Atypical organisms affecting the respiratory tract and their sequelae – a series of case studies

by

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ὅτι ἐξ αὐτοῦ καὶ δι’ αὐτοῦ καὶ εἰς αὐτὸν τὰ πάντα· αὐτῷ ἡ δόξα εἰς τοὺς αἰῶνας, ἀμήν.

Summary

The respiratory tract is exposed to a wide range of environmental constituents including potentially infective agents. This thesis presents two series of papers concerning two atypical organisms and their sequelae or potential sequelae – *Coxiella burnetii*, which causes Q fever, and flagellated protozoa, which is associated with respiratory symptoms and asthma.

The first series of papers examine a Q fever outbreak and its sequelae. They describe one of the largest Q fever outbreaks in the UK and demonstrate the benefit of facsimile cascade in supporting case searching in such an outbreak. Chronic fatigue (Chalder Fatigue scores) and depression (PHQ-9 scores) were raised in post Q fever patients six years later ($p<0.05$ in both cases). Concordance regarding serological status across three international reference laboratories was as low as 35%, indicating a major problem with international standardisation in this area. Unpublished supplementary clinical and serological findings are also presented to inform future outbreak investigation.

The second series of papers examine the role of flagellated protozoa in respiratory disease. Flagellated protozoa were shown to be present in a case series of inpatients. In a subsequent community-based case control study, protozoa were present in 67% (20/30) of induced sputum samples taken from asthmatics and 31% (4/13) of samples from non-atopic controls ($p=0.046$). In another study of inpatients, 67% of those who were on oral steroids had protozoa in their sputum, compared to 35% of those not on oral steroids. In this study, 45% of smokers/ex-smokers had protozoa in their sputum compared

to 30% of non-smokers. Unpublished data using molecular techniques to identify eukaryotes in sputum is also presented.

The findings provide a basis for a RCT of antibiotics in patients with post Q fever chronic fatigue and a trial of anti-protozoal agents to eradicate flagellated protozoa in patients with asthma.

Table of Contents

1 Introduction 1
1.1 Aims and objectives 2
1.2 Structure of this thesis 4
2 A Q fever outbreak and its sequelae 5
2.1 The wider research context 5
2.2 Original contribution made 9
2.3 Methodological considerations 12
2.4 Discussion of findings 13
2.5 Unpublished Q fever related findings 14
2.6 Summary of Q fever work 26
3 Flagellated respiratory protozoa and their association with respiratory disease 28
3.1 The wider research context 28
3.2 Original contribution made 33
3.3 Methodological considerations 34
3.4 Discussion of findings 35
3.5 Unpublished findings related to molecular analysis of sputum samples 39
4 Conclusion 42
4.1 Potential for future research 43
References 45
5 Appendix 1: Summary of contributions to co-authored papers 52
6 Appendix 2: Submitted papers 56
7 Appendix 3: Associated correspondence 108

Papers included in the thesis

SERIES ONE: A Q fever outbreak and its sequelae

1. **van Woerden HC**, Mason BW, Nehaul LK, Smith R, Salmon RL, Healy B, et al. Q Fever outbreak in industrial setting. *Emerging Infectious Diseases*, 2004; 10(7):1282-1289 (CORRESPONDING AUTHOR).

2. **van Woerden HC**, Evans MR, Mason BW Nehaul L. Using facsimile cascade to assist case searching during an outbreak of Q fever. *Epidemiology and Infection*. 2007; 135(5):798-801 (CORRESPONDING AUTHOR).

3. **van Woerden HC**, Healy B, Llewellyn M, Matthews IP. A nested case control study demonstrating increased chronic fatigue six years after a Q fever outbreak. *Microbiology Research*, 2011; 3:e11 (CORRESPONDING AUTHOR).

4. Healy B, **van Woerden HC**, Raoult D, Graves S, Pitman J, Lloyd G, Brown N, Llewelyn M. Chronic Q fever: Different serological results in three countries - results of a follow-up study 6 years after a point source outbreak. *Clinical Infectious Diseases*, 2011; 52 (8):1013-1019.

SERIES TWO: Flagellated respiratory protozoa and their potential sequelae

5. **van Woerden HC**, Gregory C, Martinez-Giron R, Burr M , Lansdown A, Matthews IP. Case series demonstrating the presence of protozoa in the sputum of a proportion of respiratory patients. *The Internet Journal of Laboratory Medicine*, 2010; 4:1 (CORRESPONDING AUTHOR).
6. **van Woerden HC**, Ratier-Cruz A, Gregory C, Aleshinloye OB, Martinez-Giron R, Matthews IP, Burr ML. Association between protozoa in induced sputum samples and asthma: a case control study. *Respiratory Medicine*, 2011; 105 (6):877-84 (CORRESPONDING AUTHOR).
7. Martinez-Giron R, **van Woerden HC**. Clinical and immunological characteristics associated with the presence of protozoa in sputum smears. *Diagnostic Cytopathology*, 2011; doi: 10.1002/dc.21752.

1 Introduction

The human lung is in contact with more than 15,000 litres of air each day (Bartsch et al., 1982). It has a surface area of around 50 m^2 (Hasleton, 1972). This leads to extensive exposure to environmental constituents including potentially infective agents. The air we breathe contains large quantities of dust and bacteria. Each breath contains around 5,000 particles of dust (Bunnell, 2002). Most of this dust comes from around 2 billion tons of dust that is lifted into the earth's atmosphere each year. This dust also contains bacteria and, on average, the dust in the earth's atmosphere contains 10,000 to 100,000 bacteria per gram of dust (Griffin et al., 2002). The 'respirable dust' fraction generally consists of particles smaller than 5µm (Choiniere and Munroe, 2001, World Health Organisation, 1999). These minute particles are not visible to the naked eye and exposed individuals are generally unaware that they are inhaling dust particles of this size. This extensive exposure to the environment means that the lungs are a common portal for infection by viruses, bacteria, fungi, protozoa and other infectious agents.

A wide range of typical and atypical respiratory infections have been described. The term 'atypical respiratory infection', or 'atypical primary pneumonia', was first used in 1944 (Marmion, 1990) and has proved useful in describing respiratory infections that are caused by atypical organisms and which present with atypical symptoms. *Coxiella burnetii*, the cause of Q fever, is one of these atypical respiratory pathogens (Saikku, 1997) and is a focus within this thesis. The other focus in this thesis is the relationship between another atypical

respiratory tract organism (flagellated protozoa) and its relationship with respiratory disease.

Historically, healthy lungs were believed to be sterile. However, there is increasing evidence that microbiota are present even in healthy lungs (Rogers et al., 2005, Rogers et al., 2006). This finding is particularly relevant to this thesis, which relates to the potential overlap between pathogenic and commensal protozoa and other microbiota in the respiratory tract. A recent study using metagenomic culture independent genomic techniques has demonstrated increased levels of *Proteobacteria*, particularly *Haemophilus*, and a decreased level of *Bacteroides*, particularly *Prevotella*, in patients with COPD (Hilty et al., 2010). The study also found that microbial communities in asthmatic airways were disordered, with pathogenic *Proteobacteria* more frequently found in the bronchi of asthmatic patients than in controls.

It is well recognised that commensal organisms can become pathogenic given the right circumstances, leading to sub-clinical or overt infection. However, there is emerging evidence that microbiota may form part of a complex causal web that results in disease, for example, by their effects on the immune system, without becoming pathogenic in the classical sense. The role of such organisms in respiratory disease is controversial, but is key to the proposal in this thesis that there is a link between atypical flagellated protozoa and respiratory disease.

1.1 Aims and objectives

This thesis presents two series of published papers relating to two atypical respiratory organisms. The first series of papers examines *Coxiella burnetii* –

the cause of Q fever, its investigation and its sequelae. This series of papers is based on the cohort exposed to a Q fever outbreak in Newport, Wales in 2002. The second series of papers examines the relationship between atypical flagellated respiratory protozoa and clinical and immunological characteristics associated with respiratory disease.

In examining the Q fever outbreak and its sequelae, the objective of each of the papers in the series is to:

- Describe the investigation of one of the largest Q fever outbreaks to have occurred in the UK
- Assess the use of facsimile cascade in supporting case finding in such an outbreak
- Assess the longer term sequelae in the exposed cohort, particularly in terms of chronic fatigue and depression
- Assess the consistency of three international reference laboratories in diagnosing chronic Q fever

In examining flagellated protozoa and their potential sequelae, the objective of each of the papers in the series is to:

- Assess whether flagellated protozoa were present in respiratory inpatients in the UK
- Assess the relationship between asthma and flagellated protozoa in the community

- Assess the relationship between clinical and immunological characteristics and the presence of protozoa in sputum smears in respiratory inpatients

1.2 Structure of this thesis

The commentary in this thesis is provided in two sections. In each section, the series of papers is treated under the following headings:

1. The wider research context
2. Original contribution made
3. Methodological considerations
4. Discussion of findings

An overarching concluding chapter then brings together the findings of the papers and considers potential lines for further research.

In line with Cardiff University's Senate Regulations for the Award of the Degree of PhD (by Published Works), information on the personal contribution to the papers is provided in Appendix 1.

2 A Q fever outbreak and its sequelae

Q fever was first described by Derrick and Burnett in Australia following the first recognised outbreak in 1935 (Cooke, 2008, Burnett and Freeman, 1937). The underlying aetiological organism (*C. burnetii*) has subsequently been shown to occur in almost every country in the world (Tissot-Dupont and Raoult, 2008). The commonest mode of infection with *C. burnetii* is inhalation of organisms from an environmental source. The organism can survive in a dormant form for many years and has an infective dose as low as one organism (Williams, 1991). Very large quantities (up to 10^9 organisms per gram) can occasionally be found in infected placenta (Welsh et al., 1953).

2.1 The wider research context

Given its relatively small population, a surprising number of outbreaks of Q fever have been documented in Wales (Anon., 1956, Salmon et al., 1982). Rural inhabitants are more exposed to *C. burnetii* and the number of previous outbreaks in Wales may be related to its rural geography and extensive livestock farming industry. In one sero-prevalence study, farm workers had a sero-prevalence of 27.2% compared to police and emergency workers who had a sero-prevalence of 10.9% (Thomas et al., 1995).

2.1.1 The initial Q fever outbreak

The Q fever outbreak reported by the author (van Woerden et al., 2004, ENCLOSED PAPER) occurred in a factory in Newport, Wales. In view of the previous rural outbreaks in Wales, the initial investigation focused on the rural

hinterland as a possible source of infection. However, the hypothesis that was finally settled upon and presented in the attached research paper was that the outbreak had been caused by infected material present in straw board, which was present within the factory itself. The renovation work on straw board in the factory is shown in Figure 1.

FIGURE 1: RENOVATION WORK INVOLVING THE STRAW BOARD CEILING (PHOTOGRAPH COURTESY OF DR R SMITH)

The outbreak presented was one of the largest to be described in the UK. The only other UK outbreak of comparable size occurred in the West Midlands in 1989 (Hawker et al., 1998). The exposed cohorts have been followed up in both outbreaks. Both follow up studies have demonstrated that chronic fatigue is present 6-10 years later in a proportion of those affected by Q fever (Wildman et al., 2002, van Woerden et al., 2011a).

A number of subsequent large outbreaks of Q fever have occurred in Europe. In particular, there has been an on-going Q fever outbreak in the Netherlands since

2007 (van der Hoek et al., 2010). A large outbreak has also been reported in Scotland (Wilson et al., 2010b).

2.1.2 The use of facsimile cascade in case finding during a Q fever outbreak

The outbreak of Q fever provided an opportunity to evaluate the effectiveness of facsimile cascade in the context of a disease outbreak (van Woerden et al., 2007, ENCLOSED PAPER). Rapid identification of as many of the cases involved in an outbreak as is possible is essential in aiding rapid identification of an outbreak's source and prevention of further cases. Although email is increasingly replacing facsimile as a means of rapid communication, many primary care physicians are overloaded with emails (Dabbish and Kraut, 2006, van der Kam et al., 2000) and communication by facsimile may be given a higher priority. The study provided some evidence that facsimile cascade was effective in triggering a response from GPs. One of the practical lessons learned from this case study was the fact that effective facsimile cascade is dependent upon contact details held in a central database being up to date. Health protection departments should ensure that their facsimile cascade contact lists are cleaned and updated on a regular basis.

2.1.3 Follow up at six years

Q fever can lead to long-term sequelae including endocarditis, as happened to one patient in this outbreak (Healy et al., 2006), and to chronic fatigue (Ayres et al., 1996, Hatchette et al., 2003, Wildman et al., 2002). For this reason, a follow up study was planned by the author at the time of the outbreak and subsequently undertaken six years later (van Woerden et al., 2011a, ENCLOSED

PAPER). This paper reports a nested case control study utilising the initial cohort of factory workers exposed to Q fever in 2002. The paper demonstrated that chronic fatigue, measured by the Chalder Fatigue questionnaire, was more prevalent in those who were defined as having acute Q fever six years previously. An analysis was also undertaken using the questionnaires that were completed and the blood samples that were taken at the time of the six-year follow-up in 2008. The results demonstrated a relationship between *C. burnetii* Phase 2 IgG and depression using PHQ-9 scores. As far as the author is aware, an association between raised *C. burnetii* Phase 2 IgG titres and depression has not previously been described.

2.1.4 International comparison of laboratory results

A subsequent paper was also based on the six-year follow-up of the cohort of factory workers exposed to Q fever. In this paper microimmunofluorescent titres from cases and non-cases were submitted to three international reference laboratories in France, Australia and the UK (Healy et al., 2011, ENCLOSED PAPER). The study demonstrated that the results of Q fever micro-immunofluorescence varied according to the centre in which the samples were analysed. Concordance between laboratories using the same set of patient blood samples was 35% (18/52) when the French results were compared to the UK results; 42% (22/52) when the French results were compared to the Australian results; and 71% (37/52) when the Australian results were compared to the UK results.

2.2 Original contribution made

The outbreak report was ground breaking in that it suggested that *C. burnetii* might have been present in the straw board for 60 years before causing the outbreak. Although spores from other species, such as anthrax, have been shown to survive for many decades, this is the first time that *C. burnetii* has been postulated to have lain dormant for so long. The organism has been shown to survive in dried form for at least eight years (Philip, 1948). It is also very resilient and can withstand pressures of up to 20,000 lb/in^2, elevated temperatures, desiccation, osmotic shock, UV light, and chemical disinfectants (McCaul and Williams, 1981).

Although straw has been implicated in other outbreaks (Jorm et al., 1990), these have been in an outdoor context, whereas the straw in this outbreak was within internal straw-board walls. This is the first study to postulate straw board as the cause of a Q fever outbreak. The study demonstrates the need for caution in the increasing trend to use natural materials such as straw in house building, as straw can potentially provide a reservoir of infective material.

The study of facsimile cascade was novel in addressing the effectiveness of the technique in the context of a disease outbreak (van Woerden et al., 2007). This question had not been previously considered in the context of outbreak management. The study presented found that, compared to the previous year, the number of samples submitted for Q fever testing rose significantly in the period just after the facsimiles were sent to GP practices (Paired Wilcoxon signed rank test $p<0.001$).

The follow up study had the advantage that a cohort of individuals could be identified all of whom had been exposed to a point source of *C. burnetii.* The benefit of this design was that all of the individuals in the follow up cohort who had evidence of previous exposure to *C. burnetii* would probably have contracted the disease at around the same time. This is because the background rate of infection with *C. burnetii* is very low. Given the size of the cohort, and considering the six-year time frame, only one individual would be expected to have developed detectable antibodies to *C. burnetii* arising from the background rate of infection in the community (based on the prevalence in Thomas et al., 1995). We can reasonably conclude that almost all of the cases in the cohort with chronic fatigue and serological evidence of exposure to Q fever were infected six years previously in the 2002 outbreak and not at a later date. In other words, this cohort study can be used to assess whether there is a temporal relationship between acute infection with Q fever and subsequent sequelae. The study published by the author (van Woerden et al., 2011a) demonstrated that when Chalder Fatigue scores for fatigue were dichotomised as 'low' (0-3) and 'high' (4-11) the 'high' fatigue scores were significantly more common in the cases compared to non-cases (Fisher's Exact Test p=0.047).

The follow up study has also provided previously unreported evidence for an association between raised Q fever serology and depression. An analysis based on the follow-up data from 2008 indicated that when depression scores (PHQ-9 scale) were dichotomised to above and below the median, raised depression scores were more common in those patients with raised Phase 2 IgG to *C. burnetii* (Mann-Whitney U=173.0; p=0.049). The mean rank in 23 individuals

with a 'low depression score' (0-3) was 19.52, whereas the mean rank in 22 individuals with a 'high depression score' (4-9) was 26.64.

The study has provided further evidence that Q fever has significant long-term sequelae and raises the question as to whether the organism is still present in the affected individuals (Devanur and Kerr, 2006) and whether trials of antibiotics might help patients with post Q fever chronic fatigue. The author believes that there is a case for a RCT to evaluate this.

The paper comparing serological results from three international reference laboratories (Healy et al., 2006) tackled a problem that has been suspected for some time but had not been addressed directly before. The only previous research in this area compared the results from a single individual whose blood was tested by a commercial laboratory and by reference laboratories in France and the USA (Ake et al., 2010).

The results are concerning. Concordance in the interpretation of micro-immunofluorescence results between different pairs of laboratories was as low as 35%. Differences were most marked in relation to the definition of chronic cases of Q fever. Comparative serological analysis revealed no 'chronic' serological profiles when the set of samples was tested in either France or Australia but 10 such serological profiles when the same samples were tested in the UK reference laboratory. This issue is of concern as the three laboratories in question are among the most important reference laboratories in the world for this disease. This work has been cited by subsequent authors who also recognise the importance of this problem (Sunder et al., 2011, Kampschreur et al., 2012).

2.3 Methodological considerations

Several of the papers presented arise out of an initial disease outbreak. Although outbreaks can be tightly defined as, "the occurrence of more cases of disease than expected in a given area or among a specific group of people over a particular period of time" (Centers for Disease Control and Prevention, 2011), the methodology underpinning the investigation of an outbreak does not have the repeatability of a scientific experiment (Gregg, 2008). However, standard study designs can be incorporated. In this outbreak, a 'cohort' and nested-case control study design was used. Although the initial Q fever investigation demonstrated a clear epidemic curve, no microbiological confirmation of *C. burnetii* in materials take from the site was achieved. Microbiological confirmation would have significantly strengthened the straw board hypothesis, which has to remain tentative in its absence (Reingold, 1998).

The facsimile study could also have been strengthened in a number of ways, for example, by a telephone survey of GPs to ascertain whether the facsimiles had been read by GPs and to determine the extent to which the facsimiles had influenced behaviour in consultations. Further work could also have been undertaken to determine the number of patients attending GP surgeries that met the case definition and the proportion for whom Q fever serology was requested.

The main weakness of the six-year follow up demonstrating chronic fatigue was the small size of the control group. This was because the response rate in controls was low. Control participants who did attend may not have been

representative of the factory workers as a whole. A second control group from another occupational setting would also have strengthened the study.

The comparison of laboratory results from three reference laboratories assessed the reproducibility of a diagnostic test (Banoo et al., 2006). However, the sample size in this study was small with only 37 discordant results. The inclusion of a larger number of samples from a range of outbreaks across the world would have strengthened the study, as there is geographical variation in the antigens expressed by different strains of *C. burnetii* and differences in the resultant immunological response.

2.4 Discussion of findings

This Q fever outbreak has been referenced in reports of subsequent outbreaks (Morovic et al., 2008, Harzing, 2007, Wilson et al., 2010a, Amitai et al., 2010). The paper on facsimile cascade has also been quoted as being one of the better studies in a systematic review of public health emergency preparedness and response (Revere et al., 2011).

The paper comparing serological results from three international reference laboratories, and demonstrating the differences obtained on the same set of samples from these laboratories has generated some discussion (Kampschreur et al., 2012, Sunder et al., 2011). The discordance has implications for the definition of chronic Q fever and for the interpretation of serology results obtained in the context of trials of different treatment regimens. There is an urgent need for the development of an agreed set of international standard

materials for *C. burnetii*, international agreement on laboratory procedures, and a system of international cross checking of a subset of samples.

2.5 Unpublished Q fever related findings

Some additional findings associated with the series of Q fever papers are presented below.

2.5.1 Unpublished findings related to symptoms reported in the Q fever outbreak

One of the challenges in outbreak investigation is distinguishing common respiratory tract infections that are present in any population at any given time from acute Q fever, based solely on clinical symptoms. The analysis below compared the symptoms of individuals with acute Q fever and individuals without Q fever, based on serological criteria. The definition of acute Q fever in this context was 'any phase 2 IgM titre, or a fourfold rise in complement fixation tests (CFT)' (van Woerden et al., 2004). The comparative group without Q fever was defined in this context as 'individuals who were in the cohort exposed to Q fever and who reported that they were unwell when completing the questionnaire during the initial outbreak investigation, but who had no Q fever antibody titre by micro-immunofluorescence or CFT, in blood samples taken at that time'. It is possible to speculate that most of these 'control' patients had incidental viral upper respiratory tract infections at the time of the outbreak. A comparison between those with acute Q fever and other inter-current illnesses is shown in Table 1 below.

TABLE 1: ODDS RATIOS FOR THE PRESENCE OF SYMPTOMS IN SEROLOGICALLY POSITIVE AND NEGATIVE CASES SAMPLED AT THE TIME OF THE OUTBREAK IN 2002

	Acute Q Fever (symptomatic and serologically positive)	**Not Q fever** (symptomatic but serologically negative)	**Odds ratio**	**p value** (Fisher's Exact test)
Fever	42	8		
No fever	15	11	3.85	0.024
Sweats	54	18		
No sweats	7	8	3.63	0.031
Headache	52	18		
No headache	7	9	3.714	0.034
Cough	26	17		
No cough	29	8	0.422	0.097
Shortness of breath	26	17		
No Shortness of breath	29	8	0.422	0.097
Joint pain	47	13		
No joint pain	8	11	4.971	0.004
Chest pain	21	7		
No chest pain	32	15	1.406	0.606
Weight loss	27	2		
No weight loss	28	27	8.196	0.003

Amongst symptomatic individuals, fever, sweats, headache, joint pain and weight loss were significantly associated with a serological diagnosis of acute Q fever ($p<0.05$). Cough was very common in both groups and therefore did not help distinguish individuals with Q fever. The questionnaire administered at the time of the outbreak did not contain questions on myalgia or fatigue, which was asked about at subsequent outpatient appointments. The addition of these two questions would be useful in future outbreaks.

Testing for viral infection would have been helpful in more definitively excluding 'possible' cases of Q fever, as acute Q fever was only diagnosed in some cases by paired sera demonstrating a rise in CFT titre. Q fever testing and virology testing are normally undertaken in the same laboratory, which would make simultaneous testing feasible. The case for simultaneously testing for a range of common respiratory viruses in Q fever outbreaks is strengthened by the fact that the price for testing for common respiratory viruses is now relatively low.

In order to assess whether symptoms predicted serological status at the time of the outbreak a binary logistic regression was explored using 'enter', 'forward' and 'backward' stepwise (likelihood ratio) approaches, in SPSS version 20. The statistically significant associations identified above - fever, sweats, headache, joint pain and weight loss were entered into the model. 'Fever' was dropped due to its association with 'sweats'. The result of the baseline model which included all the variables i.e. weight loss, headache, joint pain and sweats, is shown in Tables 2 and 3.

TABLE 2: CLASSIFICATION TABLE FOR LOGISTIC REGRESSION ANALYSIS

	Observed				
			Status		Percentage Correct
			Serology Negative but symptoms	Acute Q fever	
Step 1	Status	Serology Negative but symptoms	8	7	53.3
		Acute Q fever	4	45	91.8
	Overall Percentage				82.8

TABLE 3: VARIABLES INCLUDED IN THE COMPOSITE MODEL PREDICTING SEROLOGICALLY NEGATIVE AS OPPOSED TO SEROLOGICALLY POSITIVE PATIENTS

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	Weight loss	-2.230	1.154	3.735	1	.053	.108
	Headache	-1.622	1.046	2.407	1	.121	.197
	Joint pain	-.701	.924	.575	1	.448	.496
	Sweats	-.373	.876	.181	1	.671	.689
	Constant	3.820	1.128	11.460	1	.001	45.586

This model correctly classified 82.8% of cases in the dataset as serologically positive or negative. Other models were generated but none of these predicted a greater percentage of the cases in the dataset. A forward stepwise (likelihood ratio) model reduced to two factors 'weight loss' and 'headache', predicted 82.8% of cases, and a backward stepwise (likelihood ratio) model reduced to 'joint pain' and 'sweats', also predicted 82.8% of cases. The instability of the variables included in forward and backward stepwise models may be due to missing data and the co-linearity of the variables.

The model is open to a number of criticisms. There was variation in the time between the onset of symptoms and the time that blood tests were taken. The sample size was relatively small and the model was not tested against a separate dataset. However, a more fully tested model could prove clinically useful in distinguishing patients with acute Q fever in the context of an acute outbreak.

2.5.2 Unpublished serological findings related to the Q fever outbreak

Tests for Q fever are problematic as there is wide inter-individual variation in antibody response and variation in the antigens expressed by different strains of Q fever (Porter et al., 2011). CFT is the most widely available test but is the least sensitive and often depends on detecting a rise in titre in paired sera (Peter et al., 1985). There is no international consensus on the cut off threshold for a positive Q fever test result for tests using ELISA and immunofluorescence (Dupont et al., 1994).

The interpretation of serological tests for Q fever is complex as different individuals may be pre-symptomatic, or at different phases in their immune response when blood is taken. One patient in this outbreak had positive serology on a blood sample taken the day before he became symptomatic. To address this issue, acute Q fever was defined in this outbreak using a composite endpoint.

Both CFT and IF tests can miss cases, as is demonstrated in Table 4 below, where the relationship between Phase 2 IgM and CFT tests is shown. In the analysis presented, the composite definition of acute Q fever was "micro-immunofluorescence (IF) Phase 2 IgM greater than or equal to 80, or a fourfold rise in CFT in paired samples, or a single CFT result greater than or equal to 64".

TABLE 4: COMPARISON OF INITIAL CFT AND PHASE 2 IgM SEROLOGY, AGAINST A COMPOSITE CASE DEFINITION OF ACUTE Q FEVER, FOR 106 INDIVIDUALS

	CFT (1st sample)									
Phase 2 IgM (1st sample)	**<8**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**>512**	**Total**
Negative	2	1								3
80	***1***	***1***								2
160	***5***	***3***		***1***						9
320	1		1	2						4
640	2	1	7	4	2	1				17
1280	0			1		2		1		4
>1280	5	1	4	13	13	16	13	1	1	67
Total	16	7	12	21	15	19	13	2	1	106

A range of factors contributed to the inclusion of different subsets of cases in the above table. In the three cases shown in blue, the initial blood samples would not have led to a diagnosis of acute Q fever by either CFT or IgM. These three cases were subsequently diagnosed based on paired sera. The 11 cases in red, bold and italic font were included on the basis of raised Phase 2 IgG ≥320. If the composite definitions of Q fever is accepted as a 'gold standard', a single blood test for Phase 2 IgM would have identified 103/106 cases, a sensitivity of 97.2%. In contrast, using the same composite definition as the gold standard, initial CFT testing would only have identified 50/106 cases, a sensitivity of only

47.2% As a result, the cases shown in red would not have received a diagnosis of acute Q fever based on single CFT test. This fact is widely recognised by laboratory staff, and CFT laboratory reports recommend that a second paired sample is taken. However, the importance of this may be underestimated by clinical staff and it can be difficult to get a second sample in clinical practice.

CFT is quoted in the literature as detecting around 65% of cases during the second week after symptom onset and 90% of cases by the fourth week (Porter et al., 2011). The figure in this study was lower, as in many cases bloods were taken before the second week of infection. The poor sensitivity of CFT is likely to be a problem in many Q fever outbreak investigations.

The data in Table 4 suggests that a policy of screening for atypical pneumonia using CFT would completely miss the 23/106 (21.7%) of cases with a CFT of 8 or less. Subject to cost constraints, there is therefore a case for using immunofluorescence tests routinely as part of screening for atypical pneumonia, or where a diagnosis of Q fever is being considered.

2.5.3 Unpublished findings - 18 month follow up

The cohort of patients with acute Q fever was followed up at a routine microbiology outpatient clinic for over five years by Dr M Llewellyn and Dr B Healy to identify any patients who developed long-term complications. The author designed follow up forms that were used in the outpatient clinics to capture relevant symptoms and serological results. Some of the collated findings are presented below.

The duration of symptoms for 60 acute cases of Q fever followed up over the subsequent 18 months is presented in Table 5. Some of the patients with acute Q fever had symptoms that lasted for more than one year.

TABLE 5: DURATION OF SYMPTOMS AS REPORTED AT OUTPATIENT CLINICS FOR 60 SYMPTOMATIC CASES OF ACUTE Q FEVER FOLLOWED UP OVER 18 MONTHS

Duration (days)	Fever (%)	Sweats (%)	Headaches (%)	Cough (%)	SOB (%)	Joint pain(%)	Chest pain(%)	Fatigue (%)	Lethargy (%)	Myalgia (%)	Back pain(%)	Diarrhea (%)
1-7	64.7	60.8	66.7	52.5	51.7	53.3	33.3	29.4	29.4	55.6	50.0	72.7
8-14	25.5	25.5	22.2	26.2	20.0	20.0	33.3	17.6	17.6	19.4	11.5	27.3
15-21	7.8	5.9	8.3	9.8	15.0	13.3	19.0	3.9	3.9	11.1	15.4	0.0
22-28	2.0	3.9	2.8	3.3	6.7	3.3	4.8	3.9	3.9	0.0	7.7	0.0
29-56	0.0	0.0	0.0	3.3	0.0	0.0	0.0	3.9	3.9	0.0	0.0	0.0
57-182	0.0	3.9	0.0	1.6	0.0	0.0	4.8	11.8	7.8	2.8	3.8	0.0
183-365	0.0	0.0	0.0	1.6	5.0	3.3	4.8	3.9	5.9	5.6	0.0	0.0
365+	0.0	0.0	0.0	1.6	1.7	6.7	0.0	25.5	27.5	5.6	11.5	0.0

As expected, most symptoms such as diarrhea, headache, fever and sweats were short lived. Other symptoms were more persistent. In particular, lethargy and fatigue were present for more than one year in 25.5% of patients. Some of the persistent symptoms may represent other coincidental but common pathologies. Cough may represent asthma or other common respiratory disorders, and back pain may reflect normal levels of pathology in that occupational group. However, persistent myalgia (5.6% for more than one year)

and joint pain (6.7% for more than one year) may represent long-term morbidity associated with the outbreak.

2.5.4 Unpublished findings - five year follow up

The development of Phase 1 antibodies is associated with the risk of developing chronic Q fever and is therefore appropriate to monitor as part of the follow up of patients with Q fever. Among the 106 individuals initially identified as having acute Q fever in this outbreak, 97 had a follow up blood test for phase 1 IgG antibodies at some point over the subsequent five years. Phase 1 IgG developed in 63.9% (62/97) of these patients. Phase 1 IgG titres ranged from 160 to 10,240. In almost all cases, raised titres occurred in the first year, if they occurred at all. On this basis, a one-year period of follow up could be recommended for all cases of acute Q fever with longer follow up for those with raised Phase 1 antibodies.

It has been suggested that Phase 1 IgG ≥ 800 has a 100% sensitivity for detecting chronic Q fever (Dupont et al., 1994). This threshold is also a major criteria for the diagnosis of infective endocarditis in the context of Q fever (Fournier et al., 1996). In this cohort, eight patients met this criteria but only one has developed endocarditis so far. Figure 2 provides information on the change in titres over five years.

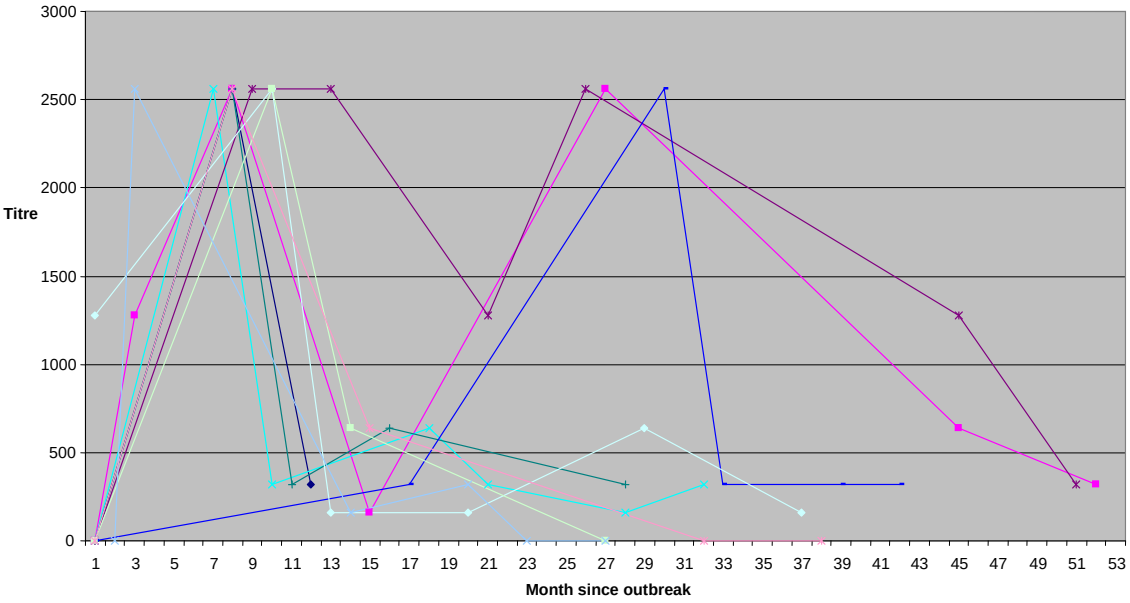

FIGURE 2: CHANGES IN PHASE 1 IgG IN EIGHT PATIENTS, WITH AN IgG OF ≥ 800 AT SOME POINT, OVER FIVE YEARS OF FOLLOW UP (GRAPH COURTESY OF DR B HEALY)

In most cases, there is a rise in titre in the first 12 months followed by a fall in titre. However, in a few cases a second peak can be observed. Ongoing surveillance of these cases is being undertaken and it is possible that some of the other cases will develop endocarditis in the future. The findings of this study would support the recommendation that patients with an IgG of ≥ 800 at one year should be provided with long-term follow up.

2.5.5 Unpublished findings - six year follow up and severity of fatigue

The follow up of the cohort of individuals exposed to Q fever six years later focused on the sequelae of chronic fatigue. Information was collected at a single clinic held by the author and Dr B Healy. The published paper (van Woerden et al., 2011a) does not provide information on the severity of the fatigue experienced by participants. Additional information on this is shown in Table 6 below.

TABLE 6: RELATIONSHIP BETWEEN SEVERITY AND DURATION OF FATIGUE, IN THE YEAR PRECEDING THE SIX YEAR FOLLOW UP, FOR 36 PATIENTS WITH PREVIOUS ACUTE Q FEVER

Question		What percent of the time do you feel tired?				Number of cases
		25%	50%	75%	100%	
If you are tired at the moment, how long has this lasted?	< 1 week	4	2	0	0	6
	< 3 months	2	4	0	0	6
	3 - 6 months	2	1	1	0	4
	>= 6 months	2	9	2	7	20
Number of cases		10	16	3	7	36

Chronic fatigue is normally defined as fatigue for six months or more (Devanur and Kerr, 2006). In this study, among those who had experienced chronic fatigue for six months or more, 90% (18/20) had been tired at least 50% of the time indicating that these patients were severely disabled by their post Q fever Chronic Fatigue.

2.5.6 Unpublished findings - six year follow up and smoking

Information gathered at the six-year follow up was also used to examine the relationship between serological abnormalities and smoking. For this analysis, serological status was assigned to each individual based on the rules provided by Dupont et al. (1994), as shown in Table 7 below.

TABLE 7: DEFINITION OF STATUS USING SEROLOGICAL CRITERIA

Status	**Definition**
Acute	Phase 1 IgM ≥ 50
Past	Phase 2 IgG detected and Phase 1 IgG <800
Negative	No antibody detected
Chronic	Phase 1 IgG ≥ 800 or Phase 1 IgA≥25
Borderline Chronic	Phase 1 IgG ≥ 400 and < 800

The results of 45 cases are presented in Table 8. The 15 Borderline Chronic/Chronic cases are compared to the 30 cases with serological evidence of 'past infection' or 'no previous infection', based on their serological status.

TABLE 8: RELATIONSHIP BETWEEN SEROLOGICAL RESULTS MEASURED IN THE UK REFERENCE LABORATORY AND SMOKING STATUS AT THE SIX-YEAR FOLLOW-UP CLINIC

		Smoking status		
		Current/ Ex-smoker	Never smoker	Total
British Results	Borderline Chronic/Chronic	12	3	15
	Negative/Past	12	18	30
Total		24	21	45

Smoking is associated with borderline chronic/chronic serological status (Odds ratio 6.0; p=0.014, Fisher's Exact Test). As in many diseases, smoking seems to increase the probability of developing a more chronic serological pattern (Morroy et al., 2012). Although this analysis does not provide unequivocal epidemiological evidence, it would be worth pointing out to patients who have acute Q fever that stopping smoking might reduce the risk of developing long term sequelae.

2.6 Summary of Q fever work

The main strength of the Q fever work presented above is that it follows a cohort of individuals over six years, starting with one of the largest outbreaks to date in the UK, and examines the sequelae in that cohort. The investigation of this cohort was also used opportunistically to investigate a number of issues such as the use of facsimile cascade and the congruence of international reference laboratories.

The unpublished work on Q fever highlighted the weaknesses of single sample CFT tests, demonstrated the severity of chronic fatigue in some patients six years later, highlighted the association between smoking and chronic Q fever symptoms, and provided a regression model that with further development could be used to help clinically distinguish Q fever from other illnesses. This might aid more rapid epidemiological analysis in future outbreaks and accelerate the pinpointing of the source of an outbreak.

Follow up to the six-year stage has identified the value of follow up for all patients for one year and the long-term follow up of patients who have raised serology suggestive of chronic Q fever. The follow up results may be suitable for publication in due course.

A number of patients have developed chronic fatigue and depression. Thought needs to be given to undertaking a trial of antibiotics in these patients. If such a trial were well designed, it would clearly be publishable.

The relationship between smoking and chronic/borderline chronic serology merits wider recognition, and should clearly be raised with any patients who develop acute Q fever as stopping smoking might reduce the risk of sequelae.

3 Flagellated respiratory protozoa and their association with respiratory disease

Prior to the author's involvement, two small studies had been undertaken in Spain examining the relationship between unidentified flagellated protozoa and respiratory disease. The Spanish studies had demonstrated that protozoa were often present in the sputum of individuals with respiratory disease (Ribas and Mosquera, 1998) and in individuals who were immuno-compromised (Ribas et al., 2005). The relationship between these unidentified protozoa and respiratory disease has received little attention by research groups elsewhere in the world. The research presented in this thesis is, therefore, contentious and requires further international validation.

3.1 The wider research context

The first of the Spanish studies referred to above identified protozoa in 15/19 patients with asthma, as opposed to 9/78 patients with other respiratory diseases, mostly COPD (Ribas and Mosquera, 1998). This was followed up by a second study that examined sputum from immuno-compromised patients, primarily patients with HIV infection, who had a productive cough. This case series found that 72/106 patients had protozoal forms present in sputum (Ribas et al., 2005).

3.1.1 A case series

To examine whether these findings could be replicated in the UK, the author undertook a small pilot study in Cardiff using spontaneously produced sputum

samples from hospital inpatients (van Woerden et al., 2010, ENCLOSED PAPER). At that time, it was unclear whether the presence of flagellated protozoa in sputum was an isolated phenomenon restricted to Spain (or perhaps to climates with high temperatures), or was a wider phenomenon that would also be present in more temperate climates such as in the UK. The case series demonstrating that protozoa were present in a significant proportion (50%) of inpatients in Cardiff indicated that the observation of protozoa in sputum was an international phenomenon. An unpublished case has been identified (email correspondence, Richard Kibbee, Environmental Microbiologist, Centre for Research on Environmental Microbiology, University of Ottawa, Canada). The photograph of the protozoa identified is shown in Figure 3 below.

FIGURE 3: PHOTOGRAPH TAKEN 27 FEBRUARY 2009, DEPICTING PRESUMPTIVE PROTOZOAL BODY; ABSOLUTE MAGNIFICATION ~1000X (RICHARD KIBBEE)

Another unpublished case was identified by correspondence with Saskia Grobben, Applied Plant Research Unit, Lelystad, Netherlands. In this case,

protozoa were identified in sputum on three consecutive days. The photographs obtained are shown below.

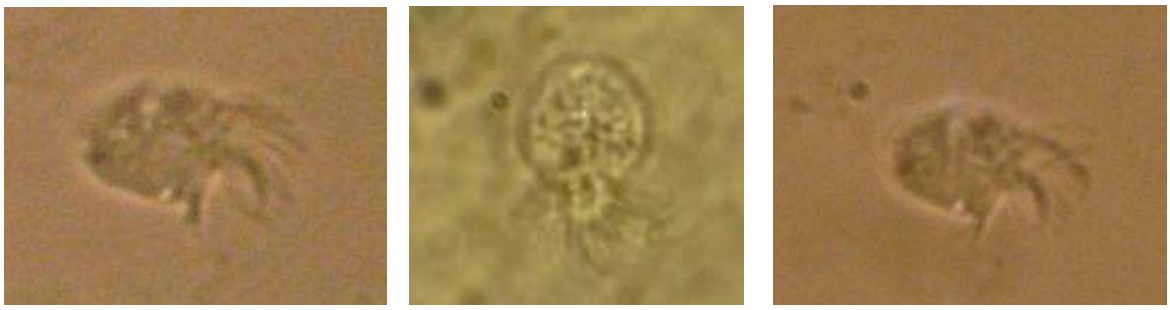

FIGURE 4: PHOTOGRAPHS OF PUTATIVE PROTOZOA IN SPUTUM (SASKIA GROBBEN, 29 DECEMBER 2011)

The photographs are similar in appearance to those taken by Spanish colleagues, with flagella covering at least a proportion of the circumference of the organism seen under light microscopy. Both samples were related to an acute exacerbation of respiratory symptoms against a background of asthma.

3.1.2 Case control study in the community

A case control study was undertaken using induced sputum comparing the prevalence of protozoa in the sputum of patients in the community with asthma and in non-atopic controls (van Woerden et al., 2011b, ENCLOSED PAPER). In this study 66.7% (20/30) of asthmatics and 30.8% (4/13) of non-atopic controls had protozoa in their sputum (p=0.046).This study provided evidence that protozoa were not only found in the sputum of patients in hospital, but were common in patients with asthma in the community.

3.1.3 Case comparison study in hospital patients

In another case comparison study, the author and colleagues demonstrated that there was an association between the presence of monocytes or small macrophages and protozoa in sputum samples (Martinez-Giron and van Woerden, 2011, ENCLOSED PAPER). Cases were defined as respiratory inpatients whose sputum contained protozoa. The comparison cases did not have protozoa identified in their sputum samples. The comparison identified that the presence of monocytes/small macrophages in the sputum sample gave a positive predictive value for protozoa of 74.3% (52/70) and a negative predictive value of 84.3% (102/121), when monocytes were absent. The study identified that 66.7% (8/12) of patients on oral steroids had protozoa in their sputum, compared to 34.6% (62/179) of patients who were not on oral steroids (Fisher's Exact test p=0.033). Similarly, in a binary logistic regression model to predict the presence/absence of protozoa, those on inhaled steroids had a reduced odds of having protozoa in their sputum compared to those on oral steroids (odds ratio 0.138; p=0.027). The commonest reason for prescribing oral steroids in respiratory inpatients with a productive cough (as in this study) is an acute asthma attack. We do not have diagnoses for the patients in this case comparison study. However, if we presume that the patients on oral steroids had been hospitalised for acute exacerbations of asthma, then asthma attacks would be associated with an increased probability of protozoa in sputum. Smokers also had a raised odds of having protozoa in sputum, compared to the combination of non-smokers and ex-smokers (odds ratio 1.859; p=0.047).

3.1.4 Other studies

The Spanish colleagues have undertaken a literature review which summarises the different species of protozoa that have been recovered from patients with respiratory infections (Martinez-Girón et al., 2008). The species of protozoa identified in the literature review represent rare organisms reported in small case series. These known species do not appear to the author to be feasible candidates for the protozoa that are prevalent in a large proportion of patients with asthma, in the studies reported by the author (van Woerden et al., 2011b).

A potentially more relevant series of papers from Eastern Europe has been identified which describe trichomonas-like protozoa in the respiratory tract. This series of papers state that these organisms are relatively common in sputum but are difficult to detect (Kutisova et al., 2005, Hersh, 1985, Mallat et al., 2004, Duboucher et al., 2007, Duboucher et al., 2008, Mantini et al., 2009).

3.1.5 Speculative hypotheses

A series of letters (see Appendix 3) have been published by the author and colleagues proposing a number of speculative hypotheses in relation to the above findings (Martinez-Giron et al., 2008, Martinez-Giron and van Woerden, 2009a, Martinez-Giron and van Woerden, 2010, Martinez-Giron and van Woerden, 2009b). The author hypothesised that these protozoa could be present in mite faeces and subsequently inhaled by some patients. Patients with asthma might be more susceptible to colonisation of the respiratory tract by such protozoa. This in turn may have an adjuvant-like effect on the immune system, increasing the response in asthma patients to other antigens such as

pollen etc. (Martinez-Giron et al., 2008, Martinez-Giron and van Woerden, 2009a).

Another speculative hypothesis has been proposed by the author and colleagues to the effect that one of the commonest allergens (Der p1), which is known to be present in the intestine of dust mites and in mite faecal pellets, could be protozoal in origin rather than secreted by the mite intestine as has widely been assumed (Martinez-Giron and van Woerden, 2009b, Martinez-Giron and van Woerden, 2010). Further work is underway to test this hypothesis.

Although these hypotheses are speculative, a similar hypothesis has been proposed in relation to Kawasaki's Disease (Hamashima et al., 1982). In this study, rickettesia-like bodies were shown to be more common in the intestines of mites taken from the homes of patients with Kawasaki's Disease. The authors of this paper hypothesised that dust mite faeces containing the rickettesia-like organisms had been inhaled and caused the disease in the affected patients.

3.2 Original contribution made

In these case studies, the author has facilitated interdisciplinary work across the disciplines of epidemiology, microbiology, and clinical medicine and provided the driving force behind the testing of the hypotheses presented in this thesis.

Protozoa have been shown for the first time to be more prevalent in induced sputum from asthma patients living in the community than in non-atopic controls. Protozoa have been shown to be common in spontaneously produced sputum from hospital inpatients. Inpatients on oral steroids have been shown to have a much higher prevalence of protozoa in their sputum (66.7%) compared

to those who are not on oral steroids (34.6%). Similarly, smokers and ex-smokers have been shown to have a higher prevalence of protozoa (45.1%) compared to non-smokers (30.3%).

The research presented has demonstrated that smokers have raised odds (1.859) of protozoa in sputum compared to non-smokers and ex-smokers. In absolute terms, 23.6% of non-smokers had protozoa in their sputum whereas 41.7% of smokers/ex-smokers had protozoa in their sputum. Similarly, the research presented has demonstrated that those who were not on either oral or inhaled steroids had reduced odds (0.267) of protozoa in sputum compared to those on oral steroids. In absolute terms, 66.7% of inpatients on oral steroids had protozoa in their sputum whereas 34.6% of inpatients with respiratory symptoms who were not on oral or inhaled steroids had protozoa in their sputum.

3.3 Methodological considerations

The author has led the development and design of the studies presented. Skills have been developed in bidding for research funding, applying to Research Ethics Committees, obtaining approval from NHS Research and Development Departments, and employing staff to facilitate patient recruitment and sputum collection.

Practical difficulties that have been encountered have been successfully resolved. For example, we found it extremely difficult to obtain induced sputum from non-atopic controls. This issue had not been highlighted in the existing

literature, but it has significant implications for the use of non-atopic controls in future studies that collect induced sputum.

The case series and case control studies would all have benefited from larger sample sizes and tighter case definitions. In particular, the diagnosis of asthma could have been more fully assessed by lung function, skin prick tests to a range of allergens or RadioAllergoSorbent Testing (RAST) for total and specific IgE to a range of common allergens. It would also have been helpful to have similar assessments undertaken on control participants. Access to more than one set of controls drawn from different populations would also have reduced the risk of bias that is inherent in case control studies.

3.4 Discussion of findings

At present, it is unclear whether the protozoa identified in the respiratory tract of asthma patients have any pathological significance. The organisms could simply be commensal organisms that have no wider significance. However, it is possible to speculate that, even in a proportion of respiratory patients, the protozoa may have a role to play, perhaps by altering the Th1/Th2 balance as part of the wider immune response to inhaled antigens such as pollen etc. (Jacquet, 2011b, Jacquet, 2011a). This could be measured in a future study. It is also possible that individuals with asthma differ from non-asthmatic individuals in terms of their genetic susceptibility to colonisation by respiratory microbiota.

The proposal that contaminating microbial compounds, present in or on dust mites, may play a role as adjuvant factors and encourage a Th2-biased allergic

response is supported by a recent review (Jacquet, 2010), which quotes previous work by the author (van Woerden, 2004). A current model of the possible role of dust mites and their ecological role in asthma, taken from this review, is provided in Figure 5 below.

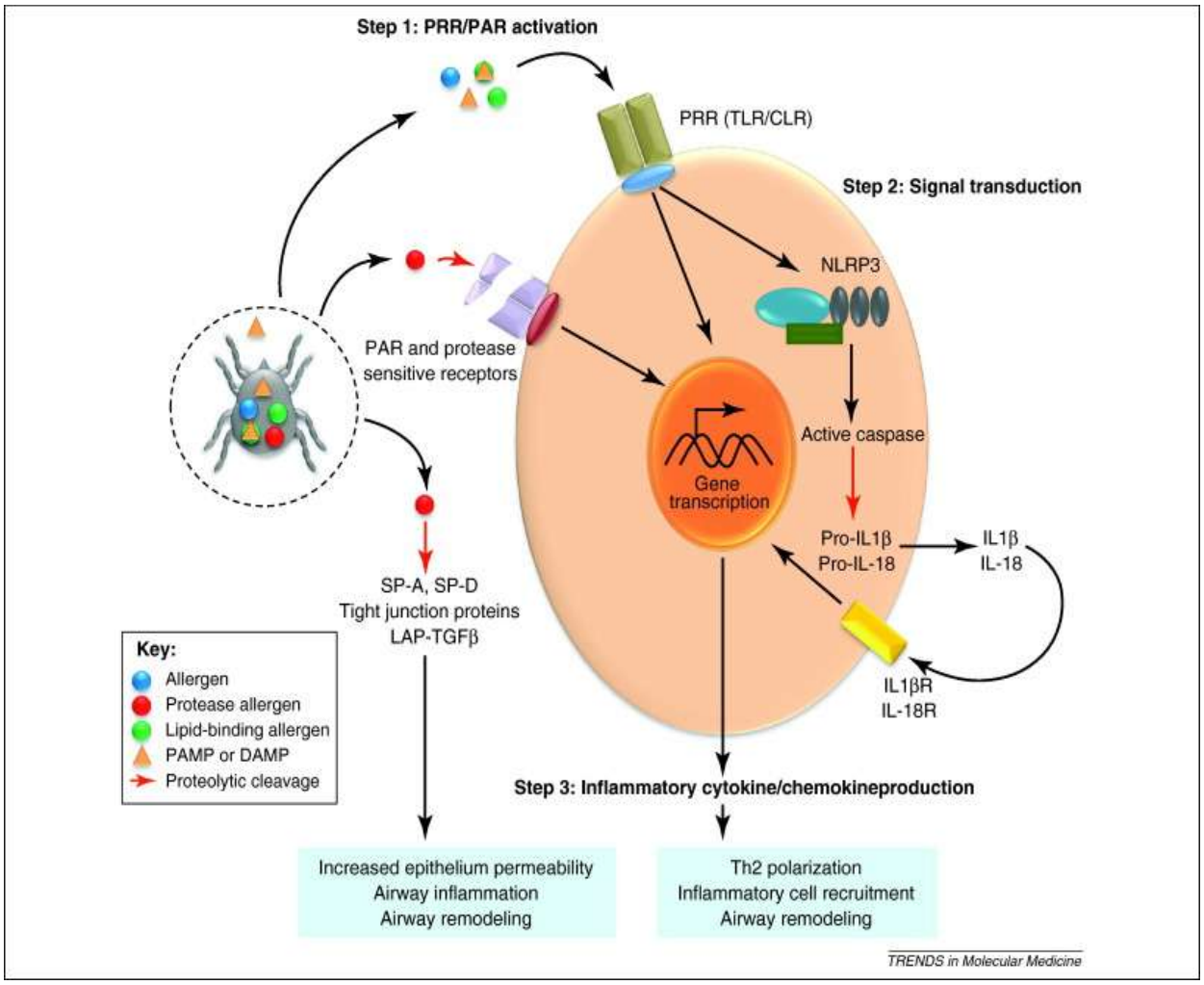

FIGURE 5: ALLERGENS AND ORGANISMS PRESENT IN AND ON MITES THAT MAY AFFECT ASTHMA (JACQUET, 2010)

The discovery that stomach ulcers were related to infection with *Helicobacter pylori* has renewed interest in the potential role that infectious agents may play as part of a causal web contributing to chronic disease (Marshall et al., 1985).

The interaction between dust mites and asthma described by Voorhorst epitomises the complex relationships that exist between human beings and other life forms (Voorhorst et al., 1967). It is possible that many similarly complex relationships are yet to be discovered.

There is increasing evidence that a large number of protozoa cannot be cultured using existing techniques and have not been described in existing taxonomies (Jeon et al., 2008, Stoeck et al., 2006). A wide range of protozoa have been discovered in studies of the biosphere using molecular techniques and have been submitted to international databases such as the National Centre for Biotechnical Information (Anon., 2011) but have not been otherwise characterised.

There is increasing recognition that human beings carry a large microbiota in the gut, on the skin and on other mucosal surfaces (Hilty et al., 2010) and that the respiratory tract has a distinct microbiota (Rogers et al., 2005, Tunney et al., 2008). These populations of microbiota interact with the immune system. It has been shown that microbiota affect immune responses in the lungs (Rogers et al., 2009, Hilty et al., 2010) and that exposure to microbiota at one mucosal surface can affect allergic responses at another mucosal surface (Cogen et al., 2008, Round and Mazmanian, 2009, Dethlefsen et al., 2006).

The research on microbiota and immune response disorders such as asthma raises the general question as to how a postulated aetiological association can be proven. Historically, Koch-Henle postulates (Inglis, 2007a, Marshall et al., 1985) were used to demonstrate that a given infectious agent had an

aetiological role in a given disease (Inglis, 2007b)[1]. Identification of organisms as the cause of disease where no culture method is known is more challenging (Evans, 1976). The emergence of molecular methods for the identification of organisms has led to the proposal of 'molecular Koch's postulates' (Inglis, 2007b). This approach builds an argument for a causal relationship based on the presence of evidence to support the following series of assertions: "congruence or reproducible correlation of a taxonomically defined life form with the clinico-pathological and epidemiological features of infection; consistency of the demonstrable biological response in the subject to an encounter with the prospective infective agent; progressive or cumulative dissonance as an explanation for pathophysiological processes at every known level of biological organization in the subject; curtailment of that pathophysiological process on the deliberate introduction of a specified biomedical intervention". This approach may be helpful in exploring the possible role of protozoa in asthma in the future. Other frameworks, such as the Bradford-Hill criteria for causality (Brownson et al., 2010), and the study design underpinning the evidence are also relevant (Sackett, 1997, Guyatt et al., 2008, Guyatt et al., 2011).

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[1] The Koch-Henle postulates are: the micro-organism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms; the micro-organism must be isolated from a diseased organism and grown in pure culture; the cultured micro-organism should cause disease when introduced into a healthy organism; the micro-organism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

3.5 Unpublished findings related to molecular analysis of sputum samples

Further work has been undertaken to assess whether the protozoa seen by light microscopy can be identified in sputum samples using molecular techniques. To date this work has been unsuccessful. Potential reasons for this include the possibility that more than one species of protozoa is present. This would result in the signal for each separate species of protozoa falling below the threshold of detection. Inhibitory compounds could be present in the samples and prevent DNA amplification. The microscopic identification of putative protozoa could even represent misidentification of other phenomena such as a combination of cell debris and fungi, although the author believes that this is unlikely.

Ethical approval was obtained by the author to allow the sputum samples collected by the author in Wandsworth, London, to be used for molecular analysis using variations in 18S rRNA to characterise different species of eukaryotes present in the samples. Sputum samples, taken from microscope slides, were combined to form one set of sputum from asthma patients and one set of sputum from control subjects. Between two and five areas were excised from each microscope slide using a Zymoresearch pinpoint system providing a total sputum sample of around 5 mm^3. DNA was extracted and then amplified using generic 18S primers and standard amplification techniques. The two samples were tagged and then analysed using a 454 pyrosequencer by a company in Texas, USA. The DNA sequences read by the pyrosequencer were compared to databases of known sequences to determine, in a semi-quantitative way, the percentage of DNA in each of the two samples derived from known

species of eukaryotic organisms. The practical laboratory work on the samples was undertaken by research colleagues in the School of Biosciences, Cardiff University (Dr C Gregory, Dr R Brown, and Dr J Marchesi).

This study has demonstrated differences in the range of fungi that were present in sputum samples taken from asthma patients and non-atopic controls. Table 9 lists the fungi found in the samples from the patients with asthma and the controls where the difference in the percent of total reads was > 4%.

TABLE 9: PERCENT OF DNA READS IN THE SPUTUM OF ASTHMA PATIENTS AND NON-ATOPIC CONTROL PARTICIPANTS WHERE THE DIFFERENCE IS > 4%

Name	Non-atopic control participants (Percentage of reads)	Asthma patients (Percentage of reads)
Eremothecium sinecaudum	41.3	1.0
Systenostrema alba	23.6	0.0
Cladosporium cladosporioides	14.5	0.1
Vanderwaltozyma polyspora	6.8	0.1
Grifola sordulenta	0.0	4.5
Termitomyces clypeatus	0.0	7.1
Malassezia pachydermatis	0.0	21.7
Psathyrella candolleana	0.0	27.3

The difference between the two samples was tested by Dr Julian Marchesi, Cardiff University using Unifrac (Lozupone et al., 2006). A statistically significant difference in the pattern of fungi that were present in the respective samples was demonstrated using the Phylogenetic (P) test ($p < 0.0001$).

The fungi *Malassezia pachydermatis*, which had a higher prevalence in patients with asthma, has a known association with atopic dermatitis (Gaitanis et al., 2012). Two of the fungi most commonly found in the sputum of asthma patients (*Termitomyces clypeatus* and *Psathyrella candolleana*) represent members of the mushroom family (Khowala et al., 1993). The latter has been found in indoor

dust (Pitkaranta et al., 2008) and one can speculate that the fungi have been inhaled from the environment. However, no obvious associations were identified in the published literature between the other fungi identified and asthma. It is possible that most of the fungi identified could have come from a single individual, or a small number of individuals, whose samples were heavily colonised by fungi.

Except for *Cladosporium,* the species identified in induced sputum are not commonly found in air samples examined using standard culture techniques (Burr et al., 2007). Analysis of air samples using molecular techniques may demonstrate that these species are commonly present in the air, but this research has not been undertaken so far. The presence of a wide range of fungi in sputum suggests that future studies of induced sputum should simultaneously collect air samples to assess the extent to which fungal species in sputum resemble those in the immediate environment.

The molecular methods used to date have not demonstrated the presence of protozoa, although it is possible to speculate that some of the genetic sequences ascribed to fungi may be protozoal in origin. To pursue this hypothesis further, there is a need to isolate significant numbers of protozoa from pooled samples and develop a sensitive and specific molecular test for one or more species of protozoa that may be present. Samples from individuals, containing very small amounts of DNA, could then be tested. A new research protocol has been drawn up using a range of techniques to physically isolate and characterise any protozoa in fresh sputum samples.

4 Conclusion

This thesis has presented two series of published papers relating to two atypical respiratory organisms. The first series of papers followed a cohort of patients exposed to *C. burnetii* in an outbreak of Q fever, examined the effectiveness of case finding, assessed the prevalence of post Q fever chronic fatigue in the exposed cohort at six years follow up, and undertook a comparison of the consistency of Q fever micro-immunofluorescent tests across three international reference laboratories. The series of papers has demonstrated a potential long lasting risk from straw board, the prevalence of post Q fever chronic fatigue, the possibility that depression is a long-term sequelae of Q fever infection, and the need for better international standards for Q fever immunofluorescent tests. There is also a need for an internationally agreed set of reference strains of *C. burnetii*, and a system of international cross-checking of samples.

The unpublished work on Q fever highlighted the weaknesses of single sample CFT tests, demonstrated the severity of chronic fatigue in some patients six years later, highlighted the association between smoking and chronic Q fever symptoms, and provided a regression model that could be used as an algorithm to help distinguish Q fever from other illnesses based on clinical symptoms. A small cohort of eight patients with serological evidence of chronic Q fever has also been identified for ongoing follow up.

The second series of papers investigated a controversial hypothesis that flagellated protozoa are more common in the sputum of patients with respiratory disease. The papers have demonstrated the presence of protozoa in the sputum

of patients in the UK as well as in Spain, an increased odds of observing monocytes or small macrophages in the sputum of patients with protozoa, an increased odds of the presence of protozoa in the sputum of patients with asthma in the community, and a raised odds for the presence of protozoa in the sputum of hospital inpatients with respiratory disease who were smokers or who were on oral steroids. Some unpublished work using molecular methods to examine sputum for eukaryotes has also been presented which identifies differences in the percentage of DNA reads for fungi present in asthma patients and control subjects.

The case series and case comparison studies presented are relatively small. In retrospect, these studies would have been strengthened by having larger sample sizes, more extensive analysis of disease characteristics, and the use of more than one set of controls to reduce the risk of selection bias.

4.1 Potential for future research

The work presented points to several lines of research on Q fever. There is a need to follow up the cohort of eight patients with serological but not clinical evidence of chronic Q fever. An RCT of the use of long-term antibiotics in patients with post Q fever Chronic Fatigue would be justified, with both predetermined serological and clinical end-points, as this is a chronic disabling condition, with no established treatment. Further work to develop international reference standards is also needed (Kampschreur et al., 2012).

The aetiological hypotheses related to flagellated protozoa are the subject of on-going research by the author using molecular methods to identify and

characterise the microbiota in the respiratory tract and examine the impact that this has on respiratory health and disease. It is hoped that this will result in the development of rapid DNA based tests for relevant species of protozoa that can be used to test the sputum of patients with respiratory symptoms or disease. Similar methods are being pursued in relation to the microbiota that exists within dust mite faeces, and to test the hypothesis that protozoa present in mite faeces could be the source of Der p1, as has been proposed in the letters attached in Appendix 3. It is fully recognised that these hypotheses are highly speculative. However, even if the hypotheses in Appendix 3 are ultimately disproved, it is likely that this line of research will yield further information regarding the microbiota in a range of environments that are relevant to human health.

Further work may well suggest that a RCT is undertaken to test the hypothesis that eradication of protozoa from induced sputum samples with an appropriate anti-protozoal agent reduces asthma symptoms (Miranda, 1980, Ribas et al., 2007).

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5 Appendix 1: Summary of contributions to co-authored papers

The material used in these papers has not been published elsewhere apart from the initial Q fever outbreak, which was used in a Part II report for the Faculty of Public Health of the Royal College of Physicians of Ireland.

The contribution of the author to the different elements of the underlying research leading to each publication is provided in the table below.

TABLE 10: SUMMARY OF CONTRIBUTIONS TO PAPERS

Paper	Concept initiation	Design	Approval processes (R&D and ethics)	Data collection	Data analysis	Paper drafting	Corresponding author
van Woerden HC, Mason BW, Nehaul LK, Smith R, Salmon RL, Healy B, et al. Q Fever outbreak in industrial setting. *Emerging Infectious Diseases*, 2004; 10(7):1282-1289.			N/A				
van Woerden HC, Evans MR, Mason BW Nehaul L. Using facsimile cascade to assist case searching during an outbreak of Q fever. *Epidemiology and Infection*. 2007; 135(5):798-801.			N/A				
van Woerden HC, Healy B, Llewellyn M, Matthews IP. A nested case control study demonstrating increased chronic fatigue six years after a Q fever outbreak. *Microbiology Research*, 2011; 3:e11.							

Paper	Concept initiation	Design	Approval processes (R&D and ethics)	Data collection	Data analysis	Paper drafting	Corresponding author
Healy B, **van Woerden HC**, Raoult D, Graves S, Pitman J, Lloyd G, Brown N, Llewelyn M. Chronic Q fever: Different serological results in three countries - results of a follow-up study 6 years after a point source outbreak. *Clinical Infectious Diseases*, 2011; 52 (8):1013-1019.							
van Woerden HC, Gregory C, Martinez-Giron R, Burr M , Lansdown A, Matthews IP. Case series demonstrating the presence of protozoa in the sputum of a proportion of respiratory patients. *The Internet Journal of Laboratory Medicine*, 2010; 4:1.							
van Woerden HC, Ratier-Cruz A, Gregory C, Aleshinloye OB, Martinez-Giron R, Matthews IP, Burr ML. Association between protozoa in induced sputum samples and asthma: a case control study. *Respiratory Medicine*, 2011; 105 (6):877-84.							

Paper	Concept initiation	Design	Approval processes (R&D and ethics)	Data collection	Data analysis	Paper drafting	Corresponding author
Martinez-Giron R, **van Woerden HC**. Clinical and immunological characteristics associated with the presence of protozoa in sputum smears. *Diagnostic Cytopathology*, 2011; doi: 10.1002/dc.21752.			N/A				

Key to table above

Minor role	
Major role	
Not applicable	N/A
Minimal role	

6 Appendix 2: Submitted papers

Q Fever Outbreak in Industrial Setting

Hugo C. van Woerden,* Brendan W. Mason,* Lika K. Nehaul,† Robert Smith,* Roland L. Salmon,* Brendan Healy,‡ Manoj Valappil,§ Diana Westmoreland,§ Sarah de Martin,† Meirion R. Evans,* Graham Lloyd,¶ Marysia Hamilton-Kirkwood,‡ and Nina S. Williams*

An outbreak of Q fever occurred in South Wales, United Kingdom, from July 15 through September 30, 2002. To investigate the outbreak a cohort and nested case-control study of persons who had worked at a cardboard manufacturing plant was conducted. The cohort included 282 employees and subcontractors, of whom 253 (90%) provided blood samples and 214 (76%) completed questionnaires. Ninety-five cases of acute Q fever were identified. The epidemic curve and other data suggested an outbreak source likely occurred August 5–9, 2002. Employees in the factory's offices were at greatest risk for infection (odds ratio 3.46; 95% confidence interval 1.38–9.06). The offices were undergoing renovation work around the time of likely exposure and contained straw board that had repeatedly been drilled. The outbreak may have been caused by aerosolization of *Coxiella burnetii* spore-like forms during drilling into contaminated straw board.

Q fever is an infection caused by the bacterium *Coxiella burnetii*. The organism is found in most parts of the world and is endemic in wild and domestic animals, rodents, and arthropods, which provide a reservoir for infection (1). Most outbreaks have been associated directly or indirectly with farms or farm animals, but urban outbreaks have been described (2,3). Infected animal birth products can cause outbreaks of Q fever, and an infected placenta can contain as many as 10^9 organisms per gram (4). *C. burnetii* produces a spore-like form, which can survive for months or years before being inhaled and causing infection (5,6). The infective dose can be as low as one organism; therefore, large outbreaks can be caused by a small source (7). A review of the literature was undertaken by one of the authors (available from H.C. van Woerden). This investigation identified 79 outbreaks reported in 48 articles in English language journals. An additional 44 papers in other languages were identified in a literature review by Williams (7) and a further 40 German outbreaks were identified in a literature review by Hellenbrand et al. (8). The literature review suggested that most outbreaks are associated with primary or secondary aerosols that arise around infected animals or contaminated fomites (5,9).

Approximately 70 cases of Q fever are identified in the United Kingdom each year as a result of routine surveillance (R. Smith, pers. comm., Zoonosis Surveillance, Communicable Disease Surveillance Centre, Wales). However, seroprevalence studies indicate that approximately 27% of farmers and 10% of the general population have antibodies, which suggests previous exposure to the organism; this finding does not appear to have changed substantially during the last 45 years (10,11). We report an investigation of an outbreak of Q fever at the premises of a manufacturer of cardboard packaging materials in Newport docks, South Wales, in the summer of 2002.

Methods

Description of the Outbreak

A possible outbreak of atypical pneumonia was reported to the local public health department on September 12, 2002, by a physician who reported that other employees at the patient's workplace had had similar symptoms. The outbreak was verified, an outbreak control team assembled, and a case definition agreed on (12). By September 15, 2002, a total 12 potential patients had been identified and the first case confirmed as Q fever. The investigation

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was begun by contacting all relevant hospital clinicians and general medical practitioners in the Gwent area and requesting that they supply blood samples from any patients who had symptoms compatible with Q fever.

Epidemiologic Investigation

Several hypotheses were explored. An outbreak could have occurred in the wider community, and employees could have been infected by contaminated straw, hay, or compost; wild or feral animals; or domestic animals, particularly pregnant or newborn animals. Contamination could have been through sources brought into the factory, which included the following: contaminated personal belongings; contact with a contaminated source on the docks, which were on the way to work; windborne spread from infected animals on nearby farms; windborne spread from goods passing through the docks; animals or animal-based feed; contaminated hay, straw, or farm vehicles; sources in the factory premises; wooden delivery pallets contaminated with chicken carcasses returned to the factory; infection passed by red mites biting infected seagulls nesting on the roof, which then may have bitten staff in the factory; airborne spread from a cat that had given birth near the factory 2 years previously; airborne spread of contaminated dust generated by the renovation work; dust previously contaminated by an infected animal, bird, rodent, or bat; or contaminated straw or straw board aerosolized during drilling or removal.

We obtained data from a variety of sources, including a questionnaire survey, laboratories, clinicians, and factory management. A list containing details of the workforce and possible, past, and confirmed cases was developed and used to construct an epidemic curve. Data on place of work provided by factory management were used to calculate attack rates. Details were also collected on persons who had been on site for a limited number of days to help pinpoint the onset of the outbreak. Employees working on the factory floor were examined to determine whether a pattern occurred in the infected patients by calculating the relative risks for employees at each machine on the factory floor.

Two clinics were held at the factory on September 23 and 30, 2002, where blood samples were obtained from and questionnaires were completed by employees and subcontractors who had worked at the factory at any time from July 15, 2002, through September 30, 2002. Data from the questionnaires were analyzed by using a nested case-control design, where cases were defined as confirmed cases and controls were defined as noncases. The questionnaire explored risk factors in three categories: possible exposure in the community, in the docks or on the route to work, and at work.

Case definitions were applied to employees and subcontractors who had worked at the factory at any time from July 15, 2002, through September 30, 2002. A confirmed case was defined as phase 2 immunoglobulin (Ig) M $\geq$320 or fourfold rise in complement fixation tests (CFT) titer or IgM 20–160 + phase 2 IgG $\geq$320. A past exposure was defined as phase 2 IgG but no phase 2 IgM. A noncase (control) was defined as a CFT of $\leq$8 + negative phase 2 IgM and IgG + either no symptoms or onset of symptoms >7 days before blood sample or (in which the onset of symptoms was within 7 days of first sample) two consecutive blood samples with a CFT of $\leq$8 + one negative phase 2 IgM and IgG. A possible case was defined as all remaining cases.

Data were analyzed with EpiInfo (v. 6.04, Centers for Disease Control and Prevention, Atlanta, GA), Excel 97 (Microsoft, Redmond, WA), and Stata (v. 7, Stata Corporation, College Station, TX) software. Possible cases in patients and persons with evidence of past exposure were excluded from the nested case-control study. The analysis also excluded responses of "not sure" from odds ratio (OR) calculations. We calculated Mantel-Haenszel OR with exact 95% confidence limits (CI) (13).

Microbiologic Investigation

Complement fixation tests for phase 1 and phase 2 antibodies were performed at the Public Health Laboratory Service, Cardiff. IgM and IgG immunofluorescent assays were carried out on the samples by the Centre for Applied Microbiological Research (CAMR), Porton Down, UK. Laboratory staff monitored all requests for Q fever serologic testing from general practitioners and hospital clinicians to identify any additional cases that might be linked with the outbreak.

Environmental Investigation

Environmental information on the factory was gathered by environmental health officers and other members of the outbreak control team during site visits on September 23, 2002, and September 30, 2002. Management representatives of several other premises in or near the docks were interviewed.

On October 11, 2002, an environmental scientist collected 17 random environmental samples of straw and dust from inside and outside the factory premises. The samples were sent for polymerase chain reaction (PCR) testing at CAMR.

Results

Epidemiologic Investigation

A total of 222 employees and 60 subcontractors were working in the factory complex from July 15 through September 30, 2002. Questionnaires were completed by 214 (75.9%) of these 282 persons. Of the 253 persons who

were tested, we identified 95 (37.5%) confirmed cases of Q fever, 42 possible cases, 8 cases of past exposure, and 108 noncases. Four persons refused blood tests but completed a questionnaire. Data for the nested case-control analysis were available on 75 (78.9%) of the 95 confirmed cases and 101 (93.5%) of the 108 noncases. The frequency and duration of symptoms are shown in Table 1 and Figure 1. Ten participants were still ill when questioned, and 5 did not provide a date of onset of symptoms. Five patients (5.3%) were admitted to the hospital with pneumonia. Some patients experienced fatigue. However, the clinical impression of one of the authors involved in follow up of patients was that very few neurologic symptoms occurred during this outbreak, compared to a previously reported U.K. outbreak (14). Further analysis of clinical symptoms is being prepared as a separate paper.

The epidemic curve for 49 confirmed cases where the date of onset of symptoms was reliably known is shown in Figure 2. A peak incidence occurs around September 1, 2002. Based on an incubation period of 5 to 40 days (1,5), these data suggest that almost all the cases can be accounted for by an exposure from August 7 to 11, 2002.

Seven confirmed patients were only present in the factory on 2 or 3 days. All these persons were present in the factory and potentially exposed to infection from August 5 through August 9, 2002.

An analysis of home postal codes of 71 participants with Q fever who completed the questionnaire showed no discernible pattern and indicates that our participants were not part of a larger Q fever outbreak with a common source in the community. Details of place of work within the factory complex were available for participants with 61 confirmed cases and 81 controls. No cases occurred among persons working exclusively outside the factory floor or office block. In addition, no cases were identified among seven participants working in a separate design office, one employee working exclusively in the dispatch building, or five sales representatives who only called into the office on an occasional basis (Table 2). The OR for having a case in office staff compared with other staff was 3.46 (95% CI 1.38–9.06). ORs for groups of staff working in all other areas were <1 (Table 2).

The relative risks of having a case of Q fever among the cohort of employees working at different machines on the factory floor are shown in Figure 3. The balcony in Figure 3 is not drawn to scale. It overhangs the adjacent machines where the relative risk to workers was zero. The relative risk for infection was greatest among people who worked in the center of the factory floor outside the shadow of the overhanging balcony; the risk for infection dropped towards the sides of the building.

Eighty-three percent of confirmed cases were in men, a similar male-to-female ratio to that of the cohort as a whole, and median age was 44 years (range 22–60 years). Questionnaire data indicated that infected employees did not own animals that had given birth or had a miscarriage nor had these employees had any contact with the birth products of animals. One subcontractor, who cleaned windows at the factory, also worked on a farm and had been in contact with animals that had given birth, but the evidence did not suggest that any of these had been infected with *C. burnetii*. Additionally, the serologic tests for this employee were negative for Q fever, and the dates on which he visited the factory suggest that his clothing or possessions could not have been the source of the outbreak.

Table 1. Frequency of symptoms in 55 symptomatic patients with confirmed cases of Q fever, Newport, Wales, August–September 2002

Symptom	Yes (%)	Not sure
Fever	41 (75)	1
Sweats	53 (96)	0
Headache	51 (93)	1
Weight loss	26 (47)	2
Cough	24 (44)	0
Shortness of breath	25 (45)	2
Joint pain	44 (80)	3
Chest pain	20 (36)	5
Jaundice	4 (7)[a]	5

[a]These responses represent a misunderstanding of the term jaundice, since none of these persons had clinical jaundice.

Case-patients were much more likely than controls (OR 5.86; 95% CI 0.55 to 291.88) to recall coming across a hay lorry entering or leaving the docks while on their way to or from work. Adjusting for cases in those whose office was refurbished reduced the OR in those who saw a hay lorry (OR 3.00; 95% CI 0.28–31.80). Employees whose offices had been refurbished were at greatest risk for infection (OR 2.60; 95% CI 0.77–9.57). Employees who described themselves as "never near an external door or window" were more likely to be infected than those who worked "near an external door or window on most days" (OR 1.98; 95%CI 0.72–5.56). Living on a farm appeared slightly protective (OR 0.35; 95% CI 0.01–4.53) as did the regular handling of compost (OR 0.14; 95% CI 0.00–1.03).

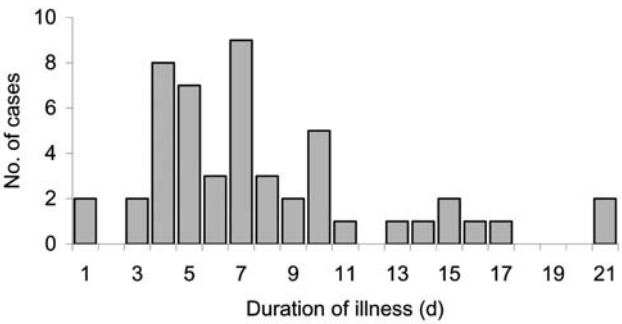

Figure 1. Duration of illness in symptomatic Q fever patients, Newport, Wales, August–September 2002.

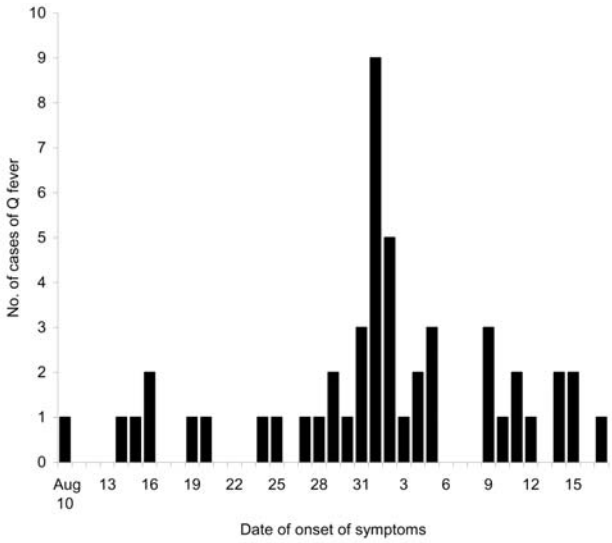

Figure 2. Epidemic curve for 49 confirmed cases in Q fever outbreak, Newport, Wales, August–September 2002.

However, none of these findings, or those in Table 3, reached statistical significance value of 5%.

The work undertaken by the seven participants with the shortest incubation times was examined for unusual characteristics. A higher proportion of those with a short incubation time were women (three of seven) when compared with the general population. Four of the seven participants worked in offices that had been refurbished, and the remaining three worked on the factory floor. Their duration of illness varied from 4 to 14 days.

Microbiologic Investigation

Two hundred and fifty-three participants (89.7%) provided blood samples. Some participants had only one sample taken and others had up to four additional samples taken from September through December 2002 at primary care or hospital clinics. A summary of CFT and IgM results is shown in Tables 4 and 5.

As a result of informing general practitioners in the area of the outbreak, more than twice the normal numbers of general practitioner requests for Q fever serologic testing were received. Hospital samples submitted for Q fever serologic testing were also monitored. Our monitoring identified one patient with a chronic case of Q fever and one patient with an acute, neither were associated with this outbreak. No *C. burnetii* was identified by PCR testing the straw board and dust samples that were obtained from the factory.

Environmental Investigation

The factory consists of several buildings. The main production area consists of a large, rectangular open-plan hanger with an elevated office block at one end of the rectangle (Figure 3). The office block was undergoing extensive renovation work at the time of the outbreak. This involved drilling >100 holes in the straw board ceiling to allow the attachment of a new suspended ceiling. Some internal walls made of straw board were also removed. A temporary corridor was created from plastic sheeting which ran through the area being renovated but did not form a complete seal. No respiratory protection was used by the contractors or the workforce at any stage. The corridor was in constant use by staff in adjacent offices. Office staff and factory floor workers who visited the offices consequently had some exposure to dust generated by the renovation work.

The layout of the factory is consistent with the possibility of disseminating contaminated dust from the renovated offices to the factory floor. The office block ran along the length of one end of the factory floor. Double-swing doors led from the second floor renovated offices onto an overhanging internal balcony 30 feet above the large open-plan factory floor (Figure 3). The factory production area had no windows and no air-conditioning system. A dust extraction system existed around some of the machines on the factory floor to collect waste cardboard. The lack of win-

Table 2. Attack rates and odds ratios (OR) for different areas of work at factory implicated in Q fever outbreak, Newport, Wales, August–September 2002

	No. of persons working in area			No. of persons working elsewhere			
Category	Cases	Controls	Attack rate (%)	Cases	Controls	Attack rate (%)	OR (95% CI)[a]
Production/factory floor	35	52	40.2	26	29	47.3	0.78 (0.36–1.57)
Dispatch	0	1	0	61	80	43.3	0 (0–71.79)
Dispatch/factory floor	4	4	50.0	57	77	42.5	0.68 (0.14–2.68)
Office	20	10	66.7	41	71	36.6	3.46 (1.38–9.06)
Production-based but sometimes in the office	1	2	33.3	60	79	43.2	0.66 (0.01–12.96)
Design	0	7	0	61	74	45.2	0 (0–0.88)
Sales representatives	0	5	0	61	76	44.5	0 (0–1.42)
Dispatch but sometimes in the office	1	0	100	60	81	42.6	Undefined
Total	61	81	43.0				

[a]CI, confidence interval.

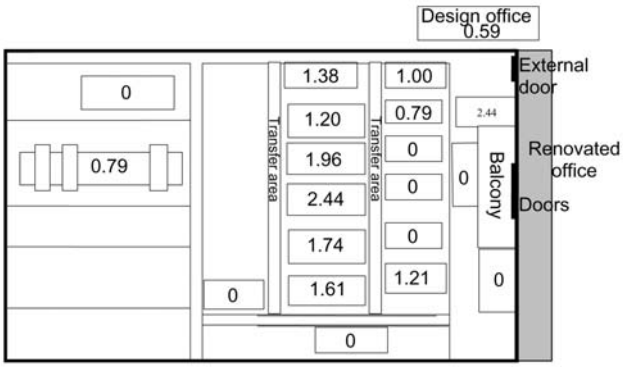

Figure 3. Relative risks for employees at various machines on the factory floor in Q fever outbreak, Newport, Wales, August–September 2002.

dows in the factory production area and the dust extraction system almost certainly caused a degree of negative pressure in the factory. This condition would draw air in through the double doors leading from the renovated office area and onto the factory floor.

Discussion

Environmental and epidemiologic evidence suggests that this outbreak was associated with the renovation of an office block within a cardboard manufacturing plant. One potential source identified was straw board in walls and ceilings disturbed by the renovation work. If straw board had been contaminated at some time in the past with a concentrated source of *C. burnetii*, drilling into this could have produced a cloud of dust containing large numbers of *C. burnetii* sporelike forms. Dust containing *C. burnetii* sporelike forms could have been sucked through the balcony doors from the renovated offices, fallen onto the workforce below, and inhaled by those infected. Workers could also have been infected when visiting the personnel or accounts offices situated adjacent to the renovation work.

No record of visits to these departments exists, which would allow this hypothesis to be further assessed. However, the hypothesis is supported by a number of factors. The pattern of relative risk for infection in groups of participants at different machines on the factory floor is consistent with this hypothesis. The highest relative risks are in the center of the factory close to the balcony, while the lowest risks are in the areas at the sides and far end of the factory floor. The overhanging balcony may have sheltered employees at some of the machines from any contaminated dust falling from above. Raised ORs for infection in employees who were decanted into neighboring offices because their offices were being renovated, and in office staff whose offices had been refurbished, also implicate the renovation work as the source of the outbreak.

The timing of the installation of the new suspended ceiling (July 17–August 9, 2002) is consistent with an outbreak source near August 5 through August 9. The raised OR in persons rarely near an open window or door compared with those often near an open window or door and the lack of cases among those who worked in the separate design office, or among sales representatives, suggest that the source of the outbreak was inside the factory.

The respirable dust fraction that is most pathogenic is generally invisible to the naked eye (15,16). We do not have a good proxy for exposure in this outbreak, and consequently the issue of a dose response has not been addressed. Exact place of work probably did not closely correlate with exposure as many staff members move around the building as part of their work.

Potential Contamination of the Straw Board

Straw board could have been contaminated either before or after manufacture. Investigating the process used to make the straw board indicated that the low pressures and temperatures involved would not kill any fungal spores present in the straw. If straw board becomes wet, these fungal spores often sprout and damage the board. The straw used to produce the board was stored in large Dutch barns and would have been accessible to rodents, cats, and other animals. Some evidence exists that a number of cases of Q fever were occurring around 1950 in the English county where the straw board was manufactured (17) and that the straw board was probably manufactured from 1950 to 1953. *C. burnetii* sporelike forms are

Table 3. Odds ratios (OR) for different risk factors in Q fever outbreak, Newport, Wales, August–September 2002

	No. of persons exposed to risk factor		No. of persons not exposed to risk factor		
Exposure at work	Cases	Controls	Cases	Controls	OR (95% CI)
Office refurbished	24	23	6	15	2.61 (0.77–9.57)
Never near an external door or window/near a window or door most days	13	10	40	61	1.98 (0.72–5.56)
Smoker/never smoked	15	35	42	48	0.49 (0.22–1.08)
Saw hay lorry on the docks	4	1	56	82	5.86 (0.55–291.88)
Live on a farm	1	3	72	76	0.35 (0.01–4.53)
Regularly handle compost	1	9	68	83	0.14 (0.00–1.03)
Contact with animal births or miscarriages	0	6	39	54	0.00 (0–1.26)

[a]CI, confidence interval.

Table 4. Summary of highest phase 2 CFT results recorded for each person in the cohort in Q fever outbreak, Newport, Wales, August–September 2002[a]

Highest CFT	AQF cases	Noncases	Past exposure	Possible cases
<8	4	104	5	35
8	4	2	3	2
16	12	1	0	2
32	14	1	0	2
64	16	0	0	1
128	21	0	0	0
256	17	0	0	0
512	5	0	0	0
1,024	2	0	0	0
Totals (253)	95	108	8	42

[a]CFT, complement fixation test; AQF, acute Q fever.

resilient. They can withstand pressures of up to 20,000 lb/in^2, elevated temperatures, desiccation, osmotic shock, UV light, and chemical disinfectants (18). However, experimental studies of the survival of *C. burnetii* sporelike forms have not demonstrated survival beyond 8 years (Table 6) (5,6). Whether experiments for longer durations were undertaken is not clear from the source documents. Although not directly comparable, *Bacillus anthracis* and *Clostridium tetani* spores are known to survive for many years. For example, *B. anthracis* spores have been recorded as surviving for 71 years on dried silk threads (19).

Alternatively, the straw board could have been contaminated after manufacture by the feces, urine, birth products, or a corpse of an infected rodent that gained access to the inner layer of a straw board. Some holes were drilled in the straw board ceiling in 1982 and 1983, which could have provided a point of entry. Rodents are considered an important potential source of *C. burnetii*, and in one U.K. serosurvey, 34% of wild brown rats (*Rattus norvegicus*) had antibodies suggesting previous exposure to *C. burnetii* (20). The placentas of common rodents can also contain large numbers of *C. burnetii* sporelike forms (21) and could contaminate straw.

Test results of environmental samples in this outbreak were, however, negative. This finding could have occurred for a number of potential reasons. The samples were collected by persons who did not have detailed knowledge of the outbreak investigation, and the samples tested were minute in comparison to the quantity of straw disrupted during the renovation work. Concentration of potential bacterial contaminants was attempted in the PCR tests, but analysis was performed on small aliquots of extract, and bacterial DNA could therefore easily have been missed. The PCR test used was also experimental, although the protocol followed was similar to that used in Australia, France, and Germany. A delay of 2 months occurred between the dates when employees were probably exposed to *C. burnetii* and when environmental dust samples were collected. Consequently, contaminated dust may have been dispersed or cleaned up in the interim. In previous outbreak investigations, test results of environmental air or straw samples for *C. burnetii* have also more often been negative (15,22–23) than positive (4,24). The environmental sampling was, therefore, like looking for a "needle in a haystack."

Other Hypotheses

We considered a range of alternative hypotheses but did not find any evidence to support them. For example, wind speeds were recorded routinely by the harbor authority but were very low during the week of August 5 through August 9, 2002, which makes windborne spread from the nearest farmland, 1 1/2 to 3 miles away, unlikely. No other potential wild or domestic animal sources were identified. Animals or animal products had not been moved through the docks in recent years. A feral cat had given birth in an adjacent building 1–2 years previously. One of the kittens had been adopted by an employee. However, the employee's serologic testing for Q fever was negative. If the feral cat had been infected with Q fever, the employee would most likely have had evidence of past exposure to *C. burnetii*. In addition, the factory strongly emphasizes controlling vermin as some of their cardboard packaging is used as secondary packaging in the food industry. No cats or other animals had been identified in or around the building for several years preceding the outbreak.

Table 5. Summary of highest phase 2 IgM results recorded for 107 persons in the cohort[a]

IgM P2 values	AQF cases	Past exposure	Uncertain status
0	1	8	
Low levels	0	0	1
80	0	0	2
<60	0	0	0
160	5	0	2
320	3	0	0
640	16	0	0
1,280	4	0	0
>1,280	65	0	0
Total	94	8	5

[a]Ig, immunoglobulin; AQF, acute Q fever.

Table 6. Survival of *Coxiella burnetii*[a]

Environment	Temperature (°C)	Survival
Wool	15–20	7–9 mo
Wool	4–6	Approx. 12 mo
Sand	15–20	4 mo
Fresh meat	Cold storage	>1 mo
Salt meat	Not recorded	5 mo
Skimmed milk	Not recorded	40 mo
Tap water	Not recorded	30 mo
Tick feces	Room	Conclusive evidence: 586 d Some evidence: 6 and 8 y
Not recorded	–20	2 y
Not recorded	–65	8 y

[a]References 5 and 6.

Contaminated fomites can produce secondary aerosols of *C. burnetii* sporelike forms (4), and several outbreaks have demonstrated the possibility of spread on fomites such as clothing, straw, hay, contaminated shoes, and building materials (22,25–36). However, unless a mechanism exists to repeatedly reaerosolize the source, fomites are likely to pose a risk even when they are not heavily contaminated, and this view is supported by the general principles that govern the dispersion and settling out of dust particles or sporelike forms (17,37).

Neither straw nor building material is a common source of outbreaks of Q fever. However, straw has been suggested as a possible source in several outbreaks (15,27,38,39). Two case reports implicate straw: a physician who contracted Q fever after clearing out straw and rubble from his new moorland home (26) and a businessman who was cleaning out a barn that had been used for keeping livestock 10 years previously but had not been properly cleaned since (24). Moldy hay from this barn, cultured using cell growth medium, grew *C. burnetii*. The renovation of buildings has also been suggested as a source of Q fever in two previous outbreaks (26,39). The widespread dispersal of spores in a building has been demonstrated both by Q fever (disseminated through a large medical school building) (23) and by anthrax (dispersed through a post office with an area of 281,387 ft^2 and a volume of approximately 7 million ft^3) (40).

One other alternate hypothesis is that the source of the outbreak was outside the factory building. Five persons mentioned having seen a hay lorry in the docks. This hypothesis was pursued because straw from farm vehicles had been implicated as a potential cause in a previous local outbreak of Q fever (2). However, the route taken by the lorries was never closer than half a mile to the factory. The lorries passed much closer to several other factories and to residential areas where several thousand persons would have had much greater exposure than the workforce at the factory. Although two Q fever cases were identified in the neighboring factory, no evidence existed of a wider outbreak involving other premises in the docks or nearby residential areas. The hypothesis that hay lorries passing through the docks could have caused the outbreak was known to a number of employees before they completed the questionnaire, and this finding may therefore be a result of diagnostic suspicion bias (41).

Control Measures

Risk assessment and risk management was undertaken by identifying groups of persons at different levels of risk and providing relevant advice, temporarily stopping work in the area of the building considered at greatest risk, and following identified patients with Q fever. The cardboard manufactured by the factory was produced at temperatures that made survival of *C. burnetii* sporelike forms impossible so customers were not considered to be at increased risk. Unlike the straw board, which was produced a very low temperatures, the cardboard is produced at temperatures that would make survival of *C. burnetii* sporelike forms impossible. In addition, the cardboard was only used for secondary packaging and was therefore not in direct contact with any food products.

Implications of the Study

Inhaled organic particles are an important source of a number of occupational diseases (17,42), and risks from exposure to occupational dust have been addressed by the U.K. Health and Safety Executive (43,44). Q fever is also a recognized occupational disease in the United Kingdom (45) and governed by existing legislation (46), although it is not a notifiable disease (47).

Straw is an increasingly popular ecologically friendly material, and >350,000 houses have been built in the United Kingdom with this particular type for straw board as internal partitions. The product has also been exported around the world. However, this outbreak is the first where straw board was suggested as a possible source of Q fever. Further research is needed to fully investigate straw board in various venues as a potential vehicle in Q fever outbreaks. Contaminated straw board represents a potential source of Q fever and should be considered in future outbreak investigations.

Acknowledgments

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Dr. van Woerden works at the National Public Health Service in Wales. His research interests include health protection and environmental epidemiology.

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SHORT REPORT
Using facsimile cascade to assist case searching during a Q fever outbreak

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SUMMARY

In September 2002, facsimiles were sent to 360 primary-care physicians alerting them to a local outbreak of Q fever. The physicians subsequently submitted serology samples on significantly more patients than in a previously comparable period in 2001. Facsimile cascade assists effective communication with primary-care physicians in an outbreak investigation.

Electronic communication is increasingly being used in the investigation and management of disease outbreaks: to alert clinicians or intensify surveillance systems [1, 2]; to collect data on cases and exposed persons [3, 4]; and to provide information for those at risk [5–8]. A range of methods has been used including facsimile, email [4], the internet [3], mobile phones and video links [9–12]. However, few of these methods have been evaluated. We reviewed specimen submission rates in order to evaluate the use of a facsimile cascade to improve the identification of cases in an outbreak investigation.

In mid-September 2002, we identified several cases of Q fever among employees of a cardboard manufacturing plant in the city of Newport, Gwent, UK [13]. Home addresses of the cases were scattered over a wide area. As part of the outbreak investigation, we decided to instigate case searching for Q fever in patients presenting to local primary-care physicians in order to exclude the possibility that a larger outbreak was occurring in the community. We used a well-established facsimile cascade system, operated on behalf of the public health department by a national telephone service provider, to send a facsimile to all primary-care practices in the area. Two facsimiles were sent to all 106 primary-care practices (representing 360 primary-care physicians) in the Gwent locality, covering an area of 600 square miles and a population of over 560 000 people on 17 and 20 September 2002. Physicians were asked to submit serum samples on any patient meeting a clinical case definition of Q fever and an association with the area where the outbreak appeared to be occurring.

To assess whether primary-care physicians had responded to these facsimiles a centralized computer database holding all laboratory records for the area was interrogated. We identified all complement fixation (CF) tests for *Coxiella burnetii* requested by primary-care physicians between 1 September 2001 and 31 October 2001 and between 1 September 2002 and 31 October 2002. Patients' dates of birth, but not their names, were used as personal identifiers. Where more than one sample had been submitted on a patient, only the first sample submitted was used in our analysis. The number of individuals tested in each week was plotted using Microsoft Excel. We compared the number of patients tested by primary-care physicians on the corresponding weeks of September and October 2001 and 2002. The paired Wilcoxon signed rank test was used to assess statistical

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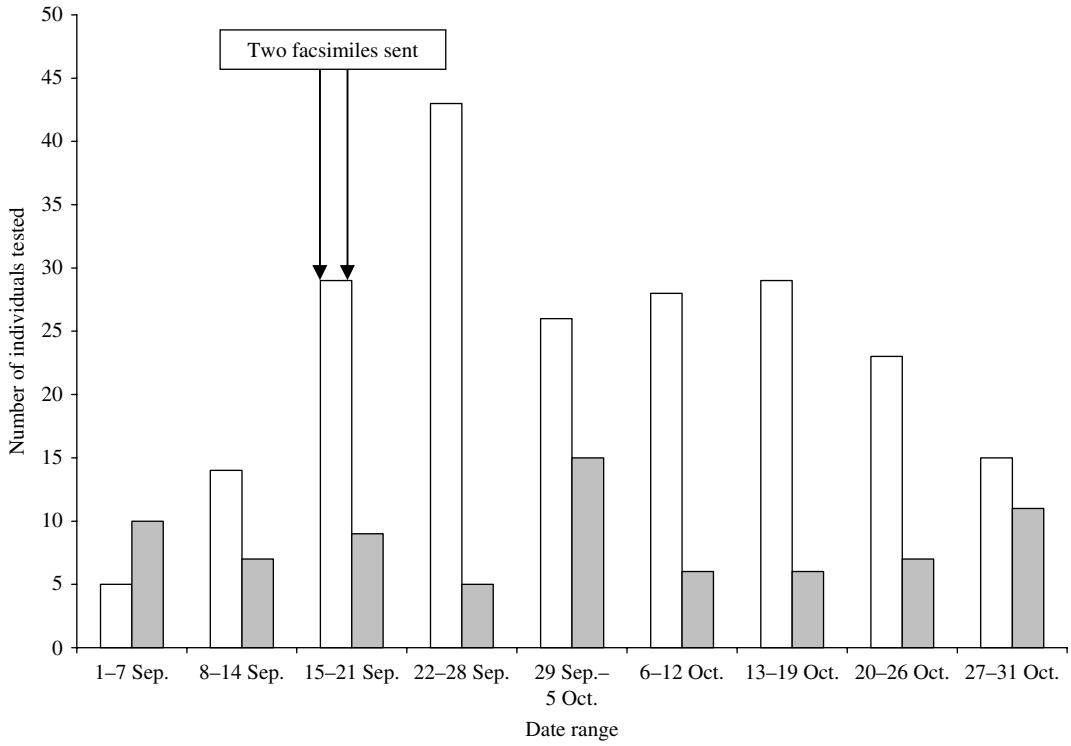

Fig. Number of patients with Q fever antibody tests, submitted by primary-care physicians in Gwent in 2001 and 2002 and analysed in South Wales laboratories. ▩, 2001 patients; □, 2002 patients.

significance. The null hypothesis tested was that there was no statistically significant difference between the number of patients tested for Q fever in the target population in each week between September and October 2001 compared to the corresponding week in September and October 2002. A second analysis comparing the number of patients tested on each corresponding day of the two time-frames was also undertaken to ensure that summarizing the data by week did not affect the results.

The locality's population did not change significantly between 2001 (563 542) and 2002 (567 315). Direct comparisons could therefore be made between the numbers of patients tested in these two years. Primary-care physicians submitted CF tests for Q fever on 69 individuals between 1 September 2001 and 31 October 2001 compared with 212 individuals between 1 September 2002 and 31 October 2002 (see Fig.). The graph demonstrates a bulge in the number of patients tested in 2002 compared to a relatively constant number of patients tested in 2001. The difference precedes the facsimile transmission but is most apparent after it. The paired Wilcoxon signed rank test for the difference in the proportion of the population tested in 2001 and 2002 was $P<0{\cdot}001$ both when data were compared on a weekly and on a daily basis. Data on the geographical pattern of samples submitted was not available. Local laboratory staff indicated that they were not aware of a rise in samples coming in from other surrounding areas during the outbreak and that the rise in the number of samples received by the laboratory did not reflect a general rise in the number of CF tests for Q fever received between 2001 and 2002. The 212 CF tests submitted in 2002 included 185 samples with a titre of $\leqslant 8$, three samples with a titre of 16, eight samples with a titre of 32, four with a titre of 64, eight with a titre of 128, three with a titre of 256, and one with a titre of 512.

One previously unrecognized case of Q fever was identified as a result of samples submitted by primary-care physicians. However, further investigation indicated that this individual was not associated with the main outbreak but represented a sporadic case of Q fever and had been exposed to recognized risk factors for the disease elsewhere. We expected to identify more previously unrecognized cases than this as a consequence of our case searching. There are several reasons why the number of new cases identified was so small. First, this outbreak was particularly localized and the at-risk group was concurrently identified by a

cohort study of those working or attending a specified factory premises. Second, CF tests were used to screen the samples submitted rather than newer, more sensitive and specific immunofluorescent tests, which might have identified a larger number of cases in the general population.

The facsimiles sent to primary-care physicians appear to have contributed to a prompt and statistically significant increase in the number of requests for Q fever serology. Test results suggest that there was no wider outbreak of Q fever, since despite the large number of community samples submitted around the time of the outbreak, only one new case of Q fever was identified.

There are a number of weaknesses and potential biases in the study that need to be considered. Factors other than receiving a facsimile would have contributed to the increase in serology requests received in September 2002. For example, local press statements (although these did not occur until after the first facsimile was sent), local peer-group networks and contacts with hospital staff would have increased awareness of the outbreak. Increased awareness of the outbreak and subsequent increased self-presentation of patients concerned about the possibility of Q fever may also have been a factor. A few GPs were phoned with a request to take blood samples of specific patients prior to the sending out of the facsimiles. Use of date of birth to remove duplicates, undertaken to maintain patient confidentiality, may have removed a few individuals who coincidentally had the same date of birth.

Anecdotal evidence from the laboratory suggests that although local GPs occasionally ask for an atypical pneumonia screen, including Q fever serology, they very rarely name Q fever serology directly on the request form. In contrast, in September 2002 a large number of the primary-care samples shown in the Figure directly requested Q fever serology. This provides indirect evidence to suggest that the facsimiles were linked to the requests for Q fever serology.

Doctors receive large quantities of correspondence [14] and vary in the quantity they read [15, 16]. We were not certain that our facsimiles would be read or acted upon. However, this study suggests that at least some primary-care physicians read and acted upon the facsimile cascade sent out as part of this outbreak investigation. It is not clear what proportion of the 360 primary-care physicians saw patients who met the case definition for Q fever but were not tested. A survey of the physicians might have produced additional useful information.

A number of previous evaluations of facsimile were identified by searching Medline 1966 to week 47, 2003 and EMBASE 1980 to week 47, 2003 for facsimile.mp limited to 'human', English language articles. In most of the 151 references identified the use of facsimile was incidental to the focus of the paper and the outcomes measured did not relate to the use of facsimile. Eight evaluations of facsimile transmission were identified of which seven suggested some benefit. One was in a communicable disease context [2]. Three papers evaluated 'one to one' transmission of clinical information by facsimile [17–19]. Two papers evaluated 'many to one' transmission by facsimile from patients or peripheral health-care workers back to a central hub [20, 21]. Three papers evaluated 'one to many' transmission (facsimile cascade) from a centre to peripheral sites [2, 22, 23]. One study provided limited evidence for the use of facsimile cascade to disseminate information and intensify surveillance during a period when there was increased risk of a disease outbreak. In 1994 a facsimile cascade was used to disseminate information to a wide range of public health officials by the Centers for Disease Control (CDC; Atlanta, GA, USA) in response to a reported epidemic of plague in India [2]. The CDC Fax Information Service subsequently sent out a further 5589 documents providing information about plague using an automated fax back system to handle requests for further information. This resulted in the identification of 13 travellers who were potentially at risk of incubating plague although none of these cases proved positive. A high percentage of primary-care physicians have access to a facsimile machine and there is some evidence that they have a preference for this method of communication [24].

In conclusion, this study provides some evidence that sending a facsimile cascade to primary-care physicians, may assist in the identification of cases during an outbreak investigation and may provide the basis for the design of future studies investigating the usefulness of facsimile for communicating with primary-care physicians.

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DECLARATION OF INTEREST

None.

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A nested case control study demonstrating increased chronic fatigue six years after a Q fever outbreak

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Abstract

The frequency and range of long-term sequelae following acute Q fever infection remains controversial. This study aimed to assess a range of potential psychological and physical sequelae in a follow up study of a large outbreak of Q fever that occurred in Newport, Wales, in 2002. A nested case control study was undertaken six years after a point source outbreak of Q fever. We invited a cohort of 211 factory workers exposed to a point source of Q fever in 2002 to attend a follow up clinic in 2008. Cases, defined as those who had clinical symptoms and serological evidence of acute Q fever in 2002, were compared to controls, who worked in the same factory but were serologically negative at the time of the outbreak. At the follow up clinic blood was taken for *Coxiella burnetii* microimmunofluorescence and questionnaires were completed including the PHQ-9, Chalder Fatigue scale, and General Health Questionnaire. Results were obtained for 32 cases and 13 controls. Chalder Fatigue scores were significantly raised in the cases (independent samples t-test: P=0.047). PHQ-9 and GHQ scores were not significantly raised in cases. However, post hoc cross sectional analysis indicated a relationship between Phase 2 IgG at follow up in 2008 and Chalder Fatigue scores (P=0.004) and PHQ-9 scores (0.049). A longitudinal association was demonstrated between acute Q fever infection and chronic fatigue six years later. In cross-sectional analysis a previously unreported relationship between depression scores (PHQ-9) and positive Q fever serology was also identified.

Introduction

A number of follow up studies of patients with acute Q fever[1-8] and serological examination of patients presenting with Chronic Fatigue Syndrome,[9] have indicated that Q fever may be associated with chronic fatigue. A relationship between previous Q fever and symptoms of depression can also be postulated.[10] The underlying aetiology of the association between Q fever and chronic fatigue remains unclear. The extent to which chronic symptoms are influenced by the strain of *C. burnetii*, psychosocial factors, immunological or genetic factors is also uncertain.[11,12] The possibility of long term effects following acute infection is supported by evidence that the aetiological organism can be detected in a proportion of patients by PCR many years later.[13-15] It is still unclear whether patients that have long standing chronic fatigue after acute Q fever gain any benefit from antibiotic treatment.[16,17]

A point source outbreak of Q fever occurred in a cardboard manufacturing plant in Newport, South Wales, in 2002.[18] This outbreak was one of the largest to have occurred in the UK. During the initial outbreak, the exposed cohort was identified and a nested case control study was undertaken to assess the difference in exposure between cases and controls to a range of potential sources of the outbreak. The cohort of factory workers, including affected and unaffected individuals provided the opportunity for a follow up study, which was conducted six years later in 2008. The serological findings of the follow up study have been published.[19] The current paper compares the symptoms of the original cases and controls in a follow up study. The aim of the study was to assess the hypotheses that i) chronic fatigue, ii) depression and iii) reduced physical function were more common in those who had acute Q fever in 2002 compared to those individuals in the cohort who worked in the same factory but did not demonstrate evidence of infection at the time of the outbreak.

Materials and Methods

From the initial cohort of 226 individuals who worked in the factory in 2002, all 131 serologically positive cases were invited to take part in the follow up study in 2008, as were a random sample of 80 of the 95 serologically negative individuals. A written invitation to participate, including a leaflet outlining the proposed study, was therefore sent to 211 potential participants.

Those individuals who agreed to take part in the study were invited to a clinic where blood was taken for microimmunofluorescence testing for presence of *Coxiella burnetii* Phase 2 IgG and IgM antibodies and Phase 1 IgG and IgA antibodies. The microimmunofluorescence assays used antigen from Patient strain *Lane* - ST12 group. The blood samples were analysed by the standard reference laboratory in the UK, the Special Pathogens Reference Unit, HPA Centre for Emergency Preparedness and Response, Porton Down, Salisbury, SP4 0JG. Questionnaires including the PHQ-9[20] which assesses the severity of symptoms of depression, the 11 item version of the Chalder Fatigue scale,[21] and the General Health Questionnaire (GHQ) which assesses physical health[22] were completed at the clinic. The questionnaire included two new single item tests for fatigue. Firstly, *If you are tired at the moment how long has this lasted?* Response options were *not reported being tired*, *less than one week*, *one week to 3 months*, *3 to 6 months*, *6 months or more*. For analysis the results were dichotomised into those who had fatigue for *six months or more* and those who had fatigue for *less than six months*. Secondly, the questionnaire asked, *What percent of the time do you feel tired?* Response options were 0%, 25%, 50%, 75%, or 100% of the time. Responses were dichotomised into those who reported being tired *less than 50% of the time* and *50% of the time or more*.

The PHQ-9 is a self-administered subset of the PRIME-MD diagnostic instrument for common mental disorders. It is a widely used and well validated measure of depression based on nine questions that start with the stem, *Over the last 2 weeks, how often have you been bothered by any of the following problems?* Answers range from *0* (not at all) to *3* (nearly every day). The Chalder Fatigue scale used in this analysis contains 11 questions covering the physical and mental symptoms of fatigue including, tiredness, need to rest, feeling drowsy, problems starting things, and difficulty concentrating. The General Health Questionnaire is a broad ranging and widely used measure of physical health. The two other questions

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Key words: Q fever, Coxiella burnettii, chronic fatigue syndrome, chalder fatigue scale, PHQ-9

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on fatigue duration and percent of time affected by fatigue were not previously validated questions.

Questionnaire data was analysed using Microsoft Excel® and SPSS®. In view of the relatively small sample sizes, the non-parametric Mann-Whitney U test for two independent samples was used to compare variables. Variables were also dichotomised and compared using the chi-square test. Fishers' exact test was used where where one cell had an expected count of less than five.

Ethical approval for the study was granted by the South East Wales Research Ethics Committee Panel C.

Longitudinal and cross sectional analyses

The data was analysed as a nested case control study, based on the status of participants in 2002 as cases or controls. In 2002 cases were defined as those members of the exposed cohort who had clinical symptoms of acute Q fever infection and serological evidence of infection as demonstrated by a Phase 2 IgM ≥80, or a fourfold rise on sequential Complement Fixation Tests (CFT). Non-cases were defined as those who had no symptoms of acute Q fever infection and no serological evidence of infection with no IgM, no CFT and no Phase 1 or 2 IgG. Three patients who did not unambiguously meet criteria as cases or controls were excluded from analysis. They had *uncertain serology* defined as no IgM, no fourfold rise in CFT but CFT ≥ 8 or Phase 1 or 2 IgG > 160. Two of the three individuals with *uncertain serology* had symptoms; the third individual did not. These three individuals were excluded from all the analysis presented in this paper.

A post hoc cross sectional analysis of the cohort was also undertaken, assessing the relationship between current clinical symptoms at the time of the clinic in 2008 and Phase 2 IgG blood results from samples taken at the same time. Phase 2 IgG titres were recorded as 0, 320, 640, 1280, or 2560. Scores for clinical symptoms were dichotomised as follows: Chalder Fatigue (0-3, 4-11); PHQ-9 scores (dichotomised at the median: 0-3, 4-9); duration of tiredness (*less than 6 months*, *6 months or more*); percent of time participants felt tired (*less than 50% of the time*, *50% of the time of more*); and GHQ scores (dichotomised at the median: 0-2, 3-12).

Results

Results for analysis were available for 45 individuals out of a total of 52 individuals who attended the follow up clinic. This represented 24.4% of cases invited to the clinic (32/131) and 16.3% of the controls invited to the follow up clinic (13/80). The characteristics of the cohort used for the nested case control study are shown in Table 1. Both groups were predominantly male, reflecting the population in the factory where the outbreak occurred. The mean age of cases was 50.18 yrs (SD 9.85, range 27-64 yrs) and the mean age of controls was 53.57 yrs (SD 8.86, range 36-68 yrs). Educational attainment was similar in both groups: 37.5% (12/32) of cases and 38.5% (5/13) of controls had remained in education after the minimum school leaving age.

Nested case control study

A table showing the relationship between status in 2002 and symptoms in 2008 is provided in Table 2. Raised Chalder Fatigue scores were more common in cases (P=0.047). The single questions analysing symptoms of *tiredness for 6 months or more* or *tiredness for 50% of the time or more* were not statistically significantly associated with being a case. Neither were PHQ-9 scores (P=0.189) or GHQ scores (P=0.743).

Cross sectional analysis

A second post hoc analysis of the cohort was

Table 1. Summary of cases and controls (bases on status in 2002) by gender.

	Status		Total
	Case	Control	
Gender			
Female	7	1	8
Male	28	13	44
Total	35	14	52

Table 2. Relationship between status as defined in 2002 (cases and controls) and clinical symptoms in 2008.

Comparison	N	Cases		Controls		Fisher's Exact test P
Chalder fatigue score		0-3	4-11	0-3	4-11	
	45	13	19	10	3	0.047
Tired for 6 months or more		< 6 months	≥ 6 months	< 6 months	≥ 6 months	
	39	11	17	8	3	0.082
Tired for 50% of the time or more		0-50%	50-100%	0-50%	50-100%	
	40	7	21	6	6	0.154
PHQ-9		1-3	4-9	1-3	4-9	
	45	14	18	9	4	0.189
GHQ		0-2	3-12	0-2	3-12	
	49	22	13	10	4	0.743

Table 3. Relationship between Phase 2 IgG in 2008 and symptoms measured at the same time.

	Comparison	N	Mean rank	Sum of Ranks	Mann-whitney U
Phase 2 IgG	Chalder Fatigue score (0-3)	23	17.91	412.00	136.000
	Chalder Fatigue score (4-11)	22	28.32	623.00	P = 0.004
	Total	45			
Phase 2 IgG	Tired for 6 months or more	19	16.45	312.50	122.500
	Not tired for 6 months or more	20	23.38	467.50	P = 0.037
	Total	39			
Phase 2 IgG	Tired less than 50% of the time	13	14.69	191.00	100.000
	Tired 50% of the time or more	27	23.30	629.00	P = 0.017
	Total	40			
Phase 2 IgG	PHQ-9 score (0-3)	23	19.52	449.00	173.000
	PHP-9 score (4 – 9)	22	26.64	586.00	P = 0.049
	Total	45			
Phase 2 IgG	GHQ score (0-2)	32	23.86	763.50	235.500
	GHQ score (3-12)	17	27.15	461.50	P = 0.406
		49			

undertaken to assess whether symptoms recorded at the follow up clinic in 2008 were associated with Phase 2 IgG results taken at the same time (Table 3). There was a clear relationship between higher *C. burnetii* Phase 2 IgG and raised Chalder Fatigue score (P= 0.004), *tiredness for 6 months or more* (P= 0.037), *tiredness for 50% of the time or more* (P=0.012), and raised PHQ-9 score (0.049), but not GHQ score (P=0.406).

Similarly, dichotomising *C. burnetii* Phase 2 IgG scores to 0-640 and 1280-2560 and assessing the relationship with symptoms using the chi-square test indicated a clear relationship to clinical symptoms of fatigue: Chalder Fatigue score (chi-square 8.049; P=0.005), *tiredness for 6 months or more* (chi-square 4.311; P=0.038), *tiredness for 50% of the time or more* (chi-square 6.686; P=0.01). Relationships with PHQ-9 score (chi-square 2.702; P=0.1), and GHQ score (chi-square 0.634; P=0.426) were not statistically significant. Four of the fourteen control subjects (based on their status in 2002) had *C. burnetii* Phase 2 IgG titres of 320 for Q fever at follow up in 2008.

Discussion

Chalder Fatigue scores were significantly raised six years later in those individuals who were diagnosed with acute Q fever at the time of the outbreak. In contrast, general health, as measured by the GHQ, was not significantly different from controls drawn from the same cohort of individuals who worked in the same factory at the time of the outbreak. There is a statistically significant cross-sectional relationship (P=0.049) at the time of follow up between raised serology by microimmunofluorescence and both Chalder Fatigue scores and depression as measured by the PHQ-9. Symptoms of fatigue and depression can overlap and can be difficult to differentiate. However, as these results were derived from the cohort of workers exposed to Q fever in the initial outbreak in 2002, there is some evidence to suggest that depression may be a associated with previous Q fever infection. This finding will need verified by future studies. An unexpectedly large proportion of the controls (28.6%) were serologically positive at follow up. This figure is much higher than the sero-prevalence of Q fever in the UK population.[23] The positive Q fever serology may represent late sero-conversion due to exposure at the time of the outbreak, later exposure to Q fever, or false positive results.

The main strength of this study was that it used validated questionnaires to assess symptoms associated with the long term sequelae of Q fever subsequent to a point source outbreak. The study was also strengthened by the fact that controls were drawn from the same environment as cases. However, some controls may indeed have been unidentified asymptomatic cases and a separate population of control subjects would have strengthened this study further. The potential inclusion of some cases in the control group would reduce the power of the study to detect an effect and should, therefore, not have introduced bias in favour of our findings.

The sample size in this study is small and the response rate is relatively low. Participants were aware whether or not they had been diagnosed as having acute Q fever in initial outbreak and this may have introduced some reporting bias when completing the follow up questionnaires. Potential problems in interpreting serological results and defining international standards for acute, past and chronic infection have been considered in a previous report.[19]

It would have been helpful to have information on other blood borne infections that could have contributed to chronic fatigue. However, we have no reason to suspect that the prevalence of such infections would be different in cases and controls. Consequently, this issue should not have materially affected our findings. It would have been interesting to assess whether there was a relationship between the nature of the work undertaken by each participant in the study and their level of fatigue. In particular, whether there was a relationship between the symptoms of chronic fatigue and the level of physical exertion undertaken on a day to day basis or the shift pattern worked by the individuals concerned. It would also have been useful to compare the frequency and duration of sick leave in cases and controls. Unfortunately, none of this information was collected. One patient from this cohort developed Q fever endocarditis and was treated with antibiotics. However, it is unclear whether or not chronic fatigue after Q fever infection should be treated with antibiotics. Raised Q fever serology is more common in patients with Chronic Fatigue Syndrome than in the background population.[9] Small studies have shown that some patients with chronic fatigue and raised Q fever serology respond to antibiotic treatment.[16,24] However, high antibody levels may indicate high responder status rather than the actual presence of the microorganism. This study points up the desirability of a trial of antibiotics in patients with post q fever chronic fatigue.

Conclusions

This study has demonstrated that chronic fatigue was more common six years later in patients who were infected with Q fever during an outbreak. The study has also raised the possibility of a relationship between raised *C. burnetii* Phase 2 IgG and symptoms of chronic fatigue and depression.

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MAJOR ARTICLE

Chronic Q Fever: Different Serological Results in 3 Countries—Results of a Follow-up Study 6 Years After a Point Source Outbreak

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Background. Acute and chronic Q fever/*Coxiella burnetii* infection is diagnosed principally by serology. The management of patients who have serological evidence of chronic Q fever but no other manifestation of chronic infection is challenging.

Methods. This paper describes a follow-up study of individuals 6 years after a point source outbreak. The study compares serological and polymerase chain reaction (PCR) results between 3 international reference laboratories in a well-defined cohort of Q fever patients.

Results. Concordance in microimmunofluorescence result interpretation from the 3 centers was only 35%. Australian and UK results had the greatest concordance and French and UK results the lowest. Serological testing revealed no chronic serological profiles when tested in either France or Australia but 10 when tested in the UK. Serological results from a patient with treated Q fever endocarditis suggested treated (France), chronic (UK), and borderline chronic (Australia) infection. PCR results on blood were universally negative.

Conclusions. This study has shown that the results from Q fever micro-immunofluorescence vary according to the center in which they are carried out. This has implications for the interpretation of such tests, raises questions regarding the validity of using serological criteria alone as a means of diagnosing chronic Q fever, and affects the interpretation of epidemiological studies. We recommend that all results are interpreted according to the clinical picture and particular caution is applied in the interpretation of chronic serological profiles. In order to further our understanding of Q fever infection we propose that an international standard of Q fever serological investigation be developed.

Q fever is an infection caused by an obligate intracellular bacterium *Coxiella burnetii*. It causes acute Q fever, the most frequent manifestations of which are a self-limiting febrile illness, hepatitis, or pneumonia. In some instances the infection is entirely asymptomatic.

The presentation of chronic Q fever is heterogeneous. The most common manifestation is Q fever endocarditis in patients with preexisting valvular heart disease [1].

Difficulties in diagnosing chronic Q fever infection clinically have led to the development of diagnostic serological criteria. These criteria are based on studies that mostly compare acute Q fever patients with those that have Q fever endocarditis. The most commonly quoted criteria [2], developed in France, are used on a worldwide basis to interpret Q fever serology.

The management of patients who only partially meet the diagnostic criteria for chronic Q fever is challenging. Although not widely published in the literature, this

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Table 1. Criteria, Intended Recruitment, and Attendance of Different Groups Defined for the Follow-up Study in 2008

Group	Criteria	Number of potential recruitable individuals	Invited to take part	Attended
Group 1	Infected with phase 1 IgG titres >800 at any time	39	39	18 (46%)
Group 2	Infected with phase 1 IgG titres consistently <800	91	91	19 (21%)
Group 3	Serologically negative	95	80	14 (17.5%)
Group 4	Q fever endocarditis—treated	1	1	1 (100%)
	Total	226	211	52 (25%)

phenomenon—patients with raised *Coxiella burnetii* antibody titres, no other clinical features of chronic infection and who do not fulfill modified Dukes criteria for endocarditis—is well recognized [3]. PCR has been proposed as a means of distinguishing those patients in this category that have Q fever endocarditis or vascular infection [4]. The significance of a negative PCR result has not been evaluated prospectively nor has PCR been evaluated on a cohort of patients with raised Q fever antibody levels (UK testing) but no other signs of chronic infection.

This paper describes a follow-up study of individuals 6 years after a point source outbreak in a cardboard box manufacturing plant in Newport, Gwent, 2002 [5]. The original outbreak consisted of 106 cases of acute Q fever (84% symptomatic) from a factory of 250 employees [5]. All infected individuals were invited to a follow-up clinic. The clinic identified 1 case of endocarditis [6] (by modified Dukes criteria) and 38 individuals with phase 1 IgG titres >800, consistent with chronic Q fever, but with no other manifestations of infection. They were afebrile, with normal C reactive protein, erythrocyte sedimentation rate (ESR), and white cell counts and did not have valve lesions diagnostic of endocarditis. The optimal management of these individuals is uncertain. This study examines the relationship between serological results and clinical features and compares serological and PCR results between 3 international reference laboratories in a well-defined cohort of Q fever patients.

Aims

The aims of this study were as follows:

1. To describe the spectrum of long-term consequences of Q fever infection in the South Wales outbreak population 6 years after exposure to point source
2. To assess the relationship between clinical symptoms and serological results 6 years after initial infection in 4 subgroups: (i) those with raised phase 1 IgG antibodies to *Coxiella burnetii*, (consistent with chronic Q fever), (ii) individuals with apparent recovery from *Coxiella burnetii* (as defined by low or absent phase 1 IgG antibodies), (iii) individuals who were potentially exposed but apparently uninfected, and (iv) 1 patient with proven Q fever endocarditis who had completed treatment.
3. To compare serological tests undertaken by 3 international reference laboratories in a well-defined cohort of Q fever patients.
4. To compare the use of PCR with serological tests in the diagnosis of chronic Q fever and examine its' role in differentiating patients into groups that have (i) active infection, (ii) those that have recovered clinically, and (iii) those who have quiescent infection.
5. To determine whether Q fever infection results in chronic persistence of the organism in the blood, as defined by PCR.

METHODS

Individuals from the exposed cohort were categorized into 4 groups, differentiated on the basis of serological tests for Q fever antibodies (UK reference laboratory results, Table 1).

Informed consent was taken prior to entry into the study and ethical approval was granted by South East Wales Research Ethics Committee Panel C. Eighty of the 95 uninfected controls were selected using a random number generator (group 3).

Blood was taken for a full blood count, urea and electrolytes, liver function tests, CRP, ESR, rheumatoid factor, thyroid function tests, thyroid peroxidase antibodies, immunoglobulin, *Coxiella burnetii* microimmunofluorescence and PCR. Serum samples were sent to 3 separate reference laboratories in the UK (Special Pathogens Reference Unit SPRU), France (Unite des Rickettsies, Marseille), and Australia (Australian Rickettsial

Table 2. Information on MIF and PCR Tests

	MIF antigen		
Reference laboratory	Phase 2	Phase 1	PCR target (reference)
ARRL, Australia	Nine mile (clone 4)	Henzerling strain	Com 1 and IS1111a [7]
SPRU, UK	Patient strain ''Lane''— ST12 group	Patient strain ''Lane''—ST12 group	IS1111a, COM1, and 16sRNA [8]
Unite des Rickettsies, Marseille, France	Nine mile (ATCC VR615) (2)	Nine mile (ATCC VR615) (2)	IS1111a x 2 targets [4]

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Table 3. Q Fever Infection Definitions Devised by Dupont et al [2] With a Borderline Category Added for Results Within One Serial Dilution of a Chronic Definition

Status	Serological criteria
Chronic	Phase 1 IgG ⩾ 800 or Phase 1 IgA⩾25
Past	Phase 2 IgG detected and Phase 1 IgG <800
Negative	No antibody detected
Acute	Phase 2 IgM ⩾ 50
Borderline	Phase 1 IgG ⩾ 400 and < 800

Reference Laboratory) for microimmunofluorescence and PCR tests (in house assays) (Table 2) [2, 4, 7–9]. Participants were asked to complete a questionnaire containing a number of validated health measures (not reported in this paper).

A serological status was assigned to each individual based on the results from each center using the most commonly accepted interpretation rules (Table 3) [2]. A "borderline" category was added as there is uncertainty in managing patients with a phase 1 immunoglobulin G (IgG) of between 400 and 800, as Q fever endocarditis has been recognized in at least 1 patient with a phase 1 IgG < 800. It is recognized that these thresholds are not universally adopted. For example, in France a serological titre of 800 would be considered borderline (D. Raoult, oral communication, 2010).

RESULTS

Of 212 individuals invited to take part in the study, 52 attended for blood testing (see Table 1) and 50 completed questionnaires.

The results from the 3 centers interpreted using recognized criteria [2] are shown in Table 4. Table 5 shows the results according to original status groups 1–4. The concordance between the test results from the 3 laboratories was 35% (18/52) overall, 35% (18/52) France cf UK, 42% (22/52) France cf Australia, and 71% (37/52) Australia cf UK (Table 6).

Concordance was greatest between Australian and UK laboratories and lowest between French and UK laboratories.

Discordant Results

Discordant results are shown in Table 7 (18 out of 52 results, 35%).

Four participants had a different status assigned by all 3 centers (Table 7, bold type, nos. 2, 3, 4, 35).

If borderline is considered as a separate category there are a further 6 participants with different serological results in each center (Table 7, italics, nos. 1, 5, 6, 12, 13, 14).

Eighteen participants had the same serological status assigned by all 3 centers, 10 negative results, 8 past infections (although 3 of these results from the UK gave a borderline chronic serological profile, nos. 15, 16, 17).

Table 4. Serological Status According to Testing Center [2]

	France	UK	Australia
Negative	34	11	17
Past	18 (1)	29 (7)	32 (2)
Chronic	0	9	0
Acute	0	3	3
Total	52	52	52

NOTE. Figures in brackets are "borderline chronic" serological results—ie, phase 1 IgG within 1 serial dilution of being ⩾800 (400 or 640).

Chronic/Borderline Serological Profile

A chronic serological profile was present in 10 individuals when tested in the UK (Table 7) but not in any tests performed in either France or Australia. Eight individuals had borderline results for chronic Q fever when tested in the UK. There were 3 borderline results from Australia (Table 6, nos. 5, 6, 15) and one borderline result from France (Table 6, no. 1).

Follow-Up Results of Serologically Negative Participants (Group 3)

Of the 12 participants from group 3 (asymptomatic and serologically negative at the time of the outbreak), 4 were serologically positive by the UK reference laboratory test, 3 by the Australian, none by the French, and 5 by any of the 3 labs (table 5). It is not possible to determine whether this represents late seroconversion, subsequent infection, or false positive serological results. The rate of seroconversion, 5 of 12 cases in 6 years, is high for a background rate of new infections, however.

Patient With Treated Endocarditis

One patient was diagnosed with endocarditis in March 2004 [6] and treated for 22 months with doxycycline and hydroxychloroquine. She was asymptomatic prior to treatment, and so treatment response was judged by a fall in her C-reactive protein (CRP) and serological titres (when tested in France). Her results from this study would suggest past/treated infection (France, Phase 1 IgG 50), chronic infection (UK, Phase 1 IgG >1280), and borderline chronic (Australia, Phase 1 IgG 400). The patient stopped treatment in July 2006 but remains under regular follow-up. Since stopping treatment, there has been no increase in her CRP and no further deterioration in her valve function.

PCR Results

PCR results on blood were universally negative in this study.

Biochemical Blood Test Results

The majority of blood results were within normal limits. There are no results from routine blood tests that suggest those patients with a chronic serological profile have ongoing infection/inflammation greater than those who have evidence of past infection serologically or those who have been consistently seronegative. Three people had mildly elevated CRP. None of these

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Table 5.

a) Results on Patients From Group 1; Infected With Phase 1 IgG Titre ≥ 800 at Any Stage *N* = 18

	Negative	Acute	Past	Chronic
UK	0	0	11 (4)	7
France	5	0	13 (1)	0
Australia	0	2	16 (1)	0

b) Results on Patients From Group 2; Infected With Phase 1 IgG Titre Consistently < 800 *N* = 19

	Negative	Acute	Past	Chronic
UK	1	2	14 (3)	2
France	15	0	4	0
Australia	6	0	13	0

c) Results on Patients From Group 3; Serologically Negative *N* = 14

	Negative	Acute	Past	Chronic
UK	10	0	4	0
France	14	0	0	0
Australia	11	1	2	0

d) Results on Patient From Group 4; Q Fever Endocarditis—Treated *N* = 1

	Phase 1 IgG	Interpretation
UK	>1280	Ongoing treatment required
France	50	Cured
Australia	400	Ongoing treatment required

NOTE. Figures in brackets are ''borderline chronic'' serological results—ie, phase 1 IgG within 1 serial dilution of being ⩾800 (400 or 640).

had a chronic serological profile, and all had serological profiles that were either negative or suggestive of past infection (UK 3/29 past infection, 0/11 negative, France 1/18 past, 2/34 negative, Australia 2/32 past, 1/17 Negative).

DISCUSSION

The main finding of this study is the discrepancy between serological tests from different reference laboratories and the consequent challenges that this presents in the interpretation of serological results.

The clinical diagnosis of chronic Q fever infection is challenging. The presentation is heterogeneous and typical markers of infection frequently absent. Q fever endocarditis, the commonest manifestation of chronic Q fever, is a slow indolent disease, and endocarditis is often only diagnosed after significant valvular damage has occurred [10]. Chronic Q fever endocarditis can be present in individuals who are afebrile [6, 11, 12], have a normal ESR [12, 13], and normal routine haematological studies [11]. Chronic Q fever endocarditis can be entirely asymptomatic [6] and can be both clinically and histologically silent [11–14].

A study by Raoult et al [15] has demonstrated that Q fever infection of heart valves does not produce the typical histological findings of endocarditis. In that study, valves removed because of infection with *Coxiella burnetii* resembled valves removed because of degenerative valve disease in almost every regard. The presence of small vegetations and focal inflammatory changes in the infected valves were the only distinguishing features. These changes however were subtle and could be easily overlooked by routine histological examination. In addition, 3 individuals had no obvious infection on histology, no vegetations, and only a slight inflammatory reaction. These findings in part explain why even transoesophageal echocardiograms perform so poorly in the diagnosis of Q fever endocarditis [1]. The long-term sequelae of these indolent infections has never been prospectively evaluated, nor has the impact of treatment of these individuals been assessed. A cohort study carried out by Swiss investigators, however, has suggested that the long-term (12 year) risk of endocarditis in a group of individuals acutely infected with *Coxiella burnetii* is no different to a noninfected control population [16]. Equally, follow-up of patients with echocardiographic evidence of valvulopathy has suggested the short-term risk of endocarditis is low [17].

Because chronic Q fever infection is extremely difficult to diagnose, the optimal management of individuals with raised titres but no other evidence of ongoing infection is uncertain. The management is further complicated by the duration and potential toxicity of the recommended treatment regimen, doxycycline and hydroxychloroquine for a minimum of 18 months.

Although local serological thresholds should ideally be developed (using Bayes theorem) and interpreted in the light of clinical information, in clinical practice published criteria are often used in isolation to interpret serological results. A variety of serological criteria to diagnose chronic Q fever infection have been published [2, 18–22] resulting in the following (sometimes contradictory) recommendations:

- Phase 1 IgG > Phase 2 IgG, Elevated Phase 1 IgG or Phase 1 IgA [19].
- Phase 1 IgA ⩾ 320 and CFT > 128 [18].
- Phase 1 IgG > 800, Phase 1 IgA not contributory [2].
- Phase 1 IgG > Phase 2 IgG + Phase 1 IgA > Phase 2 IgA [20].
- Phase 1 > Phase 2 [21].
- High Phase 1 IgG and Phase 1 IgA [22].

In these studies, the phase 1 IgG ranged from 640 to 2,097,152. In the majority of these studies, serological results of patients with chronic Q fever endocarditis were compared with those from acute Q fever patients. However, a proportion of people will develop phase 1 antibodies after Q fever infection: in 3 studies the proportion of patients who did so was 15 of 51 [23], 11 of 20 [20], and 3 of 3 [22]. These phase 1 titres are not necessarily associated with chronic illness, and it has been

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Table 6. Concordance of Tests Results Across the 3 Testing Centers

Comparison of centers	Concordance in result interpretation	Percentage
All 3 centers	18/52	35%
France cf UK	18/52	35%
France cf Australia	22/52	42%
Australia cf UK	37/52	71%

proposed that development of phase 1 IgG antibodies is part of the normal response to Q fever infection [24].

In one study, cases of Q fever endocarditis were compared with cases of acute Q fever that had been followed up for between 8 and 88 months [18]. Phase 1 IgG was present in all of the control subjects and was >800 in 2 of them. Two also had phase 1 IgA of 320 and CFT of 64.

The Duke criteria have been modified to permit improved diagnosis of Q fever endocarditis, and phase 1 IgG antibody titre ⩾ 800 to *Coxiella burnetii* is now considered a major criterion for the diagnosis of endocarditis [25, 26]. In many countries the diagnosis of chronic Q fever will be suspected if an immunofluorescence phase 1 IgG antibody ⩾ 800 is detected, and physicians may consider starting treatment based on serological results alone.

The optimal management of patients with raised phase 1 IgG levels but no other markers of chronic infection, however, is not clear [3]. It has been proposed that PCR on blood of patients with phase 1 IgG serological titres <1:25600 can identify those with active endovascular infection [4]. In that study, overall sensitivity of PCR was 27% (13/48), 39.4% (13/33) in patients with titres <1:25600, 64% (7/11) in those samples tested prospectively, and 100% (7/7) in patients with titres <1:25600 tested prospectively [4]. One hundred patients with endocarditis caused by other organisms were used as a control group. The significance of a negative PCR result has not been evaluated prospectively, nor has PCR been evaluated on a cohort of patients with raised Q fever antibody levels (UK testing) but no other signs of chronic infection.

The follow-up of patients following Q fever infection is further complicated by the finding that 88% of individuals from a previous UK outbreak [27] had bone marrow samples that were positive by PCR for Q fever 12 years after their acute infection and regardless of their clinical state at the time the samples were taken [9]. Some of these patients had ongoing fatigue, but others had made a complete clinical recovery. All attempts at culture of the bone marrow were unsuccessful [9] including PCR positive marrow/peripheral blood cell homogenates from 10 patients that were inoculated into SCID mice [28]. Although no coxiella were isolated, coxiella antigen-LPS complexes were detected by immunofluorescence in SCID mice spleens. The authors propose that noninfective, nonbiodegradable antigen-LPS complexes persist in the host, and these can provoke aberrant humoral and cell mediated immunity responses [28, 29].

Table 7. Discordant Serological Status Attributed by Different Testing Centers

	Serological status		
Number	UK	France	Australia
1	*Chronic*	*Borderline*	*Past*
2	**Chronic**	**Negative**	**Past**
3	**Chronic**	**Past**	**Acute**
4	**Chronic**	**Past**	**Acute**
5	*Chronic*	*Past*	*Borderline*
6	*Chronic*	*Past*	*Borderline*
7	Chronic	Past	Past
8	Chronic	Past	Past
9	Chronic	Past	Past
10	Chronic	Past	Past
11	Borderline	Negative	Negative
12	*Borderline*	*Negative*	*Past*
13	*Borderline*	*Negative*	*Past*
14	*Borderline*	*Negative*	*Past*
15	Borderline	Past	Borderline
16	Borderline	Past	Past
17	Borderline	Past	Past
18	Past	Negative	Negative
19	Past	Negative	Negative
20	Past	Negative	Negative
21	Past	Negative	Negative
22	Past	Negative	Negative
23	Past	Negative	Past
24	Past	Negative	Past
25	Past	Negative	Past
26	Past	Negative	Negative
27	Past	Negative	Past
28	Past	Negative	Past
29	Past	Negative	Past
30	Past	Negative	Past
31	Past	Negative	Past
32	Past	Negative	Past
33	Past	Negative	Past
34	Past	Negative	Past
35	**Acute**	**Negative**	**Past**
36	Acute	Past	Past
37	Negative	Negative	Acute

NOTE. Number 5 = Patient diagnosed and treated for endocarditis. Bold type = different results from all 3 centers. Italics = different results from all 3 centers if borderline category included.

This study has shown that there is discordance in the serological results generated by 3 reference laboratories in UK, France, and Australia. A similar disparity between American and French test results has been found in the United States [3].

It is not possible to determine the sensitivity or specificity of the results from each center as Q fever is frequently

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asymptomatic and all the individuals in the factory were potentially exposed. Any detectable titre could therefore represent past infection or exposure. In addition, it is not possible to ascertain from this study the sensitivity and specificity of the chronic serological profile in each country as Chronic Q fever is difficult to diagnose and requires more detailed investigation than carried out here.

However, it is possible to compare the results and it is apparent that the UK test produced the fewest negative results (11 cf 17 [Aus] cf 34 [France]), the most chronic serological profiles (9 cf 0 cf 0) and the most "borderline" chronic serological profiles (8 cf 3 cf 1).

The discrepancy in the results obtained by the different centers has clear implications for the interpretation of serological results.

1. The indiscriminate application of cut off points or criteria for interpretation of serological results (including the indiscriminate use of the phase 1 IgG ⩾800 cut off proposed in the modified Duke criteria) is questioned.
2. Serological evaluation of treatment response using microimmunofluorescence will vary according to the testing center being used.
3. Sero-epidemiological studies will produce different results according to the center in which the testing is carried out. As the diagnosis of Q fever infection is based primarily on serological profiles, this discrepancy hampers our understanding of the natural history of Q fever infection.

The differences in the results are surprising given that all 3 centers were using the same microimmunofluorescence method. Strain differences (Table 2), growth substrate, manufacturing technique, and use of different antigenic material are possible explanatory factors.

This study has not provided any additional evidence on the utility of Q fever PCR in establishing a diagnosis of chronic Q fever endocarditis or vascular infection. While a positive PCR in blood is undoubtedly helpful, the long-term outcome of patients with negative PCR results and a chronic serological profile (when tested in the UK) has not been established.

CONCLUSIONS

This study has shown that the results from Q fever microimmunofluorescence vary according to the center in which they are carried out. This has implications for the interpretation of such tests, raises questions regarding the validity of using serological criteria alone as a means of diagnosing Chronic Q fever and affects the interpretation of epidemiological studies. We recommend that all results are interpreted according to the clinical picture and that particular caution is applied in the interpretation of chronic serological profiles since the results from this study show that the serological diagnosis of chronic Q fever varies according to where the test is performed. In order to further our understanding of Q fever infection, we propose that an international standard of Q fever serological investigation is developed.

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Case series demonstrating the presence of protozoa in the sputum of a proportion of respiratory patients

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Abstract

A Spanish study has demonstrated the presence of protozoa in the sputum of respiratory patients admitted to hospital with acute exacerbations of respiratory disease. These findings have not previously been replicated elsewhere in the world. Sputum was obtained from two case series of patients; firstly inpatients with an acute exacerbations of respiratory symptoms, and the other consisting mainly of chronic outpatients; both at Llandough Hospital, Cardiff, South Wales. Sputum, stained using the Papanicolaou method, was examined under a microscope using previously published criteria to determine the presence of protozoa. In the first series of ten inpatients, five patients (50%; 95% CI 24 – 76%) had protozoa in their sputum. In the second series of 17 generally less acute patients, one clearly had protozoa and two possibly had protozoa in their sputum. Protozoa may have an important pathogenic role in asthma which merits further examination.

Introduction

The diagnosis of asthma is primarily based on clinical assessment rather than on definitive diagnostic tests [1]. It can be argued that asthmatic patients may include a cluster of aetiologies that produce a similar clinical pattern rather than a single disease process. This pilot study focuses on one factor that may have some relevance in a proportion of respiratory patients that have a diagnosis of asthma or COPD.

There is significant overlap in the symptoms of patients with asthma and COPD. A relevant history of smoking or occupational exposure, and spirometry can help to differentiate between the two conditions. Symptomatic improvement in response to a trial of beta-2 agonists is also a useful indicator of asthma. The aetiological distinction between asthma and COPD is further complicated by the fact that a proportion of patients have both conditions and there may be etiological factors that are common to both conditions.

Asthma appears to involve a complex interaction between environmental and genetic factors. A

wide range of allergens have been identified, which can trigger respiratory symptoms in sensitized individuals. Acute viral infections are also recognized triggers in both asthma and COPD, as are exercise, hormonal changes and stress. In addition, in some individuals, asthma and COPD are related to exposure to allergens or noxious factors that are present in the individual's occupational environment.

Infectious agents have also been proposed as having an etiological role in asthma. One of the most widely recognized hypotheses for the aetiology of asthma is the hygiene hypothesis [2] which was postulated to address the observation that children from larger families, who are presumably exposed to more infectious agents through their siblings, display less allergic symptoms than children from families with only one child. However, it is possible that infectious agents, including protozoa are more directly implicated in etiology of acute exacerbations of asthma and COPD.

This study is based on previous work exploring the role of protozoa in patients with respiratory symptoms. A number of small published studies of Spanish patients have explored the presence of protozoa in the sputum of respiratory patients admitted to hospital with acute exacerbations of disease [3,4] or patients who were immuno-compromised [5,6]. In one study [3], 15/19 patients (79%; 95% CI 35% – 92%) with asthma had protozoa in their sputum, as opposed to 9/78 patients (12%; 95% CI 6% – 21%) with other respiratory diseases, most usually COPD. The observed difference in prevalence (67%; 95% CI 43% - 81%) was statistically significant.

The aim of this pilot study was to determine whether protozoa could be identified in similar respiratory patients in the UK.

Materials and Methods

Two case series of patients had sputum collected several months apart. One case series was based mainly on inpatients and the other case series was based mainly on outpatients. The first case series comprised ten patients who were admitted to Llandough Hospital, Cardiff, South Wales, with an acute exacerbation of respiratory symptoms. The second case series comprised 17 patients, many of whom attended a COPD patient self help group, and who were generally less acutely ill than the patients in the first case series.

An information sheet was provided to all the patients involved in the study and written consent was obtained. A sterile sputum container was left with each patient. The sputum collected was transferred to two microscope slides. A small area of true sputum (not saliva), about the size of a large lentil, was taken from the expectoration using tweezers. The sample was placed on a microscope slide (frosted labeling area upwards) and, a second slide (frosted labeling area downwards) was used to make a smooth, uniform smear, gently moving both slides in opposite directions, and exerting slight pressure whist gently holding the frosted labeled area of the slide between the thumb and forefinger. Both of the slides were immediately fixed using a commercially available hair lacquer, spraying the whole surface of each slide from a distance of approximately 30 cm for a few seconds. The slides were left face up to dry to make certain that none of the material seeped out at the edges. Every effort was made to ensure that the sample observed under the microscope consisted of sputum and not of saliva.

The microscope slides were stained using a modified Papanicolaou method [7] and the slides were scanned under the microscope to identify flagellated protozoa. Protozoa were differentiated from ciliated epithelial cell remnants using the criteria in Table 1, based on previously published work [4,3].

Characteristic	Protozoal forms	Ciliocythophthoria (Ciliated epithelial cell remnants)
Red granules	Numerous, 3-8 µm, may be extracellular	Few, very small (1-2 µm), intracellular
Nuclei	Occasionally absent; if present, perinuclear clear halo, perinuclear corpuscles and prominent central kariosome	Usually absent; if present no perinuclear halo or perinuclear corpuscles, with tendency to picnosis
Cytoplasm	Friable with certain plasticity. Spindle or oval shape. Occasionally presence of cytoplasmic vacuoles	Round or columnar shape with fine granularity
Cilia or flagella	Wavy, not combed; different lengths	Cilia along 1 edge; straight, combed and same length. Discernible terminal bar
Cellular variability	Marked	Little variability
Papanicolaou stain	Eosinophilic	Amphophilic or basophilic
Ultraviolet light	Positive autofluorescence of all forms	Negative

Table 1: Morphological basis for distinguishing between protozoal forms and ciliocythophthoria

When in doubt about distinguishing between protozoa and epithelial cell remnants, the following characteristics were particularly relied on during examination under oil immersion:

1. Cytoplasmatic plasticity with ameboid-like forms
2. Irregular insertion of numerous flagella around all the cellular border and absence of terminal bar
3. Size about 12-20 microns
4. Background with red granules (1-3 microns in diameter), and a characteristic eosinophylic material as a cloud

Quality assurance of the process involved: clear explanation to patients on how to produce a good-quality sample of sputum; rapid fixing of slides; a robust protocol for labeling of slides with unique patient identifiers; transport using approved containers for such samples; and assessment of sputum quality under the microscope based on the presence of macrophages in the smear.

Ethical approval for the study was obtained from the South East Wales Local Research Ethics Committee Panel C (Ref 05/WSE03/134) and informed consent was obtained in line with the Helsinki declaration.

Results

In the first case series, protozoa were observed in the sputum of five of the ten inpatients (50%; 95% CI 24 – 76%). All the patients in the sample had been admitted with an acute exacerbation of

respiratory disease and were over 18 years of age. Other demographic details were not available for this case series.

In the second case series of 17 patients, mainly made up of outpatients, one case had protozoa and two had possible protozoa in their sputum. Some of the characteristics of this case series are given in Table 2.

Taking both case series together six of 27 patients (22%; 95% CI 11% - 41%) with respiratory disease had protozoal forms in their sputum.

Diagnosis	Gender	Age	Acute/Chronic	Duration of daily phlegm production	Protozoa seen
Bronchiectasis	F	65	Chronic	60 years	No
COPD/alpha1 anti-trypsin deficiency	M	55	Chronic	30 years	No
COPD	M	71	Chronic	7 years	No
COPD	M	78	Acute	1 to 2 weeks	No
Bronchiectasis	F	64	Chronic	2 years	No
COPD/asthma	M	78	Acute	1 week	No
COPD	M	69	Acute	5 weeks	No
COPD	M	73	Acute	1 week	No
Emphysema	M	49	Chronic	16 years	No
COPD	M	73	Chronic	12 months	No
Bronchiectasis	F	74	Chronic	2 years	No
Bronchiectasis	M	70	Chronic	40 years	No
Cystic Fibrosis (Bronchiectasis)	F	27	Chronic	5 years	No
Cystic Fibrosis	M	18	Chronic	50 years	No
Asthma	F	25	Chronic	Years	Possibly
COPD	F	83	Chronic	Years	Possibly
COPD	M	75	Chronic	2 years	Yes

Table 2: Characteristics of the second case series of 17 respiratory patients tested for the presence of protozoa

An example of a protozoal form in sputum is shown in Figure 1.

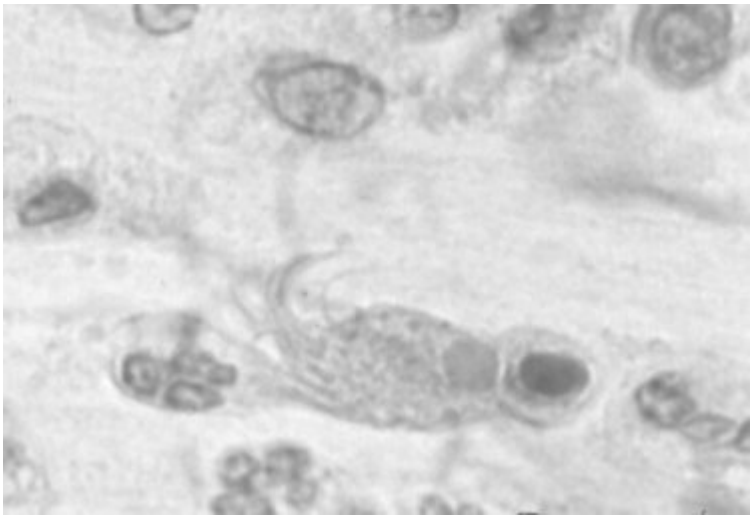

Figure 1: Example of protozoa in sputum

Discussion

This small pilot study provides some evidence that protozoa are present in a proportion of patients in the UK who have acute exacerbations of respiratory disease. This pilot study confirms previous observations regarding the presence of protozoa in the sputum of Spanish patients, but in case series in patients living in a more temperate climate. The higher rate of infection in inpatients than in outpatients may indicate that protozoal infection was in some way associated with acute exacerbations of disease.

It is unclear whether a single species of protozoa is being observed in all patients. It is also unclear whether these organisms are commensals or have any pathogenic significance whatsoever. Although the respiratory tract has historically been considered free from micro-organisms, in the absence of acute infection, there is some emerging evidence that organisms may be present in the respiratory tract without having a clear pathological role.

There is relatively little published literature on protozoa in the sputum of patients with asthma or COPD. A number of studies that have identified protozoa in the sputum of respiratory patients and infections related to protozoa have recently been reviewed [8]. Several studies have demonstrated chlamydia in the sputum of a proportion of patients with asthma [9,10] and a number of case reports from Chinese researchers have also identified protozoal organisms in patients with asthma [11,12].

Protozoa are often difficult to culture and previously reported attempts to culture the protozoa identified in sputum have been unsuccessful [3]. Culture of the protozoa would allow the examination of the effect of the protozoa on respiratory epithelial cell cultures. There is also the potential to undertake PCR amplification of 18S rDNA to determine whether the protozoa present in sputum is a known species, or is similar to a known species. Characterisation of the protozoa would allow us to determine whether the same species of protozoa is appearing in this diverse group of patients with respiratory symptoms. These techniques would allow the development of a rapid diagnostic technique to identify the presence of protozoa in sputum samples.

There is also a need for further work on the natural history of protozoa in the lungs, to determine whether the protozoa are present in patients over a prolonged period of time or only appear transiently. The relationship between acute exacerbations of respiratory illness, recovery from illness, and the presence or absence of protozoa in the respiratory tract also needs to be determined.

If the protozoa can be cultured, their antibiotic sensitivity could be determined and a randomized control trial could be undertaken to determine whether clearance of the protozoa in symptomatic patients resulted in a more rapid recovery from an acute exacerbation of disease, or alternatively whether treatment of patients with chronic respiratory disease resulted in long term improvement in symptoms. Successful treatment of a very small number of individuals with metronidazole has suggested that this may be an appropriate antibiotic to test in a larger therapeutic trial, in individuals who have an acute exacerbation of asthma/COPD and where protozoa can be demonstrated in the sputum.

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

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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

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using genetic techniques based on 18S ribosomal RNA. Once tis is established it would be worthwhile to determine if asthma symptoms improve when treated by anti-protozoal agents.
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Background

A wide range of aetiological factors have been suggested as possible causes of asthma[1,2] including the hygiene hypothesis[3] 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[4,5] and some authors have suggested that asthma may even be an infectious disease.[6] There is growing interest in the potential contribution of a number of atypical infectious agents to the aetiology of asthma, particularly *Mycoplasma* and *Chlamydia*.[4,7,8]

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[9–11] and in patients who were immuno-compromised.[12,13] 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 samples is that flagellated protozoa are easily mistaken for ciliated epithelial cells.[14] The cyto-pathologists involved in this study have described a set of morphological and staining criteria to differentiate flagellated protozoa from ciliated cell fragments.[9,10]

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 home 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.[15–17] 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[18] 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.[19] This score incorporates four questions: Is there any visible mould growth on your house? Is there any odour of mould or cellar-like fusty 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.[20–23] 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,

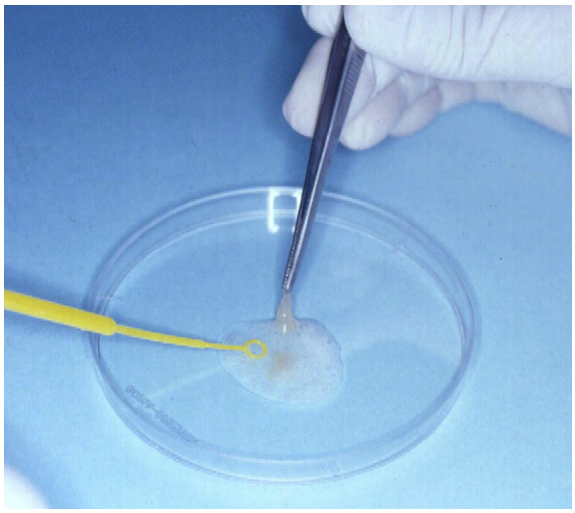

Figure 1 Selecting sputum, as opposed to saliva, using the pick technique.

muco-cellular clumps at least 1.5 × 3.0 mm in size had been collected.[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® spray. The microscope slides were stained using previously described techniques[9] and examined for the presence of flagellated protozoa by a cyto-pathologist using a previously described set of morphological criteria.[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.

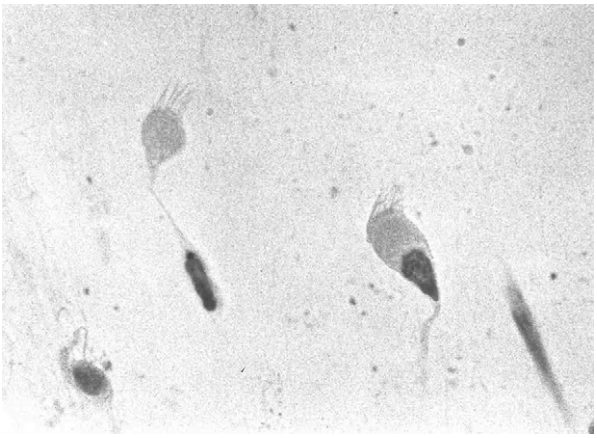

Figure 2 Degenerative changes in ciliated bronchial cells (Magnification × 1000).

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)

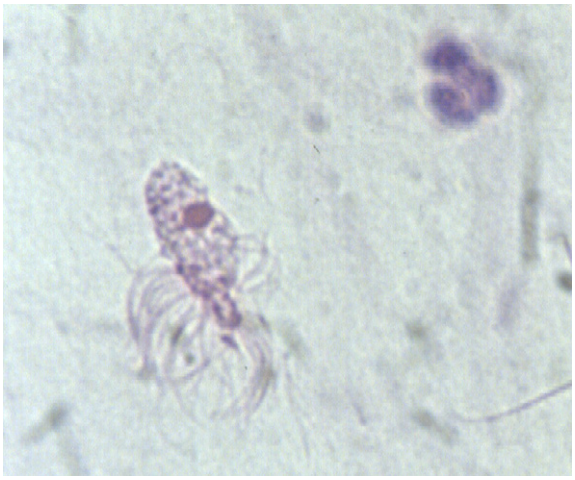

Figure 3 Flagellated protozoa from the sputum of an asthma patient clearly demonstrating the presence of "capitullum" and irregular flagella (Magnification × 1000).

Table 1 Summary of the presence or absence of protozoa in study participants.

	Protozoa present	Protozoa absent	Unclear whether protozoa present	Inadequate sample (saliva)	No sputum obtained	Total
Asthma patients	20	10	3	1	20	54
Controls	4	9	2	0	27	42
Total	24	19	5	1	47	96

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/macropages or neutrophils observed in sputum smears when the differences were assessed using the Mann—Whitney test.

Were protozoa more common in asthmatics than in non-asthmatics?

The prevalence of protozoa in asthma patients was 66.7% (20/30) and in controls, it was 30.8% (4/13). Fisher's Exact Test comparing the proportion of asthma patients and control subjects in which protozoa were clearly 'present' or 'absent' gave a p value of 0.046, relative risk of 1.58 (95% CI 1.00—2.51). The data supports the hypothesis that protozoa are more prevalent in patients diagnosed with asthma than in comparable controls.

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 2 Comparison of the presence or absence of protozoa against the use of a steroid inhaler in the preceding two weeks, among 30 asthma patients.

		Protozoa in sputum		Total
		Yes	No	
Have you taken a steroid/preventative inhaler in the last 2 weeks?	Yes	10	8	18
	No	10	2	12
Total		20	10	30

Table 3 Comparison of the presence or absence of protozoa against evidence of damp/mouldy in the home.

	Protozoa in sputum		Total
	Yes	No	
No evidence of damp/mould	18	14	32
Evidence of damp/mould	6	5	11
Total	24	19	43

Table 4 Observed duration of the presence of protozoa in participants.

Patient or Control	First visit	Later visit	Interval (days)
Asthma patient	Protozoa	Protozoa	103
Asthma patient	Protozoa	Protozoa	88
Asthma patient	Protozoa	Protozoa	80
Asthma patient	Protozoa	Protozoa	46
Asthma patient	Protozoa	Protozoa	37
Asthma patient	Protozoa	Protozoa	17
Asthma patient	Protozoa	Protozoa	14
Asthma patient	Protozoa	Protozoa	8
Asthma patient	Protozoa	Protozoa	2
Asthma patient	Protozoa	Protozoa	2
Asthma patient	Protozoa	Protozoa	2
Control	Protozoa	Protozoa	18

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 symptoms[2,25] 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[27] 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

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Competing interests

None declared.

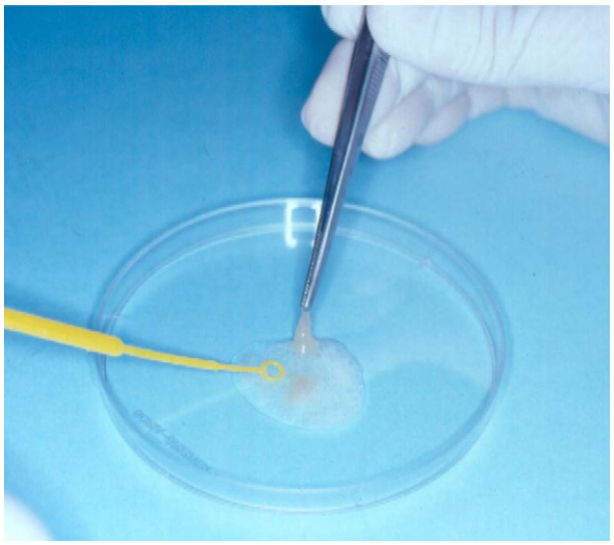
Acknowledgements

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.

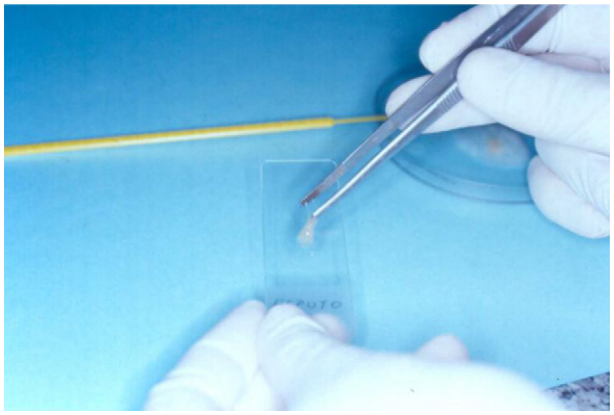
Appendix 1. Instructions for obtaining sputum smears

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

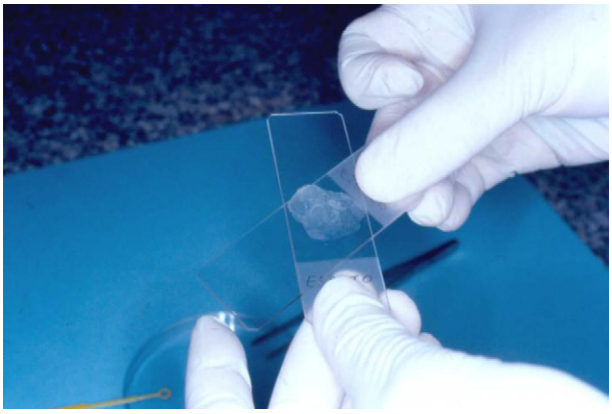


Step 2

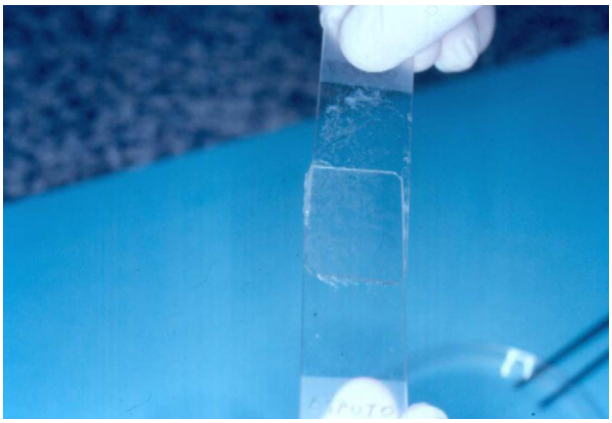


3. Place the sample on a slide (frosted edge upwards) and, with a second slide (frosted edge downwards) make a smooth, uniform smear. It is not appropriate to crush the sample, but rather to move both slides in opposite directions, exerting slight pressure. To facilitate this operation, take the frosted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

Step 3

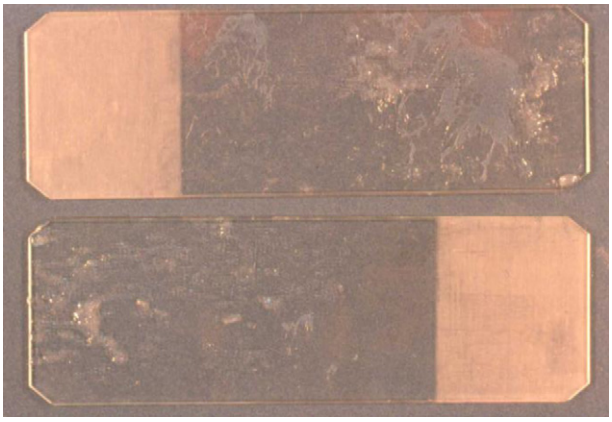


Step 4



4. Immediately fix the smears on both slides with commercially available hair lacquer, spraying the whole surface from a distance of approximately 30 cm. Make sure to spray the surface of the slides where the smears are (that corresponding to the frosted area of the edges). Before introducing the fixed slides into their containers for dispatch, leave them face up for a while until certain that none of the material seeps out at the sides.

Step 5



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ORIGINAL ARTICLE

Clinical and Immunological Characteristics Associated With the Presence of Protozoa in Sputum Smears

Rafael Martínez-Girón, M.D., Ph.D.,[1*] and
Hugo Cornelis van Woerden, M.B.Ch.B., M.P.H., F.F.P.H., M.R.C.G.P.[2]

The objective of this study is to assess the relationship between protozoa in spontaneously expectorated sputum samples and a range of clinical and immunological variables. Clinical details including age, gender, smoking status, and use of oral or inhaled steroids were recorded for a cohort of 199 patients whose spontaneously expectorated sputum samples were submitted to a Cytology Laboratory in Spain between January 2005 and December 2006. Slides were scanned for protozoa under light microscopy and scanned for monocytes/small macrophages highlighted by immunocytochemistry (CD68 monoclonal antibody). One hundred ninety-one patients provided adequate sputum samples, of whom 70 had protozoa in their sputum. There was a strong relationship between the presence of protozoa and monocytes/small macrophages identified under light microscopy ($P < 0.001$). A binary logistic regression model also indicated a relationship between protozoa and both smoking status and steroid use. The diagnoses in those with protozoa included infection (including tuberculosis), chronic obstructive pulmonary disease (COPD), lung fibrosis, asthma, chronic liver disease, immunosuppression, cancer, pancreatic or renal disease, heart failure, and AIDS. The identified association between protozoa and monocytes/small macrophages in sputum suggests an immune response and warrants further investigation to clarify whether or not these organisms have any pathological significance in this wide range of conditions. Diagn. Cytopathol. 2011;00:000–000. © 2011 Wiley-Liss, Inc.

Key Words: monocytes/small macrophages; protozoa; sputum smears; respiratory disease

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Microscopy of spontaneously expectorated sputum provides a simple, noninvasive technique for identifying malignant and inflammatory cells in the respiratory tract.[1–3] The pattern of inflammatory cells present in a sputum sample can predict response to treatment. Thus, the presence of eosinophils in sputum predicts corticosteroid response in some asthma patients.[4] This suggests that the relationship between other inflammatory cells and respiratory disease may also be worth investigating.

Monocytes and small sputum macrophages are associated with a range of respiratory diseases.[5,6] As the precursors of alveolar macrophages, monocytes are recruited into the lungs[7] and contribute to the respiratory system's defence against a range of pathogens.[8] The presence of monocytes/small macrophages can be readily assessed on the basis of morphological features and antibody staining.[9]

In a number of previous reports, we have identified multiflagellated protozoa in sputum from patients with a range of disorders.[10–14] During those investigations, an anecdotal observation was made that monocytes/small macrophages were more commonly present in samples that contained protozoa. The purpose of this study was, therefore, first to quantify the relationship between the presence of protozoa and monocytes/small macrophages in sputum and second to assess the relationship between the presence of protozoa and range of other available clinical variables.

Materials and Methods

Information was prospectively recorded on a consecutive cohort of patients whose spontaneously expectorated sputum samples were submitted to the Cytology Laboratory, in the Central University Hospital, Asturias, Oviedo,

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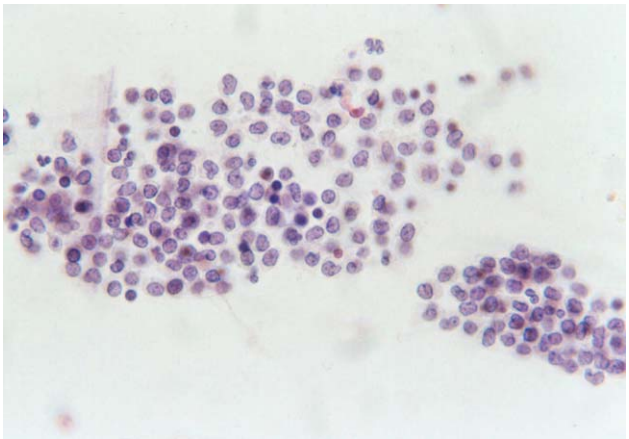

Fig. 1. Clusters of monocytes/small macrophages in a sputum smear (Papanicolaou, ×400). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Spain between January 2005 and December 2006. Clinical details including age, gender, smoking status, and use of oral or inhaled steroids was recorded for each patient. Sputum samples were collected over a standard 3-day period,[15] smeared onto two glass slides using a "pick-and-smear" technique, fixed for 30 minutes in 96% ethanol, and stained by the Papanicolaou method. Inadequate samples, consisting primarily of saliva, were excluded from further analysis.

The presence of monocytes/small macrophages in a smear (Fig. 1) was confirmed based on the presence of standard morphological features under light microscopy including:

- cellular diameter of about 12–14 μm;
- characteristic nucleus with a bean-like appearance;
- scanty cytoplasm;
- low nuclear/cytoplasm ratio; and
- positive immunocytochemistry using a commercially available antibody anti-CD68 (1:50 dilution, Dako EnVision™, Denmark).

Fig. 2. **A** and **B**: Multiflagellated protozoa in a sputum smears. Note the distinctive flagellar arrangement around the irregular cytoplasmic outline (Papanicolaou, ×1,000. Scale bar = 10 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The variable depth and quantity of mucus on the slides means that counting of the monocytes/small macrophages would probably not have provided counts that could be reliably compared. Instead, monocytes/small macrophages were deemed to be "present" if a minimum of two areas were identified on a slide where at least 10 monocytes/small macrophages were present.

Protozoa were identified using previously described criteria.[15] In summary, this involved scanning of sputum slides under light microscopy for 10–20 minutes using the Papanicolaou stain and identifying and distinguishing multiflagellated protozoa (Figs. 2A and B) from ciliated epithelial fragments or other cellular debris (Fig. 3). The first six criteria in Table I were routinely used to distinguish protozoa from ciliated epithelial cells or cell fragments. The additional criteria in the table were occasionally used to confirm the presence of protozoa. A monoclonal antibody to cytokeratin (CAM 5.2) can also be used to stain ciliated epithelial cells or cell fragments and thus distinguish them from protozoa[16] (Fig. 4).

The χ^2 test was used to assess the relationship between the presence of protozoa and monocytes. A binary logistic regression model (backward stepwise—likelihood ratio) was run in SPSS 14.0 (Chicago, IL) to examine whether the available clinical variables predicted the presence of protozoa. A similar model was run to assess the relationship between the presence of monocytes/small macro-

Fig. 3. Ciliated epithelial fragment in a sputum smear (Papanicolaou, ×1,000). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

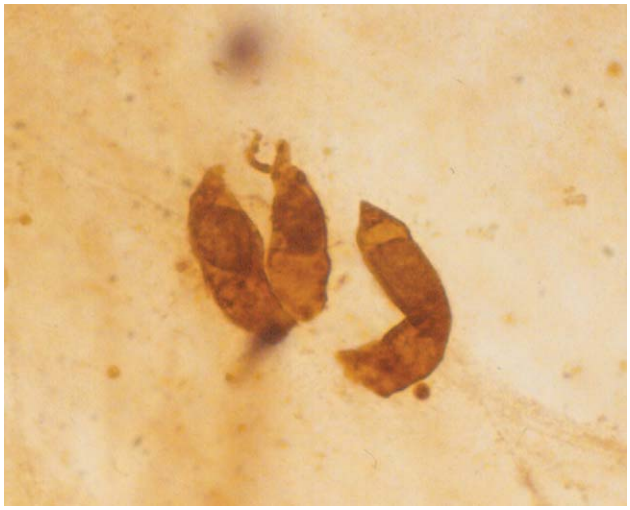

Fig. 4. Sputum smear. Bronchial epithelial cells positive for CAM 5.2 immunostaining (×1,000). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Morphological Basis for Distinguishing Between Protozoal Forms and Ciliocytophthoria

Characteristic	*Protozoal forms*	*Ciliocytophthoria (ciliated epithelial remnants)*
Red granules	Numerous 3–8 μm; may be extracellular	Few, very small (1–2 μm), intracellular
Nuclei	Occasionally absent; if present perinuclear clear halo, perinuclear corpuscles, and prominent central karyosome	Usually absent; if present no perinuclear clear halo, perinuclear corpuscles, with tendency to pyknosis
Cytoplasm	Friable with certain plasticity; spindle or oval shape; occasional presence of cytoplasmic vacuoles	Round or columnar shape with fine granularity
Cilia or flagella	Wavy, not combed; different lengths	Cilia alone one edge; straight, combed and the same length; discernable terminal bar
Cellular variability	Marked	Little variability
Papanicolaou stain	Eosinophilic	Amphophilic, eosinophilic, or basophilic
Under ultraviolet light	Positive autofluorescence of all forms	No fluorescence
Heindenhain's iron haematoxylin stain	Positive in nuclei and internal structures	Negative
Acridine orange stain	Positive	Negative
Wheatley's Trichome stain	Positive, magenta with granularity and red nuclei	Greenish cytoplasm
Motility in wet mounts of fresh sputum samples (<1 hour)	Wavy shaft, nonsynchronized movement	Straight, short, metachronal rhythm

phages in our sputum samples and available clinical variables: age, gender, smoking status (nonsmoker, ex-smoker, and current smoker), and steroid use (oral or inhaled).

The data presented in this article were collected as part of the routine work of a laboratory. Formal ethical approval was, therefore, not obtained. The study was undertaken in full compliance with the principles laid out in the Declaration of Helsinki.

Results

A consecutive series of 199 patients with respiratory disease each provided sputum samples. Eight patients (seven men and one women, aged 47–86 years) were excluded from the study as their samples were deemed "inadequate." Data on smoking status were missing for 22 (11.5%) cases, but information on all the other variables was complete. The mean age in the study was 66.9 years (SD 14.6, range 28–90 years). The cohort consisted of 167 men (87.4%) and 24 women (12.6%).

Summary diagnostic information for the 70 patients who had protozoa in their sputum is shown in Table II. Four patients with cancer, or possible cancer, also had COPD. "Query cancer" was the commonest provisional diagnosis, present in 24/70 (34%) patients with protozoa. Unfortunately, similar information on clinical diagnosis was not collected from those individuals in the cohort who did not have protozoa in their sputum.

As shown in Table III, there was a strong relationship between protozoa and monocytes/small macrophages (χ^2 65.2, $P > 0.001$).

A binary logistic regression model to predict the presence/absence of protozoa was produced using the following clinical parameters: age, gender, smoking status (nonsmoker, ex-smoker, and current smoker), and steroid

Table II. Relationship Between Provisional Diagnosis and the Presence or Absence of Monocytes/Small Macrophages in 70 Patients with Protozoa in their Sputum

Main diagnosis	*No monocytes/ small macrophages present*	*Monocytes/ small macrophages present*[a]	*Total*
AIDS	2	2	4
Asthma	1	1	2
Cancer or possible cancer being considered	4	20	24
COPD	2	6	8
Heart failure	0	3	3
Immunosuppression	0	1	1
Infection	4	6	10
Liver, pancreatic or renal disease	1	3	4
Lung fibrosis	0	2	2
Other	2	3	5
Tuberculosis	2	5	7
Total	18	52	70

[a]At least two groups of at least 10 monocytes/macrophages present

Table III. Relationship Between the Presence of Monocytes/small Macrophages and Protozoa in Sputum Samples of 191 Respiratory Patients

	Monocytes/small macrophages		
	No	*Yes*	*Total*
Protozoa			
No	52	18	70
Yes	19	102	121
Total	71	120	191

use (oral or inhaled). The Nagelkerke R^2 for the model was 0.073, indicating that only 7.3% of the variance in the presence/absence of protozoa was explained by the covariates in the model (see Table IV). Based on the classification table (not shown), this model would correctly predict 96.7% of those without protozoa but only 11.4% of those with protozoa. When a variable for the presence/absence of monocytes/small macrophages was added into the model all the other variables dropped out (see Table V). The presence of monocytes/small macrophages explained 40.5% of the variance and correctly predicted 84.3% of cases where protozoa were absent and 74.3% of cases where protozoa were present. The presence of monocytes/small macrophages in sputum samples was therefore a better predictor of the presence of protozoa than the available clinical variables.

A similar binary logistic regression model to predict the presence of monocytes/small macrophages, based on available clinical parameters, showed statistically significant relationships with age, smoking status, and steroid use, although the model only explained 13.4% of the variance.

Table IV. Coefficients for Binary Logistic Regression Model Predicting the Presence of Protozoa in Sputum Smears

	B	*S.E.*	*Wald*	*df*	*Sig.*	*Exp(B)*
Steroids			5.503	2	0.064	
Steroids (none)[a]	−1.320	0.641	4.239	1	0.040	0.267
Steroids (inhaled)[a]	−1.980	0.897	4.867	1	0.027	0.138
Known nonsmoker	0.620	0.312	3.959	1	0.047	1.859
Constant	0.451	.629	0.514	1	0.473	1.570

Exp(*B*) is the predicted change in odds for a unit increase in the covariate.
[a]Compared to those on oral steroids.

Table V. Binary Logistic Regression Model (When Monocytes/Small Macrophages Covariate was Added) Predicting the Presence of Protozoa in Sputum Smears

	B	*S.E.*	*Wald*	*df*	*Sig.*	*Exp(B)*
Monocytes/small macrophages	−2.741	0.370	54.768	1	<0.001	0.064
Constant	1.007	0.268	14.106	1	<0.001	2.737

Variables entered: monocytes/small macrophages, age, sex, inhaled corticosteroids, oral steroids, and smoking status.

Discussion

This study has demonstrated a significant relationship between the presence of monocytes/small macrophages and the presence of multiflagellated protozoa ($P < 0.001$). Relationships between protozoa and smoking, and protozoa and oral steroid use were also demonstrated.

These findings are significant in the light of a growing body of evidence for the presence of multiflagellated protozoa, in both sputum and nasal extrusions from patients suffering bronchial asthma and allergic rhinitis.[17–19] Our findings are also consistent with a number of reports indicating that protozoa lead to the recruitment of monocytes as part of the host's immune response to infection.[20–23]

A relationship between smoking and the activation/stimulation of human monocytes is widely recognized.[24–27] This relationship was also observed in this study.

The release of monocytes by bone marrow is associated with air pollution[28] and future studies should consider the extent to which the patients with protozoa and monocytes/small macrophages have been exposed to air pollution.

The study has a number of potential weaknesses. The cytologist who examined the microscope slides was not blinded in any way and was aware of the underlying hypothesis. This could have introduced some bias by inadvertently increasing the scrutiny for protozoa in samples that had high numbers of monocytes/small macrophages. Ideally, all the microscope slides should have been assessed for protozoa and monocytes/small macrophages by two independent observers who were blinded to each other's analysis. No gold standard currently exists against which to assess the presence or absence of protozoa. Attempts at culturing the organisms have been unsuccessful

(unpublished data), but nonculture methods may be developed in the future to address this issue.

The cohort of patients in this study was somewhat atypical in that it is primarily male. However, a retrospective review of 25 more recent cases where sputum samples were submitted to our laboratory for cytology, and where protozoa and monocytes/small macrophages were not identified, indicated that a similar percentage were male, 20/25 (80%). The preponderance of samples from men in this laboratory may in part be due to the high smoking rate in men in Spain, and perhaps due to a tendency for clinicians to request sputum cytology more often in men, as lung cancer is relatively common in Spanish men. A weakness of this study is the absence of diagnostic information in the individuals in the cohort who did not have protozoa in their sputum. Future studies should collect greater clinical detail including information on fever, respiratory symptoms, radiographic and laboratory abnormalities, any treatments and response.

The diagnoses summarized in this study were also provided on the laboratory request form, rather than representing discharge diagnosis and therefore need to be interpreted with caution.

A case–control study on an association between protozoa and asthma has been reported,[29] and it is possible to speculate that some of the 12 patients recorded as taking oral steroids may have had acute exacerbations of asthma, although this was not recorded on their laboratory request form.

Although the protozoa we have observed may simply be commensal organisms, our tentative finding of an association with an inflammatory response by monocytes/small macrophages leads us to believe that, although this line of research does not have immediate clinical application, the role of protozoa in respiratory disease does warrant further investigation. A randomized controlled trial demonstrating an improvement in symptoms and a fall in the number of monocytes/small macrophages after eradication of the protozoa by an appropriate antibiotic would strengthen the hypothesis that these protozoa are of pathological significance.

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7 Appendix 3: Associated correspondence

INSTITUT PASTEUR

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Letter to the editor

Could inhaled mite faeces introduce pathogens to the lungs?

Mites are small arthropods to which we are regularly exposed. They are commonly present in bedrooms and living rooms (house dust mites), kitchens (storage mites) and greenhouses (predatory mites). Several species including *Dermatophagoides*, *Blomia*, *Lepidoglyphus*, *Tyrophagus*, *Tetranychus* have strong associations with allergic and occupational diseases such as rhino-sinusitis, conjunctivitis, bronchial asthma and atopic dermatitis.

House dust mites are sources of multiple potent allergens in the indoor environment, and although many aspects of its biology are not yet understood [1–3], it is widely accepted that inhalation of mite antigens such as Der P1, found in dust mite faeces, contributes to the aetiology of asthma. However, mites may play a wider role in the pathogenesis of human disease.

The presence of microbes in and on house dust mites has been described in a number of papers. One paper identified bacterial sources of endotoxin in mite extracts and provided evidence that house dust mites contain *Bartonella* and other Gram-negative species. DNA sequence analysis has also shown the widespread presence of *Bartonella* species and uncharacterized α-proteobacteria, in both *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* [4]. Another paper, examining intestinal extracts from house dust mites, has demonstrated the existence of flagellated protozoal forms inside dust mites [5].

Other arthropods, such as termites and cockroaches, have also been shown to contain flagellated and ciliated protozoa in their hindgut, where they establish a symbiotic relationship and are necessary to the regulation of digestive processes [6] and metabolic activities [7]. Moreover, ectosymbiont bacteria are attached to the surface of these protozoa providing them with some motility [8].

These observations may be of pathogenic significance in the human respiratory tract. Infected sputum samples indicate the entry of bacteria into the lower respiratory tract of patients with chronic respiratory diseases, where the release of antigens, including endotoxin, produces potent inflammatory effects that are well described [9]. Some evidence on the pathogenic effects of *Bartonella* in human lungs has been reported [10,11]. Uncommon flagellated protozoa have also been found in the respiratory tract of patients suffering from respiratory disease [12–14]. Similarly, in a small unpublished case series of ten patients in South Wales, UK, with respiratory disease, five patients had evidence of protozoa in their sputum.

The potential for synergistic, concurrent exposure to allergenic epitopes from different sources, including organisms, has long been postulated as associated with asthma [15] and increasing evidence is emerging for this. For example, there is evidence that epitopes from helminths modulate allergic processes [16,17].

Protozoal lipopolysaccharide might also act as an adjuvant. Intranasal sensitization to ovalbumin has been shown to develop because of contaminating low levels of endotoxin suggesting that allergic sensitization may develop when aeroallergens are admixed with microbial products that act as adjuvants by activating toll-like receptors [18,19].

Inhalation of mite faeces containing Der P1 and protozoa is feasible. The aerodynamics of Der P1 inhalation have been assessed, both by measurement of indoor air and at the entrance to the nose. Der P1 has been shown to be present, attached to a range of particles of different sizes, including mite faeces. These particles are of a size that can be inhaled deep into the lungs [20].

Our conclusion is not that protozoal infection itself is the cause of asthma, but that the presence of flagellated protozoa together with Der P1 may play a synergistic role or a modulating role in acute exacerbations of respiratory disease and that further investigation is needed to explore whether there is a clinically significant relationship between the presence of flagellated protozoa and mite faeces Der P1 in the respiratory tract. The epidemiology (frequency, duration, and associated characteristics) of respiratory patients who have flagellated protozoa in their sputum needs to be further investigated. There is also a need to see whether eradication of any protozoa present aids the resolution of respiratory symptoms or reduces recurrent exacerbations of disease. DNA sequencing to characterise the flagellated protozoa seen in microscopy of the sputum of patients with respiratory disease is also needed.

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Is the Dust Mite Allergen Der p1 Protozoal in Origin?

To the Editor:

The major allergen in house dust mites faeces, Der p1, is believed to have a role in digestion of the mite's main food supply (human epithelium). We postulate that the digestion of human epithelial scales by dust mites may rely on a symbiotic relationship between protozoa and mites, whereby the Der p1 required for mite digestion is produced by protozoa or other intestinal commensals rather than being of mite origin.

Der p1, a cysteine protease [1], affects matrix remodelling and immuno-regulation [2]. It is a potent inducer of proinflammatory cytokines, disrupts intercellular tight junctions and facilitates the transfer of allergens across the respiratory epithelial barrier [3].

There are important protozoal parallels. In parasitic organisms, including protozoa, cysteine proteases play important roles in pathogenic mechanisms including: cell invasion, immunoevasion, encystment, etc. Flagellated protozoa, including *Trichomonas vaginalis* and *Giardia lamblia*, display these phenomena when parasitizing human mucosal cavities. Cysteine proteases have been implicated as virulence factors utilized by *T. vaginalis,* including cytototoxicity by extracellular matrix proteins degradation [4], epithelial cell detachment and apoptosis [5]. *G. lamblia* cysteine proteases damage mucosa in giardiasis [6] and may increase antigen penetration [7].

The Der p1 gene, also called Peptidase 1 (mite), contains two active enzymes, a cysteine protease inhibitor and a C1A cysteine peptidase. Der p1's cysteine peptidase falls within the clan CA, family C1, sub-family C1A, which includes around 1300 sequenced enzymes (http://merops.sanger.ac.uk/cgi-bin/make_frame_file?id=c1a;action=tree). The C1A subfamily includes papin and cathepsin like enzymes that are distributed widely across many life forms including plants, nematodes, viruses, fungi, protozoa and mammals. The difference in protein sequence between different protozoal C1A enzymes, using tools such as BLAST, is not greater than the difference between Peptidase 1 and, for example, a C1A enzyme found in *Tetrahymena thermophilia*. This moderately high level of sequence homology indicates that a protozoal origin for Der p1 cannot be excluded, although the sequence is not identical to that of any know protozoa.

The studies that identified the Der p1 protein structure/gene sequence used mite faeces or crushed samples of whole mites as their starting point [8]. They did not distinguish between gut contents and mite tissue and cannot therefore exclude a protozoal origin for Der p1. In contrast, Der p5 has been identified in secretory granules of the midgut of epithelial cells of house dust mites indicating that it is of mite origin [9].

Protozoal forms have been found in intestinal extracts of dust mites [10]. Multiflagellated protozoa have also been demonstrated in the sputum of a proportion of asthmatics and we have previously postulated that the inhalation of mite faeces could introduce protozoa to the lungs.

It is conceivable, therefore, that Derp1 could be of protozoal, rather than of mite origin, as is currently assumed. This question is important because if it is found that Der p1 has a protozoal origin, then a number of existing anti-protozoal chemotherapeutic agents could be explored as treatments for Der p1 related allergic conditions.

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Disruption of Airway Epithelium in Asthma Pathogenesis: Are Protozoa Responsible?

To the Editor:

In a recent paper published in this journal by Holgate and colleagues (1), the authors state that asthma is primarily a defect of epithelial barrier function, which allows greater access for environmental allergens, toxicants, and microbiota to infiltrate airway tissues. The innate immune response to viral infection, particularly with human rhinoviruses, has been proposed as the trigger for subsequent breakdown of the epithelial barrier. However, an alternative hypothesis is that secondary infection by other organisms plays a role (2).

In a different context, *in vitro* analysis has demonstrated that the flagellated protozoa *Trichomonas vaginalis* disrupts urogenital epithelial monolayers, causing epithelial damage, and may predispose to secondary HIV viral infection (3). In a respiratory context, the identification of multiflagellated protozoa in the sputum of patients with asthma and immune-depressed patients has been reported (4), and it has been hypothesized that the source of these protozoa may be the inhaled fecal pellets of dust mites (5).

A marked increase in the shedding of bronchial epithelial cells is found on examination of sputum from patients with asthma, signaling that the epithelial barrier of the airways is damaged. We have suggested that proteolytic enzymes, secreted by inhaled protozoa, could break the bonds between bronchial epithelial cells, accelerating cellular sloughing, and compromising the effectiveness of the airway epithelium as a barrier (6). Thus, viral respiratory infection could facilitate opportunistic overgrowth of normally nonpathogenic protozoa, leading to a cycle of increasing damage to airways and increased bronchial hyperresponsiveness.

In conclusion, we agree with the assertion by Holgate and colleagues that the integrity of respiratory epithelium is crucial to both the origin and progression of asthma, as well as playing a key role in asthma exacerbations. Further studies are needed to determine whether interplay exists between viral infections and the opportunistic overgrowth of other microbiota, including protozoa, which could play a role in disrupting airway epithelium and contribute to the pathogenesis of asthma.

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From the Author:

I was most interested to read the letter from Dr. Rafael Martínez-Girón regarding the possibility that an interplay may exist between viral infections and the opportunistic overgrowth of other microbiota, including protozoa, that could contribute to disrupting airway epithelium by breaking tight junctions through the release of proteolytic enzymes, thereby contributing to the pathogenesis of asthma. This is a possibility.

Indeed, there is increasing evidence that in addition to innately unstable epithelial tight junctions in asthma, there are a wide variety of environmental factors that possess proteolytic activity and capable of cleaving tight junction proteins. These include dust mite, pollen, fungal and cockroach allergens, and occupational exposures (e.g., proteolytic enzymes in washing powders) (1–5). There is also mounting evidence that this epithelial perturbation serves as a danger signal in activating and directing mucosal dendritic cell differentiation and activation (6, 7). Together, these interacting factors could be considered as part of the altered innate immune response of asthma as well as being involved in tissue injury and repair.

In general, viruses and other microbial interactions are assuming greater importance not only in the causation of exacerbations, but also in the origins and progression of certain types of asthma (8). There is clearly a need for further research in this field.

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