Thorax Online First, published on March 3, 2006 as 10.1136/thx.2005.056291

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Non-random distribution of pathogenic bacteria and viruses in induced sputum or pharyngeal secretions of adults with stable asthma

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Running title: Asthma and infections

Key words:
Asthma, rhinovirus, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Bordetella pertussis*

Word count: 3856

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Abstract

Background: Respiratory infections are well known triggers of asthma exacerbation but their role in stable adult asthma has remained unclear.

Methods: 103 asthmatics and 30 control subjects were included. Sputum was induced by inhalation of 3% NaCl solution. Oropharyngeal swab specimen was obtained from the posterior wall of the oropharynx. Respiratory specimens were analyzed 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 of 2/30 (6.7%) healthy controls, 5/53 (9.4%) mild asthmatics and 8/50 (16.0%) in the moderate asthma group were positive for rhinovirus. The rhinovirus-positive asthmatics had more asthma symptoms and lower FEV1 (79% predicted) compared to rhinovirus-negative cases (93.5% predicted), p=0.020. *C. pneumoniae* PCR was positive in 11/30 (36.6%) of healthy controls, 11/53 (20.8%) mild asthmatics and 11/50 (22%) of moderate asthmatics and PCR positive asthmatics had lower FEV1/FVC than negative cases (78.2% and 80.8%, respectively; p=0.023). *B. pertussis* PCR was positive in 30 cases: 5/30 (16.7%) of healthy controls, 15/53 (28.3%) mild asthmatics and 10/50 (20%) of moderate asthmatics. *B. pertussis* positive individuals had lower FEV1/FVC (77.1% versus 80.7%, p=0.012) and more asthma symptoms than *B. pertussis* negative cases.

Conclusions: Rhinovirus as well as *C. pneumoniae* and *B. pertussis* can be found in the sputum or pharyngeal swab specimen of an asthmatic 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.

Abstract word count: 247

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Introduction

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 both in adults and in children. Viral respiratory infections are considered the most common precipitating factors of acute asthma and have been shown to be associated to over 80% of asthma exacerbations in school children. [1] In that study, picornaviruses, mostly rhinoviruses, accounted for two thirds of the viral infections, coronavirus being the next but causing less severe asthma exacerbations than other respiratory viruses. Thumerelle et al. [2] confirmed the high incidence of viral infections, especially enteroviruses or rhinoviruses, in children hospitalised with 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 [3] 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. [4] The association between *C. pneumoniae* infection and adult-onset asthma was first described by Hahn at al in 1991. [5] There is also evidence that *C. pneumoniae* may be involved in the pathogenesis of chronic stable asthma: serologic studies suggest a dose response between *C. pneumoniae* antibody levels and the severity of asthma [6] and reactivation of C. pneumoniae infection during acute asthma. [7] 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. [8] The role of *M. pneumoniae* in stable chronic asthma has been studied recently by Kraft et al. [9] and the treatment with claritromycin has led to a significant improvement in lung function in the PCR-positive subjects.

Bordetella pertussis, the causative agent of whooping cough is capable to produce various toxins, which cause damage to 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. [10]

The potential role of asymptomatic viral infections in asthma is poorly defined. In a study of asymptomatic asthmatic children, even over 80% virus-positivity has been reported by PCR, while 5% of swabs from healthy controls were virus-positive. [11] In children with acute expiratory wheezing, after the infection viral RNA may take up to 5 weeks to disappear from the nasal mucus. [12]

In the present study, we hypothesised that respiratory viruses as well as *C. pneumoniae*, *M. pneumoniae* and *B. pertussis* can be present in the airways of asthmatics and that these infectious agents can 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

103 asthmatics with disease severity ranging from mild (n = 53) to moderate (n = 50) and 30 control subjects were included during the period of 1999 (table 1.).

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Table 1. Demographic features and lung function of study groups.

	Healthy controls N=30	Mild asthma N=53	Moderate asthma N=50	P-value
Age, mean (SD)	39 (14)	43 (13)	45 (12)	0.075[a]
Sex, male:female	10:20	14:39	17:33	0.668[c]
Smoking, non:current	27:3	48:5	38:12	0.097[d]
Atopy[g], % (n)	25% (8)	42% (22)	54% (27)[e]	0.034[c]
Inhaled corticosteroids ug/d, Md (IQR)		500 (400-800)	900 (475-1600)	0.006[f]
Duration of asthma years, Md (IQR), range		1 (0.5-1.5), 0.1-39	3 (0.5-9), 0.1-31	0.010[f]
FEV1, mean (SD)	3.56 (0.78)	3.41 (0.82)	2.7 (0.61)[b]	<0.001[a]
FEV1%predicted, mean (SD)	97.6 (11)	101 (11)	80.5 (13)[b]	<0.001[a]
FVC, mean (SD)	4.33 (0.98)	4.2 (1.03)	3.57 (0.82)[b]	0.001[a]
FVC%predicted, mean (SD)	99.0 (11)	103 (12)	87.1 (12)[b]	<0.001[a]
FEV1/FVC%, mean (SD)	82.6 (5.9)	81.6 (5.1)	76.4 (8.1)[b]	<0.001[a]
FEV1/FVC%predicted, mean (SD)	98.1 (7.2)	97.9 (7.4)	92 (8.9)[b]	<0.001[a]

[a] Analysis of variance
[b] The mean difference is significant at the 0.05 level vs healthy controls, Dunnett t-test
[c] Chi-squared test
[d] Fisher's exact test
[e] p=0.010 between moderate asthma and healthy controls, Chi-squared test
[f] Mann-Whitney U-test between mild and moderate asthma groups
[g] Anamnestic information of prick test positivity

The clinical severity of asthma was classified according to the GINA guidelines. [13] Asthmatics were recruited from the outpatient clinic of Oulu University Hospital and Department of Allergy, Helsinki University Central Hospital. Asthma patients fulfilling the study criteria were asked to participate this study, when they came to the lung function laboratory to perform spirometry during the time period of 7.12.1998-16.12.1999, the summer months excluded. 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. Mild asthma group included 33 asthmatics fulfilling the American Thoracic Society asthma criteria and 20 subjects with mild intermittent asthma: asthma symptoms, bronchial hyperreactivity, exclusion of other lung diseases but less than 15% FEV_1 reversibility. Both controls and asthmatic patients had been free from respiratory infection for at least 4 weeks. Asthma symptoms during the last month (cough, sputum production, shortness of breath, wheezing or cough at exercise, and disturbed sleep) were recorded on a structured questionnaire and graded on a scale ranging from 0 (asymptomatic) to 3 (the most severe discomfort) and the frequency of asthma symptoms from 0 (no asthma symptoms) to 3 (several times per day) and the results were summarized to form a asthma severity score.

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Sputum induction

Sputum was induced by inhalation of 5 ml of 3% NaCl solution using an ultrasonic nebulizer (Omron U1, Omron, Germany). Both healthy controls and asthmatics were given salbutamol 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. [14] 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 mild asthma and 24 moderate asthma), cytospin slides were made for the assessment of the cell differential count.

Pharyngeal swab

Oropharyngeal swab specimen was obtained from the posterior wall of the oropharynx with a sterile cotton-tipped swab and placed in Chlamydia Transit medium and frozen in -70° C.

Measurement of CRP concentration

Serum CRP levels were measured with a rapid two-site ultra-sensitive assay based on time-resolved immunofluorometry. [15]

RT-PCR-hybridisation method for rhino- and entrovirus

RNA was extracted from 100 µl of clinical specimen using a commercial kit (RNeasy®, Qiagen, GmbH, Heidelberg, Germany) and the specimens were analyzed for rhinovirus and enterovirus RNA by a modification of the previously described RT-PCR-hybridisation method [16] using two different lanthanide-labelled probes designed for detection of amplicons representing enteroviruses or rhinoviruses, respectively. [17] A positive result in both two parallel wells was required to score a specimen positive. If only one well was positive, the specimen was reassayed. The specimen was regarded rhinovirus positive, if the rhinovirus probe yielded a positive result and enterovirus positive, if the enterovirus probe alone was positive. [16] [17]

RT-PCR for respiratory syncytial virus (RSV)

RSV detection by RT-PCR was a modification of methods described before. Briefly, purified RNA was reverse transcribed and the resulting cDNA was amplified using the oligonucleotide primers described by Osiowy. [18] The 5 end of the reverse primer was labelled with biotin. The RSV type was identified by a liquid-phase hybridisation method [16] using RSV A- and RSV B-specific lanthanide-labelled probes.

PCR for *C. 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 precence of C. pneumoniae 16S rDNA in the samples. [19] 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. Sample volume was 8 µl and a negative control was included as every 7th sample.

PCR for *Mycoplasma pneumoniae* and adenovirus

The PCR methods for detection of adenoviruses and M. pneumoniae were performed as described. [20] [21]

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PCR for *B. pertussis*

Details of PCR have been described earlier and the assay has proven to be specific for *B. pertussis*. [22] 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 visualized after staining with ethidium bromide. The sensitivity of the PCR assay was ~ 5 bacteria per reaction tube. [22]

Statistical methods

Results are expressed as means and standard deviations (SD) or as medians (Md) and ranges or interquartile ranges (IQR) for continuous variables and as frequencies or percentages for categorical variables. Analysis of variance followed by Dunnett post-test was used to compare continuous variables between the three study groups. In the case of the categorical variables, the groups were compared using Chi-squared test or Fisher's exact test, when appropriate. The variables representing the use of inhaled corticosteroids and the duration of asthma were skewed and applicable only for two asthma groups; therefore the comparisons between the groups were performed by non-parametric Mann-Whitney U-test. Comparisons of lung function and CRP between negative and positive cases in patients were done by Mann-Whitney U-test due to small data sets. CIA-program was used to calculate 95% confidence intervals for median differences.

Ethical considerations

The study protocol was approved by the Ethics Committee of the University of Oulu and Oulu University Hospital and by the Ethics Committee of Helsinki University Central Hospital. All subjects gave their informed consent.

Results

The microbiological findings in induced sputum of asthma patients and controls are presented in Table 2.

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Table 2. Microbiological findings in induced sputum

	Healthy controls N=30	Mild asthma N=53	Moderate asthma N=50	P-value*
Adenovirus	0	0	0	
Enterovirus	2 (6.7%)	0	1 (2%)	0.118
RSV	0	0	0	
Rhinovirus	2 (6.7%)	5 (9.4%)	8 (16.0%)	0.438
Chlamydia pneumoniae	9 (30%)	10 (18.9%)	8 (16%)	0.304
Mycoplasma pneumoniae	1 (3.3%)	0	0	0.226
Bordetella pertussis	3 (10%)	7 (13%)	3 (6%)	0.561

*Chi-squared test or Fisher's exact test when appropriate

Four asthmatics were both rhinovirus and *C. pneumoniae* positive, and three healthy controls and seven asthmatics were both *B. pertussis* and *C. pneumoniae* positive. Adenovirus and RSV were not found in the sputa of our patients or controls. Two healthy controls and one asthma patient had enterovirus in their sputa. One healthy control had *M. pneumoniae* DNA in the cellular fraction of her sputum. All other cases were mycoplasma-negative. Sputum cell differential count was available for 17 controls, 26 cases with mild asthma and 24 cases with moderate asthma. The percentage of eosinophils was higher in asthmatics compared to healthy controls (mean 0.24% SD (0.73) in controls, mean 6.06% SD (15.42) in mild asthma, and 4.36% SD (7.14) in moderate asthma), p=0.005. In individuals positive for *B. pertussis* PCR there was mild lymphocytosis in induced sputum cell differential count (0.4% in B. pertussis negative cases and 5.2% in positive cases, p=0.039). There was no correlation in cell differential count and positivity for other studied infectious agents.

Rhinoviruses were found by PCR in altogether 15 sputum samples (9 cellular fractions, 4 supernatant and 2 both fractions, Table 3).

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

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

NT = not tested

*The positivity rate for B. pertussis, pharyngeal swab and cellular fraction (p=0.001, McNemar Test), supernatant or pharyngeal swab (0=0.017, McNemar Test).

2/30 (6.7%) of healthy controls, 5/53 (9.4%) mild asthmatics and 8/50 (16.0%) of patients in the moderate asthma group. Rhinovirus-positive cases had more often moderate asthma but this trend was not statistically significant (p=0.179). The rhinovirus-positive asthma patients had lower FEV1 (median 79% predicted compared to 93,5% predicted, p=0.020), whereas in healthy controls no differences were seen between rhinovirus-positive and negative individual. Rhinovirus PCR positive cases had also higher symptom score: asthma symptom score was over median in 80% of rhinovirus positive cases compared to 47.4% rhinovirus negative cases, p=0.018. There was a trend for higher CRP in rhinovirus positive group (Md 3.31 mg/l vs 1.96 mg/l), but the difference was not statistically significant (p=0.092). 40% of rhinopositive cases were atopic compared to 43% of rhinovirus negative cases (p=0.812).

C. pneumoniae PCR was positive in 33 cases: 11/30 (36.7%) of healthy controls, 11/53 (20.8%) mild asthmatics and 11/50 (22%) of moderate asthmatics (p=0.230). Most often the positive sample was induced sputum (31 positive findings: 17 cellular fraction, 14 supernatant and in four cases both fractions were positive, Table 3). The pharyngeal swab was positive only in six cases. *C. pneumoniae*-positive asthma patients had more obstructive airways (Md FEV1/FEV 78.3% versus 80.8%, p=0.023 and 94% versus 98% of predicted, p=0.041) and the duration of asthma tended to be shorter (median 0.5 versus 1 years, p=0.089). Interestingly, C-reactive protein levels in *C. pneumoniae*-positive healthy controls, but not in asthma patients, were significantly lower than in negative ones (Md 0.42 versus 1.28, p=0.004).

B. pertussis PCR was positive in 30 cases: 5/30 (16.7%) of healthy controls, 15/53 (28.3%) mild asthmatics and 10/50 (20.0%) of moderate asthmatics were positive for *B. pertussis* (p=0.894) (Table 2). Most often the positive sample was pharyngeal swab (25 positive findings vs. 5 sputum/cellular fraction and 8 sputum/supernatant). In one case, all samples were positive. In healthy controls, 3/5 positive findings were pharyngeal swabs compared to

12/15 mild asthma and 7/10 in moderate asthma. *B. pertussis* positive healthy controls had lower FEV1/FVC (Md 92.0% versus 98 % of predicted, p=0.019, Table 4).

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Table 4. Comparison of lung function and CRP between rhinovirus, *Bordetella pertussis, Chlamydia pneumoniae* positive and negative cases in asthma group.

	N	FEV1(l/s)		FEV1 (%predicted)		FEV1/FVC (%)		FEV1/FVC (%predicted)		CRP (mg/l)	
		Median (range)	Median difference (95% CI *)	Median (range)	Median difference (95% CI)	Median (range)	Median difference (95% CI)	Median (range)	Median difference (95% CI)	Median (range)	Median difference (95% CI)
Rhinovirus											
Positive	13	2.79 (2.03, 3.57)	-0.23 (-0.61, 0.18)	79 (58, 111)	-10.0 (-18.0, -2.0)	80.5 (72.0, 88.3)	1.0 (-2.3, 4.3)	98 (82, 105)	1.0 (-3.5, 5.0)	3.31 (0.86, 12.11)	1.04 (-0.18, 2.75)
Negative	90	3.03 (1.59, 5.52)		93.5 (40, 130)		80.0 (47.6, 91.4)		97 (67, 110)		1.96 (0.01, 24.41)	
P value [†]		0.243		0.020[‡]		0.568		0.651		0.092	
B. pertussis											
Positive	25	2.93 (1.69, 5.47)	-0.05 (-0.38, 0.31)	93 (40, 127)	0.0 (-8.0, 8.0)	78.4 (47.6,87.9)	-2.5 (-5.5, 0.5)	95 (67, 109)	-2.0 (-6.1, 2.0)	2.21 (0.07, 24.41)	0.35 (-0.59, 1.37)
Negative	78	2.99 (1.59, 5.52)		92 (57, 130)		80.4 (60.2, 91.4)		97 (72, 110)		2.07 (0.01, 19.65)	
P value [†]		0.838		0.926		0.105		0.3		0.472	
C.pneumon.											
Positive	22	2.94 (1.81, 4.41)	0.03 (-0.32, 0.35)	89 (58, 127)	-4.0 (-11.0, 4.0)	78.2 (61.2, 89.5)	-3.5 (-6.1, -0.4)	94 (74,110)	-3.9 (-7.0, 0.0)	2.47 (0.07, 7.31)	-0.17 (-1.09, 0.94)
Negative	81	2.94 (1.59, 5.54)		92 (40, 130)		80.8 (47.6, 91.4)		98 (67, 110)		2.01 (0.01, 24.41)	
P value [†]		0.888		0.376		0.023[‡]		0.041[‡]		0.815	

* 95% confidence interval, [†] Mann,Whitney U-test, 2-tailed asymptomatic significance, [‡] Statistically significant

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The asthma symptom score was higher in *B. pertussis* positive cases: 66% of cases in positive group and 47% in negative group had symptom score over median (p=0.053). CRP level did not differ between negative and positive groups (Md 1.69 mg/l vs 1.62 mg/l, p=0.588). 37 % of *B. pertussis* positive cases were atopic compared to 45% of negative cases (p=0.436).

Discussion

Our study showed, that asthmatics had an association between pathogen-positivity and clinical symptoms: presence of genetic material of 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 sputa or pharyngeal swab specimens of asthmatic persons 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 searched for from respiratory tract specimens obtained from healthy persons or from patients with stable asthma. Corne et al [23] followed 76 couples in which one person had atopic asthma and one was healthy by taking nasal aspirates every second week and rhinoviruses were found in 10.1% and 8.5% of specimens obtained from asthmatic and healthy persons, respectively. In our study, asthmatic patients tended to have higher rhinovirus-positivity than healthy persons. Interestingly, in the present study we could find rhinoviruses in induced sputum, which can be considered to represent lower respiratory tract. Rhinovirus is an important cause of exacerbations of multiple types of pre-existing airways disorders [24] and recent studies have shown that it can inhabit the lower respiratory tract even in the cases where upper respiratory tract specimens have been rhinovirus-negative. [25] [26]

Human rhinovirus has been implicated as the principal virus associated with asthma episodes both in children [1] as well as in adults. Respiratory virus infections may also contribute to allergic sensitisation to aeroallergens and the development of asthma. Experimental rhinovirus 16 infection has been found to cause airway obstruction in subjects with atopic asthma [27] and to increase intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment. Experimental rhinovirus infections have shown deficient type1 cytokine response in asthmatics [28] and correlation to symptom severity as well as impaired viral clearance and an impaired innate responses to rhinovirus infection in primary bronchial epithelial cell culture from asthmatics has been found. [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 give an answer to this question.

In the present study, we also showed that *C. pneumoniae* DNA can be present in the lower airway secretions of both asthmatics and healthy controls without symptoms of ongoing

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respiratory infection. *C. pneumoniae* seems to be more common inhabitant of the lower than the upper respiratory tract: it was found only in 4.9% of the nasopharyngeal specimens compared to 16% of induced sputa. Biscione et al. [30] have shown previously that *C. pneumoniae* is more commonly present in the nasal secretions of asthmatic than nonasthmatic healthy persons (6.4 vs 2.3 %). In our study, the upper respiratory tract carriage rate for *C. pneumoniae* agrees with that of Biscione et al., [30] although we could not demonstrate any difference between asthmatics and healthy persons. Wu et al. [31] have studied lung tissue samples by immunohistochemical staining for *C. pneumoniae* and they could find 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 *C. pneumoniae*-positive, but the number of *C. pneumoniae*-positive cells was significantly higher in the patiets with chronic obstructive pulmonary disease. Thus, *C. pneumoniae* seems to be commonly present in the lower respiratory tract of healthy persons and of those with respiratory diseases. Interestingly, in the healthy controls, CRP levels were lower in *C. pneumoniae*-positive than in negative cases, but the cituation was *vice versa* in asthmatics suggesting that the carriage as such does not lead to systemic inflammation. It is possible that genetic and environmental factors determine the outcome and sequelae of the persistent *C. pneumoniae* infection in the host.

It is known that in many countries whooping cough is being increasingly recognized as an important cause of respiratory disease in adults. [32] In Finland, between 1995 and 2004, 23 to 40% of diagnosed pertussis cases as defined by culture, PCR and EIA serology were found in adults. The present study was carried out in the year 1999 when a nationwide epidemic of pertussis was noticed in this country. It remains to be shown whether *B. pertussis* DNA found in airway secretions in these study subject is due to transient colonization of *B. pertussis*.

B. pertussis respiratory infection has been shown to selectively induce T helper 1 type responses in humans [33] and hypothetically reduce the development of asthma later in life. In murine model of allergic asthma, Bordetella infection before ovalbumin sensitisation led to increased bronchial hyperreactivity and exacerbation of allergic asthmatic response, but in already sensitised animal whole-cell B. pertussis could inhibit allergic airway reaction induced by ovalbumin. [34] In our study asthmatics did not have more or less *B. pertussis* positivity in their airway secretions compared to healthy controls but those who had, were more symptomatic and with more obstructive airways and in our study group presence of *B. pertussis* DNA in airways did not have a protective effect against asthma symptoms or signs. The difference in FEV1/FVC found between the healthy controls who were PCR positive and negative for B.pertussis was small, and this finding should be interpreted tentatively.
Although adenoviral DNA has been found in bronchial samples of asthmatics as well as control subjects, [35] no adenoviral DNA was found in the induced sputum samples of our study material. The cells in induced sputum are mainly alveolar macrophages and polymorphonuclear cells, with only occasional bronchial epithelial cells. It might be possible that in stable asthma adenovirus DNA exists only in bronchial epithelial cells and thus cannot be found in induced sputum.

The association between RSV infection and asthma is somewhat controversary but the available data supports the suggestion that RSV infection can facilitate or enhance allergic sensitisation and the development of allergic airway disease, at least in some asthmatic children. [36] In this study, no RSV RNA was found in sputums.

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Our study showed, that rhinovirus as well as *C. pneumoniae* and *B. pertussis* are found in the sputum or pharyngeal swab specimen of an asthmatic 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. Asthmatics have been shown to have an increased risk of invasive pneumococcal disease.[37] The presence of two pathogens in 14 individuals is an interesting finding and the significance of the co-presence of several pathogens, host factors leading to different responses to pathogens as well as potential effects of microbial presence on the baseline respiratory function remain to be shown by further studies.
The sample size is small due to the limited period of study material collection time. It is possible, that some effects have been missed due to low power and the results need to be cautiously interpreted.

Acknowledgments
This work was supported by a grant from the Finnish Anti-Tuberculosis Association Foundation, the Finnish Lung Health Association, and the University of Oulu Scholarship Foundation. We thank Maritta Möller and Tuula Rantasalo for excellent technical assistance (pertussis).

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