positivity is associated with lower lung function and more frequent asthma symptoms.

The aetiology of asthma involves interactions between genetic susceptibility, allergen exposure, and external aggravating factors such as air pollution, smoking and respiratory infections. Both clinical and experimental evidence suggest an important role for respiratory infections as triggers of asthma attacks in adults and children. Viral respiratory infections are considered the most common precipitating factors of acute asthma and have been shown to be associated with over 80% of asthma exacerbations in school children.1 In that study picornaviruses (mostly rhinoviruses) accounted for two thirds of the viral infections, with coronavirus causing less severe asthma exacerbations than other respiratory viruses. Thumerelle et al6 confirmed the high incidence of viral infections (especially enteroviruses or rhinoviruses) in children admitted to hospital with an asthma exacerbation. Persistent clinical symptoms were more commonly associated with atypical bacterial infections.

Atypical bacteria, Chlamydia pneumoniae, and Mycoplasma pneumoniae infections may precede asthma onset or exacerbate asthma,2 and these bacteria might be involved in chronic asthma. In vitro and animal studies suggest that atypical agents play a role in the pathogenesis of the disease.2 The association between C pneumoniae infection and adult onset asthma was first described by Hahn et al in 1991.3 There is also evidence that C pneumoniae may be involved in the pathogenesis of chronic stable asthma: serological studies suggest a dose response between C pneumoniae antibody levels and the severity of asthma4 and reactivation of C pneumoniae infection during acute asthma.5 Further evidence of a possible role of C pneumoniae in asthma is derived from observations that some subjects treated with antichlamydial antibiotics reported improvement in their asthma symptoms.4 The role of M pneumoniae in stable chronic asthma has been studied recently by Kraft et al5 and treatment with clarithromycin led to a significant improvement in lung function in the polymerase chain reaction (PCR) positive subjects.

Bordetella pertussis, the causative agent of whooping cough, produces toxins which cause damage to the epithelium and depress the defence mechanisms. The role of B pertussis in asthma is not known, but it has been shown that asthmatics with whooping cough suffer longer from cough and have an increased risk for sinusitis.6

The potential role of asymptomatic viral infections in asthma is poorly defined. In a study of asymptomatic asthmatic children over 80% virus positivity has been reported by PCR, while 5% of swabs from healthy controls were virus positive.7 In children with acute expiratory wheezing, viral RNA may take up to 5 weeks after the infection to disappear from the nasal mucus.8

We hypothesised that respiratory viruses as well as C pneumoniae, M pneumoniae, and B pertussis are present in the airways of asthmatics, and that these infectious agents may be found in induced sputum and/or pharyngeal secretions of...
asthmatics. Sensitive assay for C-reactive protein was used as a marker of systemic inflammation.

**METHODS**

**Study population**

One hundred and three asthmatics with disease severity ranging from mild (n = 53) to moderate (n = 50) and 30 control subjects were included in the study during 1999. Their demographic characteristics and lung function are shown in table 1.

The clinical severity of asthma was classified according to the GINA guidelines. Patients with asthma were recruited from the outpatient clinic of Oulu University Hospital and the Department of Allergy, Helsinki University Central Hospital. Asthma patients fulfilling the study criteria were asked to participate in the study when they came to the lung function laboratory to perform spirometric tests between 7 December 1998 and 16 December 1999, excluding the summer months. Induced sputum and oropharyngeal specimens were obtained from individuals at the same session/visit. Healthy controls were volunteers with no lung disease and normal lung function. The mild asthma group included 33 asthmatics ranging from mild (n = 53) to moderate (n = 50) and 30 control subjects were included in the study during 1999. Their demographic characteristics and lung function are shown in table 1.

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Pharyngeal swab

Oropharyngeal swab specimens were obtained from the posterior wall of the oropharynx with a sterile cotton tipped swab and placed in Chlamydia Transit medium and frozen at −70°C.

Measurement of CRP concentration

Serum CRP levels were measured with a rapid two-site ultrasensitive assay based on time-resolved immunofluorometry.

RT-PCR hybridisation method for rhinovirus and enterovirus

RNA was extracted from 100 μl of clinical specimen using a commercial kit (RNasy, Qiagen, GmbH, Heidelberg, Germany) and the specimens were analysed for rhinovirus and enterovirus RNA by a modification of the previously described RT-PCR hybridisation method using two different lanthanide labelled probes designed for detection of amplimers representing enteroviruses or rhinoviruses, respectively. A positive result in two parallel wells was required to classify a specimen as positive. If only one well was positive, the specimen was re-assayed. The specimen was regarded rhinovirus positive if the rhinovirus probe yielded a positive result, and enterovirus positive if the enterovirus probe alone was positive.

RT-PCR for respiratory syncytial virus (RSV)

RSV detection by RT-PCR was a modification of the methods described before. Briefly, purified RNA was reverse transcribed and the resulting cDNA was amplified using the oligonucleotide primers described by Osowy. The 5 end of the reverse primer was labelled with biotin. The RSV type was identified by a liquid phase hybridisation method using RSV A and RSV B specific lanthanide labelled probes.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic features and lung function of study groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy controls (N = 30)</td>
</tr>
<tr>
<td>Mean (SD) age (years)</td>
<td>39 (14)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>10:20</td>
</tr>
<tr>
<td>Smoking (non-current)</td>
<td>27.3</td>
</tr>
<tr>
<td>Atopy % (n)††</td>
<td>25% (8)</td>
</tr>
<tr>
<td>Median (IQR) inhaled corticosteroids (µg/day)</td>
<td>70 (28)</td>
</tr>
<tr>
<td>Median (IQR) duration of asthma (years), range</td>
<td>0.1–39</td>
</tr>
<tr>
<td>Mean (SD) FEV₁</td>
<td>3.56 (0.78)</td>
</tr>
<tr>
<td>Mean (SD) FEV₁ (% predicted)</td>
<td>96.7 (11)</td>
</tr>
<tr>
<td>Mean (SD) FVC</td>
<td>4.33 (0.98)</td>
</tr>
<tr>
<td>Mean (SD) FVC (% predicted)</td>
<td>99.0 (11)</td>
</tr>
<tr>
<td>Mean (SD) FEV₁/FVC%</td>
<td>82.6 (5.9)</td>
</tr>
<tr>
<td>Median (IQR) FEV₁/FVC% (n = 30)</td>
<td>98.1 (7.2)</td>
</tr>
</tbody>
</table>

*Analysis of variance.
††The mean difference is significant at the 0.05 level vs healthy controls (Dunnett t test).
‡‡Fisher’s exact test.
*Fisher’s exact test between moderate asthma and healthy controls (Fisher’s exact test).
**Mann-Whitney U test between mild and moderate asthma groups.
††Anamnestic information of prick test positivity.

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0.2 mg as a dry powder inhalation 15 minutes before sputum induction (Buventol Easyhaler 0.1 mg/dose, Orion Pharma, Espoo, Finland). The sputum was processed as previously described. The filtered suspension was centrifuged and the cell pellet and supernatant were stored at −70°C for later assays. In 67 cases (17 controls, 26 patients with mild asthma and 24 with moderate asthma) cytospin slides were made for the assessment of the cell differential count.

Pharyngeal swab

Oropharyngeal swab specimens were obtained from the posterior wall of the oropharynx with a sterile cotton tipped swab and placed in Chlamydia Transit medium and frozen at −70°C.

Measurement of CRP concentration

Serum CRP levels were measured with a rapid two-site ultrasensitive assay based on time-resolved immunofluorometry.

RT-PCR hybridisation method for rhinovirus and enterovirus

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RT-PCR for respiratory syncytial virus (RSV)

RSV detection by RT-PCR was a modification of the methods described before. Briefly, purified RNA was reverse transcribed and the resulting cDNA was amplified using the oligonucleotide primers described by Osowy. The 5 end of the reverse primer was labelled with biotin. The RSV type was identified by a liquid phase hybridisation method using RSV A and RSV B specific lanthanide labelled probes.
PCR for *Chlamydia pneumoniae*

DNA from nasopharyngeal swab and sputum specimens was isolated using Qiamp DNA Mini kits (QIAGEN). Quantitative LightCycler real time PCR assay was used to detect the presence of *C pneumoniae* 16S rDNA in the samples.\(^{18}\) As a standard, a dilution series of 80 000 to 8 genome equivalents of *C pneumoniae* DNA extracted from *C pneumoniae* elementary bodies (strain Kajaani 7) was used. The sample volume was 8 µl and a negative control was included as every seventh sample.

**PCR for Mycoplasma pneumoniae and adenovirus**

The PCR methods for detection of adenoviruses and *M pneumoniae* were performed as described elsewhere.\(^{20,21}\)

**PCR for Bordetella pertussis**

Details of the PCR have been described earlier and the assay has proved to be specific for *B pertussis*.\(^{15}\) Primers targeting insertion sequence 481 of *B pertussis* were used and 40 cycles were carried out. The DNA extracted from the prototype strain of *B pertussis* was used as the positive control, and all reagents except template DNA were included in the negative control tube. Both positive and negative controls were included in each PCR run. The amplified products were separated by 1.5% agarose gel electrophoresis and the bands were visualised after staining with ethidium bromide. The sensitivity of the PCR assay was ~5 bacteria per reaction tube.\(^{22}\)

**Statistical methods**

The results are expressed as mean (SD) or as median (range) or (interquartile range, IQR) for continuous variables and as frequencies or percentages for categorical variables. Analysis of variance followed by the Dunnett post-test was used to compare continuous variables between the three study groups. In the case of categorical variables, the groups were compared using a 2 test or Fisher’s exact test as appropriate. The variables representing the use of inhaled corticosteroids compared using a x² test or Fisher’s exact test, as appropriate.

Mann-Whitney U test. The Mann-Whitney U test was used for comparisons of lung function and CRP between negative and positive cases because of the small data sets. The CIA program was used to calculate 95% confidence intervals for median differences.

**RESULTS**

The microbiological findings in induced sputum of asthma patients and controls are shown in table 2. Four asthmatics were positive for both rhinovirus and *C pneumoniae*, and three healthy controls and seven asthmatics were positive for both *B pertussis* and *C pneumoniae*. Adenovirus and RSV were not found in the sputum of patients or controls. Two healthy controls and one asthma patient had enterovirus in their sputum. One healthy control had *M pneumoniae* DNA in the cellular fraction of her sputum. All other cases were mycoplasma negative.

Sputum cell differential counts were available for 17 controls, 26 cases with mild asthma, and 24 cases with moderate asthma. The mean (SD) percentage of eosinophils was higher in asthmatics than in healthy controls (0.24 (0.73)% in controls, 6.06 (15.42)% in patients with mild asthma, and 4.36 (7.14)% in patients with moderate asthma; p = 0.005). In individuals positive for *B pertussis* PCR there was mild lymphocytosis in the induced sputum differential cell count (0.4% in *B pertussis* negative cases v 5.2% in positive cases, p = 0.039). There was no correlation between the differential cell count and positivity for other studied infectious agents.

Rhinoviruses were found by PCR in 15 sputum samples (nine cellular fractions, four supernatants, and two both fractions, table 3) in two of 30 healthy controls (6.7%), five of 53 mild asthmatics (9.4%), and eight of 50 patients with moderate asthma (16.0%). Rhinovirus positive cases more often had moderate asthma, but this trend was not statistically significant (p = 0.179). Rhinovirus positive asthma patients had lower FEV₁ than rhinovirus negative patients (median 79% predicted v 93.5% predicted, p = 0.020), whereas in healthy controls no differences were seen between rhinovirus positive and negative individuals. Rhinovirus PCR positive cases also had higher asthma

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**Table 2** Microbiological findings in induced sputum

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (N = 30)</th>
<th>Mild asthma (N = 53)</th>
<th>Moderate asthma (N = 50)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.118</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>2 (6.7%)</td>
<td>0</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>RSV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>2 (6.7%)</td>
<td>5 (9.4%)</td>
<td>8 (16.0%)</td>
<td>0.438</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>9 (30%)</td>
<td>10 (18.9%)</td>
<td>8 (16%)</td>
<td>0.304</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>1 (3.3%)</td>
<td>0</td>
<td>0</td>
<td>0.226</td>
</tr>
<tr>
<td>Bordetella pertussis</td>
<td>3 (10%)</td>
<td>7 (13%)</td>
<td>3 (6%)</td>
<td>0.561</td>
</tr>
</tbody>
</table>

RVS, respiratory syncytial virus.*x² test or Fisher’s exact test as appropriate.

**Table 3** Microbiological findings (rhinovirus, *Bordetella pertussis* and *Chlamydia pneumoniae*) in different fractions of induced sputum and nasopharyngeal swabs

<table>
<thead>
<tr>
<th></th>
<th>Rhinovirus (N = 133)</th>
<th>Bordetella pertussis (N = 127)</th>
<th>Chlamydia pneumoniae (N = 130)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharyngeal swab</td>
<td>NT</td>
<td>22 (17.3%)*</td>
<td>6 (4.9%)</td>
</tr>
<tr>
<td>Induced sputum, supernatant</td>
<td>6 (4.5%)</td>
<td>8 (6.3%)*</td>
<td>14 (11.1%)</td>
</tr>
<tr>
<td>Induced sputum, cellular fraction</td>
<td>11 (8.3%)</td>
<td>5 (3.8%)*</td>
<td>17 (13.1%)</td>
</tr>
</tbody>
</table>

NT, not tested.

*Positivity rate for *B pertussis* in pharyngeal swab and cellular fraction (p = 0.001, McNemar test), supernatant or pharyngeal swab (p = 0.017, McNemar test).
symptom scores (above the median in 80% of rhinovirus positive cases compared with 47.4% of rhinovirus negative cases, p = 0.018). There was a trend for higher CRP levels in rhinovirus positive subjects (median 3.31 mg/l v 1.96 mg/l), but the difference was not statistically significant (p = 0.092). 40% of rhinopositive cases were atopic compared with 43% of rhinovirus negative cases (p = 0.812).

*Chlamydia pneumoniae* PCR was positive in 33 cases: 11 healthy controls (36.7%), 11 mild asthmatics (20.8%), and 11 moderate asthmatics (22%, p = 0.230). The positive sample was most often induced sputum (31 positive findings; 17 cellular fraction, 14 supernatant, and in four cases both fractions were positive, table 3). Pharyngeal swabs were positive in only six cases. *Chlamydia pneumoniae* positive asthma patients had more airways obstruction (median FEV/FVC 78.3% v 80.8%, p = 0.023 and 94% v 98% of predicted, p = 0.041) and the duration of asthma tended to be shorter (median 0.5 v 1 years, p = 0.089). Interestingly, CRP levels in *C pneumoniae* positive healthy controls, but not in asthma patients, were significantly lower than in negative individuals (median 0.42 v 1.28, p = 0.004).

*Bordetella pertussis* PCR was positive in 30 cases: five healthy controls (16.7%), 15 mild asthmatics (28.3%), and 10 moderate asthmatics (20.0%, p = 0.894; table 2). Pharyngeal swabs were most often the positive sample (25 positive findings v five sputum/cellular fraction and eight sputum/supernatant). In one case all samples were positive. In healthy controls 3/5 positive findings were in pharyngeal swabs compared with 12/15 patients with mild asthma and 7/10 with moderate asthma. *Bordetella pertussis* positive healthy controls had lower FEV/FVC (median 92.0% v 98% predicted, p = 0.019, table 4).

The asthma symptom score was higher in *B pertussis* positive cases than in negative cases (66% v 47% had symptom scores above median, p = 0.053). CRP levels did not differ between negative and positive groups (median 1.69 mg/l v 1.62 mg/l, p = 0.588). 37% of *B pertussis* positive cases were atopic compared with 45% of negative cases (p = 0.436).

**DISCUSSION**

Our study has shown that there is an association between pathogen positivity and clinical symptoms in patients with asthma: the presence of genetic material of the studied pathogens was associated with lower lung function and more frequent asthma symptoms. Rhinovirus RNA as well as *C pneumoniae* and *B pertussis* DNA are commonly present in the sputum or pharyngeal swab specimens of asthmatic patients without ongoing infection or asthma exacerbation, as well as in healthy controls. Adenovirus and RSV were not found in any of the induced sputum samples by the PCR methods used, and enterovirus and *M pneumoniae* were present only occasionally.

Several respiratory viruses are known triggers of asthma episodes, and 70–85% of asthma exacerbations are associated with upper respiratory tract infections, of which 60% are caused by rhinoviruses.1 There are only a few studies in which rhinoviruses have been sought from respiratory tract specimens obtained from healthy persons or from patients with stable asthma. Corne et al23 followed 76 couples in which one person had atopic asthma and one was healthy by taking nasal aspirates every second week; rhinoviruses were found in 10.1% and 8.5% of specimens obtained from asthmatic and healthy subjects, respectively. In our study, asthmatic patients tended to have higher rhinovirus positivity than healthy controls. Interestingly, we found no rhinoviruses in induced sputum, which can be considered to represent the lower respiratory tract. Rhinovirus is an important cause of exacerbations of a number of pre-existing airways disorders,24...
and recent studies have shown that it can inhabit the lower respiratory tract even in cases where upper respiratory tract specimens are rhinovirus negative.\textsuperscript{25, 26}

Human rhinovirus has been implicated as the principal virus associated with asthma episodes in children\textsuperscript{1} and in adults. Respiratory virus infections may also contribute to allergic sensitisation to aeroallergens and to the development of asthma. Experimental rhinovirus 16 infection has been found to cause airway obstruction in subjects with atopic asthma,\textsuperscript{27} and to increase intercellular adhesion molecule 1 expression in the bronchial epithelium of asthmatics, regardless of inhaled steroid treatment. Experimental rhinovirus infections have shown a deficient type 1 cytokine response in asthmatics\textsuperscript{28} and correlation with symptom severity, as well as impaired viral clearance. An impaired innate response to rhinovirus infection in primary bronchial epithelial cell culture from asthmatic subjects has also been found.\textsuperscript{29} Rhinovirus PCR positive individuals may either have a chronic infection or a slowly resolving acute infection. Following PCR positive individuals by sputum sampling at several time points would provide an answer to this question. In our study we also showed that \textit{C pneumoniae} DNA can be present in the lower airway secretions of both asthmatics and healthy controls without symptoms of ongoing respiratory infection. \textit{Chlamydia pneumoniae} seems to be more common in the lower respiratory tract than in the upper respiratory tract, occurring in only 4.9% of nasopharyngeal specimens and 16% of induced sputum. Biscione et al\textsuperscript{30} have shown that \textit{C pneumoniae} is more commonly present in the nasal secretions of asthmatic patients than in non-asthmatic healthy subjects (6.4% vs 2.3%). In our study the presence of \textit{C pneumoniae} in the upper respiratory tract agreed with the finding of Biscione et al,\textsuperscript{30} although we could not find any difference between asthmatics and healthy controls. Wu et al\textsuperscript{31} studied lung tissue samples by immunohistochemical staining for \textit{C pneumoniae} and found positive staining in 44% of lung tissues obtained from young persons (mean age 32 years) who had died accidentally, although the number of positive cells was low. In addition, they showed that all lung tissues from elderly persons were \textit{C pneumoniae} positive, but in five of \textit{C pneumoniae} positive cases the number of \textit{C pneumoniae} positive cells was significantly higher in patients with chronic obstructive pulmonary disease. Thus, \textit{C pneumoniae} seems to be common in the lower respiratory tract of healthy persons and of those with respiratory diseases. Interestingly, in healthy controls CRP levels were lower in \textit{C pneumoniae} positive cases than in negative cases, but the opposite was true for asthmatics, which suggests that the presence of \textit{C pneumoniae} as such does not lead to systemic inflammation. It is possible that genetic and environmental factors determine the outcome and sequelae of persistent \textit{C pneumoniae} infection in the host.

It is known that in many countries whooping cough is increasingly being recognised as an important cause of respiratory disease in adults.\textsuperscript{32} In Finland, 23–40% of diagnosed pertussis cases—as defined by culture, PCR and enzyme immunoassay serology—were found in adults in 1995–2004. The present study was carried out in the year 1999 when there was a nationwide epidemic of pertussis in the country. It remains to be shown whether \textit{B pertussis} DNA found in airway secretions in the study subjects was due to transient colonisation of \textit{B pertussis}. \textit{Bordetella pertussis} respiratory infection has been shown to selectively induce T helper 1 type responses in humans,\textsuperscript{33} and hypothetically to reduce the development of asthma later in life. In a murine model of allergic asthma, \textit{Bordetella} infection before ovalbumin sensitisation led to increased bronchial hyperreactivity and exacerbation of the allergic asthmatic response but, in already sensitised animals, whole cell \textit{B pertussis} inhibited the allergic airway reaction induced by ovalbumin.\textsuperscript{34} In our study asthmatics did not have more or less \textit{B pertussis} positivity in their airway secretions than healthy controls, but those who had \textit{B pertussis} were more symptomatic and had more airways obstruction. The presence of \textit{B pertussis} DNA in the airways did not have a protective effect against asthma symptoms or signs. The difference in FEV\textsubscript{1}/FVC between healthy controls who were PCR positive and negative for \textit{B pertussis} was small; this finding should be interpreted tentatively.

Although adenoviral DNA has been found in bronchial samples from asthmatic and control subjects,\textsuperscript{35} no adenoviral DNA was found in the induced sputum samples of our study material. Cells in induced sputum are mainly alveolar macrophages and polymorphonuclear cells, with only occasional bronchial epithelial cells. It might be possible that, in subjects with stable asthma, adenovirus DNA exists only in bronchial epithelial cells and is not therefore found in induced sputum.

The association between RSV infection and asthma is somewhat controversial, but the available data support the suggestion that RSV infection can facilitate or enhance allergic sensitisation and the development of allergic airway disease, at least in some asthmatic children.\textsuperscript{36} In this study no RSV RNA was found in sputum samples. Our study showed that rhinovirus as well as \textit{C pneumoniae} and \textit{B pertussis} are found in the sputum or pharyngeal swab specimens of asthmatic subjects without concurrent symptoms of infection or asthma exacerbation, as well as in some healthy controls. Positivity is associated with lower lung function only in asthmatics and more frequent asthma symptoms. Patients with asthma have been shown to have an increased risk of invasive pneumococcal disease.\textsuperscript{37} The presence of two pathogens in 14 individuals is an interesting finding, and the significance of the occurrence of several pathogens, host factors leading to different responses to pathogens, and the potential effects of the presence of these organisms on the baseline respiratory function remain to be examined in further studies.

The sample size was small due to the short period of time over which study material was collected. It is possible that some effects have been missed due to low power and the results need to be interpreted with caution.

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