Association between protozoa in sputum and asthma: A case-control study

Hugo C. van Woerden a,b,*, Adriana Ratier-Cruz c, Olabode B. Aleshinloye c, Rafael Martinez-Giron d, Clive Gregory b, Ian P. Matthews b

a Community Health Sciences, St George’s University of London, Cranmer Terrace, London SW17 0RE, UK
b Department of Primary Care and Public Health, Cardiff University, Neuadd Meirionnydd, Heath Park, Cardiff, CF14 4XN, UK
c Public Health Department, Wandsworth Teaching PCT 3rd floor Wimbledon Bridge House, Wimbledon, London SW19 3RU, UK
d INCLINICA Foundation for Clinical, Pneumological and Carcinogenic Research, Calvo Sotelo, 16, 3º Dcha. 33007 Oviedo, Spain

Received 1 March 2010; accepted 14 November 2010
Available online 8 December 2010

KEYWORDS
Asthma; Protozoa; Respiratory tract infections; Case-control study

Summary
Background: Atypical infectious agents have been proposed as potential contributors to asthma. A novel set of morphological and staining criteria permit the identification of flagellated protozoa in sputum. This case-control study was designed to use this novel method and to assess: (1) are protozoa more common in asthmatics than in non-asthmatics; (2) is the presence of protozoa associated with the use of steroid inhalers; and (3) is the presence of protozoa associated with living in damp housing?

Methods: Induced sputum samples were collected from asthma patients and local non-atopic, non-smoking controls. Questionnaires assessed asthma severity and housing conditions. Sputum was examined for flagellated protozoa using a previously described staining technique.

Results: 96 participants were recruited for this study; 54 asthma patients and 42 controls, age range 21–62 years, 70% female participants. Limiting results to those who were clearly positive or negative for flagellated protozoa, 66.7% (20/30) of asthmatics and 30.8% (4/13) of controls had protozoa ($p = 0.046$). Among the asthma patients, prevalence of protozoa was not significantly different between those who had (10/18), and those who had not (10/12), used steroid inhaler in the preceding two weeks ($p = 0.11$). Similarly, the prevalence of protozoa was not significantly different between those who did (6/11) and those who did not (18/32), live in damp homes ($p = 0.92$).

Conclusions: This case-control study demonstrates an association between flagellated protozoa in sputum and asthma. It is now necessary to confirm and characterise the protozoa.
Background

A wide range of aetiologic factors have been suggested as possible causes of asthma, including the hygiene hypothesis, which postulated that differential exposure to infectious agents might have an aetiological role in asthma. Viral infections of the respiratory tract are widely accepted as an important factor in acute exacerbations of asthma, and some authors have suggested that asthma may even be an infectious disease. There is growing interest in the potential contribution of a number of atypical infectious agents to the aetiology of asthma, particularly *Mycoplasma* and *Chlamydia*.

A number of case series have previously demonstrated the presence of flagellated protozoa in the sputum of patients with respiratory symptoms admitted to hospitals in Spain and Wales with acute exacerbations of disease and in patients who were immuno-compromised. These studies have demonstrated that flagellated protozoa are present in a proportion of respiratory patients, particularly those with asthma, when using a set of staining techniques that have not previously been utilised in the field of sputum cytology to identify protozoa. Another reason why flagellated protozoa have not been widely recognised in sputum cytology to identify protozoa is that flagellated protozoa are easily mistaken for ciliated epithelial cells. The cyto-pathologists involved in this study have described a set of morphological and staining criteria to differentiate flagellated protozoa from ciliated cell fragments.

It is unclear whether the protozoa observed in the respiratory case series above are more common in patients with asthma than healthy controls; whether their presence is related to other factors such as the use of steroid inhalers; or whether their presence is related to living in damp housing conditions. It can be argued that steroid inhalers could dampen the immune system and facilitate the growth of commensal protozoa. Dampness, mould and other indoor bioaerosols have also been associated with a variety of respiratory symptoms including Building Related Illnesses, and bioaerosols including microbes, fungi, viruses, protozoa, pollens, dander and mite-related debris are present in indoor air in significant quantities. The smaller sized particles in a bioaerosol remain airborne for long periods of time, and also fall within the respirable size fraction. It was therefore, considered important to consider whether living in a damp home was associated with the presence of protozoa in sputum.

This case-control study was designed to test three hypotheses arising from these considerations: (1) are protozoa more prevalent in asthmatics than in non-asthmatics, (2) is the presence of protozoa associated with the use of steroid inhalers, and (3) is the presence of protozoa associated with living in damp housing?

Methods

Asthma patients and control subjects were recruited from two GP practices in Battersea, south west London and from Wandsworth Primary Care Trust, south west London. Control subjects were non-smokers who did not have a history of asthma, eczema, or hay fever. For this study the definition of asthma was: physician diagnosed asthma, currently under active management by the patient’s GP, as recorded in the practice’s computer records.

To recruit asthma patients, a letter was sent to all the patients on the asthma register of both GP practices explaining the study design and inviting them to participate. Notices were also placed in the waiting rooms of the GP’s surgeries inviting individuals who did not smoke and who did not have asthma, eczema or hay fever to volunteer as controls. Some participants were recruited by word of mouth. Patients and control subjects were paid a small amount in recognition of the time involved in participating in the study. Participants were subsequently asked to complete a questionnaire gathering demographic details, oral or inhaled steroid use in the preceding two weeks, and asthma symptoms in the preceding two weeks. A previously published asthma score, AS-2 was used to assess the severity of asthma. This score is based on four questions: How many days did you cough in the past 2 weeks? How many days were you wheezy in the past 2 weeks? How many days were you short of breath in the past 2 weeks? How many days were you awakened at night due to your asthma in the past 2 weeks? The questions are marked on a scale of 1–4, where 1 is "Not at all", 2 is 1–3 days, 3 is 4–7 days and a score of 4 is 8–14 days. The average score of the four questions forms the AS-2 score.

The presence of damp in the home was assessed using a previously published score. This score incorporates four questions: Is there any visible mould growth on your house? Is there any odour of mould or cellar-like dusty air in your house? Is there any moisture stains in your house? Is there any water/moisture damage in your house? A positive answer to any of these questions was taken to indicate a "damp" home.

Participants then attended a clinic held at their GP surgery where an induced sputum sample was collected. Any individuals who had been on oral steroids in the preceding two weeks, or had suffered from a respiratory tract infection in the preceding two weeks, or who had a baseline Peak Expiratory Flow Rate (PEFR) of less than 70% their predicted value (based on gender, height and age) were asked to return at a later date. A standard protocol, using normal saline (0.9%) as the nebulised solution, was used to induce sputum production based on widely used sputum induction methods. Participants were pre-medicated with three puffs of Salbutamol via a spacer device to reduce the risk of inducing bronchospasm. Peak flows (best of three attempts) were checked at 30 s, 2 min, 6 min, 10 min, 14 min, and 18 min, giving a maximum nebulisation time of 18 min. Nebulisation was stopped after a shorter interval if an adequate sputum sample had been produced. The procedure was terminated if the peak flow fell by 20% of the baseline PEFR, if the participant became wheezy, or if the participant wished at any point to terminate the procedure. Sputum samples were collected in sterile petri dishes. Samples were considered adequate when at least two or more opaque, using genetic techniques based on 18S ribosomal RNA. Once this is established it would be worthwhile to determine if asthma symptoms improve when treated by anti-protozoal agents.

© 2010 Elsevier Ltd. All rights reserved.
muco-cellular clumps at least 1.5 × 3.0 mm in size had been collected.\textsuperscript{22,24} Samples of true sputum (not saliva), were taken from the petri dish using sterile disposable tweezers to minimise salivary contamination of the samples using a standard ‘pick technique’ (Fig. 1). The sputum sample was placed on a microscope slide and, a second slide was used to make a smooth, uniform smear, gently moving both slides in opposite directions, without exerting undue pressure. The two microscope slides obtained from each participant were immediately fixed using Cytofix\textsuperscript{10} spray. The microscope slides were stained using previously described techniques\textsuperscript{9} and examined for the presence of flagellated protozoa by a cyto-pathologist using a previously described set of morphological criteria.\textsuperscript{9,10,14} All the slides were scanned in a systematic manner under the microscope. Slides that had only scanty squamous cells or scanty white cells were classed as ‘inadequate’ as it was presumed that these slides contained little true sputum. Differential cell counts were calculated for each individual in the study based on a sample of 100 cells. Duplicate counts were undertaken on a subset of the samples to confirm the accuracy of the initial cell counts. Fig. 2 provides an example of degenerative phenomena in ciliated bronchial cells and Fig. 3 provides an example of protozoa in the sputum of a patient with asthma, although not a participant in this study.

Participants were informed if protozoa were found in their sputum, although they were advised that the significance of this finding was currently unknown. Any willing participants, particularly those with protozoa, were invited to re-attend the clinic on more than one occasion to provide an additional induced sputum sample.

For the purposes of statistical analysis, participants who had sputum collected on more than one occasion were classed as “positive for protozoa” if protozoa were detected on any occasion. Participants were particularly encouraged to return to provide another sample where the first sample was classified as “inadequate” or “unclear”. This was because participants’ technique improved over time, and better quality sputum samples were often produced at the second visit. To assess whether our collation of the positive results was reasonable, a sensitivity analysis was undertaken using only sputum collected at the first visit. This did not materially change the direction of any finding, although the smaller sample size reduced the statistical significance of the findings (data not shown).

Data were entered and analysed using SPSS 14 for Windows. EpiInfo Version 6 was used to calculate Fisher’s Exact Test. The study protocol was approved by Camden and Islington community local research ethics committee (Reference 08/H0722/540).

**Results**

**Demographic data**

A total of 96 individuals, 15 male and 39 female asthma patients, and 14 male and 28 female controls subjects participated in the study. There were no significant differences between the mean age of asthma patients (39.4 yrs; range 16—64 yrs) and the mean age of controls (37.1 yrs; range 21—62 yrs). Amongst the asthma patients, 87\% (47 individuals) were non-smokers, 7.4\% (4 individuals) were current smokers, and 5.3\% (5 individuals) had a history of smoking.

![Figure 1](image1.png) Selecting sputum, as opposed to saliva, using the pick technique.

![Figure 2](image2.png) Degenerative changes in ciliated bronchial cells (Magnification × 1000).

![Figure 3](image3.png) Flagellated protozoa from the sputum of an asthma patient clearly demonstrating the presence of “capitullum” and irregular flagella (Magnification × 1000).
were current smokers, and 5.6% (3 individuals) were ex-smokers. Control subjects were selected based on being non-smokers and having a low probability of being atopic, by using a set of screening questions to identify a history of asthma, eczema and hay fever. None of the control subjects had asthma, although in five cases the information available could not fully exclude the possibility of previous eczema or hay fever. The 96 participants made 137 visits to the clinic and attempts to collect sputum were successful in 58.4% of attempts (80/137).

A breakdown of the results in patients and controls is provided in Table 1. One participant withdrew from the study, and nebulisation was stopped in one patient who became wheezy. There was one inadequate sample, where only saliva rather than muco-cellular content was present on the microscope slides. In five cases, the cyto-pathologist was uncertain over the presence or absence of protozoa in the sample provided. Forty-six participants did not produce any sputum. Protozoa were identified in 20 asthma patients and 4 control subjects.

No statistically significant relationship was observed between the presence or absence of protozoa and the proportion of eosinophils, monocytes/macrophages or neutrophils observed in sputum smears when the differences were assessed using the Mann–Whitney test.

Was the presence of protozoa associated with the use of steroid inhalers?

To determine whether the presence of protozoa was associated with the use of steroid inhalers, we compared the proportion of asthma patients with and without protozoa in their sputum against self-reported use of a steroid inhaler in the preceding two weeks. The prevalence of protozoa in the sputum of asthma patients who had not used a steroid inhaler in the preceding two weeks (83%, 10/12), was higher than the prevalence in those who had used a steroid inhaler in the preceding two weeks (55.5%, 10/18). However, this difference was not statistically significant (chi-square 2.50, \( p = 0.114 \)). See Table 2. These findings do not support the hypothesis that an increased prevalence of protozoa in sputum is associated with recent use of steroid inhalers.

Was the presence of protozoa associated with living in a damp home?

The presence or absence of protozoa in sputum was compared against the presence of damp in the home. The score was dichotomised (‘no evidence of damp’ against ‘any evidence of damp’) as it had been in the paper from which this set of questions were taken. See Table 3. Analysis was limited to those who had clear positive or negative result for protozoa in their sputum. The prevalence of protozoa was not significantly different between those who did (54.5%, 6/11) and those who did not (56.3%, 18/32), live in damp homes (\( p = 0.92 \)). In summary, the data do not support the hypothesis that an increased prevalence of protozoa in sputum is associated with living in damp housing.

Over what duration did protozoa persist in the sputum of an individual?

Some information was obtained on the duration over which protozoa persist in the sputum of an individual. Table 4 summarises the information on 12 participants who had

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of the presence or absence of protozoa in study participants.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protozoa present</td>
</tr>
<tr>
<td>Asthma patients</td>
<td>20</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of the presence or absence of protozoa against the use of a steroid inhaler in the preceding two weeks, among 30 asthma patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protozoa in sputum</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Have you taken a steroid/preventative inhaler in the last 2 weeks?</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Comparison of the presence or absence of protozoa against evidence of damp/mouldy in the home.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protozoa in sputum</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>No evidence of damp/mould</td>
<td>18</td>
</tr>
<tr>
<td>Evidence of damp/mould</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
</tr>
</tbody>
</table>
sputum samples taken on two separate visits to the clinic. The mean interval between the two visits was 34.75 days (range 2–103 days).

Three patients had protozoa in their sputum at one visit but not at the other visit. The change in PEFR before induced sputum was collected, comparing the two visits, was around 10 l/min in all three cases, with the higher PEFR readings occurring on the occasions when protozoa were identified. This sample size is too small to determine whether asthma symptoms were significantly different but merits further investigation in a larger sample.

### Do symptoms and PEFR differ between asthma patients with and without protozoa in their sputum?

To assess whether protozoa were more likely to be present in the milder or more severely affected asthma patients, the distribution of the AS-2 asthma scores in those asthma patients who had, and those who did not have, protozoa were compared. The median AS-2 score in those with protozoa was 1.25 and in those without protozoa the median score was 1.75. The Mann–Whitney U statistic was 59.5, \( p = 0.068 \). Although the asthma patients with protozoa in their sputum generally had milder asthma than those who did not have protozoa, the difference was not statistically significant.

The relationship between peak flow and the presence or absence of protozoa in the sputum of asthma patients was assessed using the Mann–Whitney test, with equal variances not assumed. There was no relationship between peak flow (best of three blows at the start of the procedure) and the presence of protozoa (\( p = 0.252 \)). Similarly, there was no relationship between the presence or absence of protozoa and the lowest peak flow recorded during the sputum induction procedure (\( p = 0.14 \)).

### Discussion

This case-control study demonstrates an association between the presence of protozoa in induced sputum samples and a previous GP diagnosis of asthma. No association was found between the presence of protozoa and the use of steroid inhalers or the presence of protozoa and living in damp housing.

The prevalence of protozoa in asthma patients (66.7%) was broadly similar to that seen in a previous Spanish study.9 The Spanish study also examined patients with other respiratory diseases, mostly COPD, but did not study health controls.

Asthma may represent a cluster of conditions with similar symptoms2,25 and it is possible to speculate that the presence of protozoa represents a relatively mild infection which produces asthma like symptoms. The relatively high proportion of non-asthmatic individuals who had protozoa in their sputum also suggests that in many individuals these protozoal organisms do not have a pathogenic role. However, a number of recent papers have demonstrated that a range of organisms are present in the healthy lung but are more common in COPD or asthma.26,27 There is clearly growing interest in the role of infective organisms in asthma, but their role and significance is still unclear. However, it is possible to speculate that the protease enzymes that protozoa produce could act in the same way as Der P1 to breakdown the tight junctions between epithelial cells, increase the shedding of epithelial cells in the respiratory tract, and facilitate the penetration of allergens into local tissues. It is also possible to speculate that degenerative remnants from the cytoplasm or cell membrane from dead protozoa could act as an ‘adjuvant’, magnifying the immune response to concomitantly presented allergens.28–30 If asthma is viewed as a symptom cluster caused by range of underlying aetiologies, respiratory tract infection with protozoa could be a contributory factor in a subset of asthma patients.

Comparing the demographic characteristics of patients and controls does not suggest that our approach to the recruitment of cases and controls has introduced significant bias. In addition, the cyto-pathologist examining the sputum samples was blinded as to whether each sample came from an asthma patient or a control subject. Analysis of data was also undertaken independent of data collection.

The main weakness of the study is its relatively small size. The study would also have been strengthened by further classification of asthma, including a more detailed assessment as to whether patients had intrinsic or extrinsic asthma and an assessment of other end points such as exhaled nitric oxide levels. The study could have been strengthened by having the sputum slides examined by two independent cyto-pathologists, as the characteristics used to define the presence or absence of protozoa, have not been externally validated by an independent laboratory. Consequently, we currently have no estimate of the false positive or false negative rate for the assessment of the microscope slides by our cyto-pathologist.

We chose to use normal saline in the ultrasonic nebuliser as it tastes more pleasant and is less likely to trigger bronchospasm.22,24 This may have contributed to the relatively low proportion of control patients in whom sputum was obtained (35.7%, 15/42). The proportion of participants who produced sputum might have been higher had we had used hypertonic saline. If the prevalence of protozoa in the sputum of those control participants from whom sputum was not obtained was zero, then the incidence of protozoa could have been as low as 9.5% (4/42). It is possible to...
speculate that the control subjects who did not produce any sputum after nebulisation may have been less atopic and had lower bronchial reactivity. Similarly, further testing (for example, by skin prick tests) of the four controls that had protozoa to detect any underlying atopic tendency would have been helpful.

It is possible for induced sputum samples to be contaminated by oral microbiota. However, a study comparing the bacteria present in oral, induced sputum and bronchial lavage samples taken from the same individuals has provided some reassurance that this is not a major issue. The 'pick technique' that we have used should also have ensured that most of the material smeared across a slide comes from the inside of the gelatinous sputum globules that were individually picked up and placed on microscope slides. Although the surface of such globules would have some contact with saliva, this would only form a small proportion of each sputum sample as each sample was smeared on a microscope slide. There is evidence that protozoa live in the biofilms that form dental plaque.31–33 As the oral and respiratory mucosa form one continuous surface, it is clearly possible that there could be a link between oral and respiratory tract protozoa.

**Conclusions**

This case-control study supports the hypothesis that the presence of protozoa in sputum is statistically associated with a clinical diagnosis of asthma. It provides sufficient evidence to suggest that this hypothesis requires further exploration to determine whether these organisms are playing any primary or secondary pathogenic role, or whether their presence is merely an incidental finding.

It would be helpful if the organisms could be characterised using molecular techniques, for example, based on the characterisation of 18s RNA, as at present the species of protozoa observed is unknown and we cannot determine whether the same species of organism is present in different samples. Once this is established, a trial of anti/protozoal agents would also be worthwhile, to determine whether asthma symptoms improve when these protozoa are killed by an anti/protozoal agent. Similarly, trials of antibiotics have been used to assess the impact of the treatment of *Chlamydia* infection in patients with asthma.34

**Funding**

This project was funded by Wandsworth Primary Care Trust.

**Competing interests**

None declared.

**Acknowledgements**

We are grateful to the two GP practices that assisted us in this study: Battersea Fields Practice, and Lavender Hill Group Practice, and also to Wandsworth PCT who funded the study.

**Appendix 1. Instructions for obtaining sputum smears**

1. The sputum should be deposited in a sterile container.
2. A small area of true sputum (not saliva), about the size of a large lentil, should be taken from the expectoration using tweezers and scissors.

**Step 1**

3. Place the sample on a slide (frosted edge upwards) and, with a second slide (frosted edge downwards) make a smooth, uniform smear. It is not appropriate to crush the sample, but rather to move both slides in opposite directions, exerting slight pressure. To facilitate this operation, take the frosted edge of each slide between thumb and forefinger.
References