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False positive legionella serology in campylobacter infection: campylobacter serotypes, duration of antibody response and elimination of cross-reactions in the indirect fluorescent antibody test

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SUMMARY

Sera from 83 patients with campylobacter gastroenteritis were examined for the presence of legionella antibodies by indirect immunofluorescence. Twenty-one patients (25%) had positive titres ($\geqslant 16$) including 11 patients with titres of $\geqslant 128$. Legionella seropositivity persisted in 5 of 9 patients (55%) studied for 6–9 months. Campylobacter isolates were serotyped by the Penner scheme. Isolates associated with legionella seropositivity included Penner types 1, 2 and 4, the common endemic serotypes in England. Campylobacter blocking fluids were prepared from a range of Penner reference strains. The blocking fluid prepared from Penner type 11 was the most efficient at inhibiting the false-positive legionella titres. Using this absorption step legionella titres were inhibited from 24 of 26 patients (92%) with campylobacter but not from 8 patients with culture-proven legionnaires' disease. We recommend that this method is incorporated into routine diagnostic legionella serology in order to eliminate false-positive reactions due to campylobacter.

INTRODUCTION

Using formolized yolk-sac antigen of *Legionella pneumophila* serogroup 1, the Indirect Fluorescent Antibody Test (IFAT) is generally thought to be highly specific for the serological diagnosis of legionnaires' disease [1, 2]. We have recently reported however that legionella seropositivity can occur in patients with campylobacter gastroenteritis due to a cross-reaction between *L. pneumophila* and campylobacter antibodies [3, 4].

We have now measured the legionella titres of sera from a larger number of patients with campylobacter infection in order to study the relationship between legionella seropositivity and campylobacter serotype together with the duration of the antibody response. In addition we have devised a method for inhibiting these false positive titres using an absorption step in the legionella IFAT.

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PATIENTS AND METHODS

Sera were obtained from 83 patients with gastroenteritis whose stool cultures yielded *Campylobacter* species. Where possible, sera were drawn 10 days or more after the onset of symptoms to allow for the antibody response. Further sera were requested after 4–8 months from those who were legionella seropositive on initial testing. Cross-reacting sera were also available from five patients included in the original study [4].

Campylobacter isolates were saved and subsequently sent for serotyping by the Penner typing scheme [5] at the Campylobacter Reference Laboratory, Manchester Public Health Laboratory, Withington Hospital, Manchester M20 8LR.

Legionella IFA tests were performed using formolized yolk-sac antigen of *L. pneumophila* serogroup 1 (Division of Microbiological Reagents, CPHL, Colindale) as previously described [1].

Preparation of campylobacter blocking fluid

A random selection of campylobacter Penner type strains, including the common serotypes endemic in England, was obtained from the Reference Laboratory (Penner types 1, 2, 3, 4, 5, 6, 10, 11, 13, 16, 18, 19, 21, 23). Each strain was grown on ten 5% horse blood agar plates (Advanced Protein Products) in a microaerophilic atmosphere for 48 h at 42 °C. The growth from all plates was then harvested and dense suspensions of each serotype prepared in 10 ml of phosphate buffered saline (PBS) pH 7·2. These suspensions were heated at 100 °C for 60 min, cooled and then centrifuged at 1200 g for 12 min. The pellets were discarded and the supernatant, containing soluble heat-stable antigens, stored at 4 °C with the addition of sodium azide to inhibit bacterial growth (final concentration 0·08%).

Absorption of cross-reacting antibodies

Each campylobacter blocking fluid was tested in turn for its ability to inhibit legionella titres in cross-reacting sera. One in 16 and 1 in 32 dilutions of sera were made using the blocking fluid as diluent. The same dilutions were also made in PBS. These were incubated at 37 °C for 60 min and then at 4 °C for 18 h after which further doubling dilutions were made from the 1 in 32 dilutions using PBS. Absorbed and unabsorbed sera were then tested in parallel by legionella IFAT.

After initial evaluation with cross-reacting sera from nine patients, one campylobacter blocking fluid (Penner type 11) was chosen to perform all subsequent absorptions. This blocking fluid was also tested with sera from ten patients with legionnaires' disease (*L. pneumophila* serogroup 1 cultured from sputum, 8 patients; *L. pneumophila* serogroup 1 antigen detected in urine, 2 patients) in order to ensure that genuine legionella antibodies were not absorbed. Sera from 12 other patients with suspected legionnaires' disease (pneumonia together with positive serology) were also tested.

RESULTS

A total of 99 sera were obtained from 83 patients. There were 22 acute sera (day 4-9 after onset of symptoms) and 68 early convalescent (day 10-60). Overall 21 patients (25%) had a positive legionella IFAT titre ($\geqslant 16$) in one or more sera.

Table 1. *Legionella IFAT titres in late convalescent sera from nine patients*

Initial titre	Convalescent sample: Time after onset (months)	Convalescent sample: Titre
256	9	< 16
32	6	< 16
⩾ 512	6	16
64	7	< 16
⩾ 512	7	32
32	8	16
64	7	32
2048	7	512
64	7	< 16

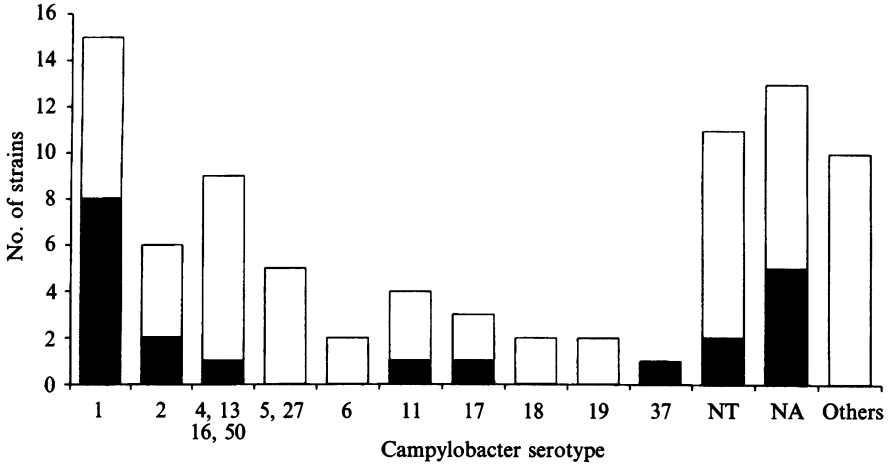

Fig. 1. Distribution of campylobacter serotypes and relationship to legionella seropositivity. NT, not typable; NA, not available. □, Seronegative; ■, seropositive.

Three patients showed a rising titre from < 16 to 64, one patient a rise from 32 to 64 and one patient a fall from ⩾ 512 to 32. Six patients had single titres between 16 and 64 and ten patients single titres of ⩾ 128. All other patients had negative titres (< 16).

Late convalescent sera (6–9 months) were obtained from 9 of the 21 positive patients. These results are shown in Table 1. Five patients (55%) were still legionella seropositive although the titres had fallen to 16 or 32 in four cases. One patient still had a high titre of 512. The other four patients were seronegative.

Campylobacter serotypes

Campylobacter isolates were available for serotyping from 70 of the 83 patients. Fig. 1 shows the distribution of serotypes and the relationship to legionella seropositivity. The commonest serotype was Penner type 1 (15 strains) followed by type 4, 13, 16, 50 (10 strains) and type 2 (6 strains). Eleven strains were not typable by the range of typing sera used. Other serotypes isolated were 5, 27, 6, 10, 11, 15, 17, 18, 19, 23, 29, 37, 40, 41, 53 and 55.

Table 2. *Reduction in legionella titres after absorption with specific Penner type campylobacter blocking fluids*

Patient	Penner type of isolate	IFAT titre (No absorption)	Penner type of campylobacter blocking fluid													
			1	2	3	4	5	6	10	11	13	16	18	19	21	23
1	NA*	256/512	32	16	16	32	32	256	32	< 16	32	512	512	16	512	32
2	NA	128	64	32	32	64	16	128	< 16	< 16	16	128	128	16	128	64
3	NA	⩾ 512	< 16	< 16	< 16	< 16	32	⩾ 512	< 16	< 16	< 16	⩾ 512	⩾ 512	16	⩾ 512	⩾ 512
4	NA	⩾ 512	32	< 16	16	< 16	32	⩾ 512	16	16	32	⩾ 512	⩾ 512	16	⩾ 512	⩾ 512
5	1	256	256	256	32	32	32	256	128	32	64	256	256	32	256	32
6	1	⩾ 512	16	< 16	16	32	128	⩾ 512	32	< 16	64	⩾ 512	⩾ 512	16	⩾ 512	128
7	2	⩾ 512	256	16	16	128	64	⩾ 512	32	16	64	⩾ 512	⩾ 512	64	⩾ 512	⩾ 512
8	11	256	< 16	< 16	< 16	< 16	32	256	< 16	< 16	16	256	256	16	256	128
9	17	32/64	16	16	< 16	16	< 16	16	< 16	< 16	16	32	32	< 16	16	32

* NA, not available.

Serotypes associated with legionella seropositivity were types 1, 2, 4, 50, 11, 17 and 37 together with two strains that were not typable. Seropositivity in particular was associated with Penner type 1 (8 of 15 strains) whereas only 1 of 9 patients with type 4, 13, 16, 50 was positive. However the number of patients with each serotype was relatively small and it was difficult to draw any conclusion between campylobacter serotype and legionella cross-reaction. Unfortunately the isolates were not available for serotyping from 13 patients of whom 5 were legionella seropositive.

Absorption of cross-reacting sera

Campylobacter blocking fluids were each tested for their ability to absorb cross-reacting sera. Sera from the 21 positive patients together with stored sera from 5 of the patients previously reported were available for testing (26 patients in total).

Initially 14 blocking fluids were tested against sera from 9 patients. Table 2 shows the legionella titres before and after absorption with each blocking fluid. The serotype of the clinical campylobacter isolate associated with each serum is also shown. The different blocking fluids varied in their ability to absorb these sera. Penner types 6, 16, 18 and 21 failed to produce any significant absorption whereas types 1 and 23 produced variable absorption depending on the sera tested. Interestingly type 1 failed to absorb the serum from patient 5 who had been infected with a type 1 strain. Types 2, 3, 4, 5, 10, 13 and 19 produced significant absorption in most sera ($\geqslant$ 4-fold reduction) although some sera were unaffected. Type 11 completely absorbed six sera (post absorption titre < 16) and produced $\geqslant$ 8-fold reductions in the other three. Thus it was decided that Penner type 11 would be used to perform the absorptions with sera from the remaining patients and that the demonstration of a $\geqslant$ 4-fold reduction in titre after absorption constituted a false positive initial test.

Table 3 shows the titres before and after absorption with type 11 blocking fluid in the sera from the remaining 17 patients (including the late convalescent sera from 5 patients). The titres were significantly reduced in sera from 15 of these patients. In 18 out of 24 sera tested the post-absorption titre was < 16. A further 2 sera gave 8- and 16-fold reductions respectively.

Combining the results from Tables 2 and 3, campylobacter blocking fluid Penner type 11 consistently eliminated false-positive legionella titres in 24 of the 26 patients studied (92%).

There were two patients where absorption did not occur. Patient 17 showed a two-fold reduction from a titre of 32 to 16 (acute serum) and no reduction in the late convalescent serum. The same results were obtained using Penner type 2 blocking fluid even though this was the Penner type of the patient's campylobacter isolate. Patient 22 had no reduction in titre after absorption of either the early or late convalescent sera. Unfortunately the serotype of the infecting campylobacter was not available for this patient. As this patient had a high legionella titre ($\geqslant$ 512) we decided to test the other campylobacter blocking fluids against this serum. Blocking fluids from Penner types 1, 2, 3, 4, 5, 10, 13, 19 and 23 were each tested. In addition the absorption method was modified by using the blocking fluid as diluent for all dilutions rather than using PBS to dilute beyond the 1 in 32 dilution. None of these blocking fluids had any effect on the legionella titre from

Table 3. *Absorption of cross-reacting antibodies by campylobacter blocking fluid type 11*

Patient	Campylobacter serotype	Time after onset	Legionella IFAT titres	
			Pre-absorption	Post-adsorption
10	NT*	8 days	⩾ 512	< 16
11	4,50	14 days	⩾ 512	32
		6 weeks	32	< 16
		7 months	32	< 16
12	1	10 days	32	< 16
13	1	10 days	⩾ 512	< 16
		6 months	16	< 16
14	37	12 days	128	< 16
15	11	4 weeks	32	< 16
		6 weeks	64	< 16
16	1	11 days	64	< 16
17	2	8 days	32	16
		8 months	16	16
18	NA†	8 days	16	< 16
19	2	11 days	64	< 16
20	1	10 days	64	< 16
21	1	10 days	64	< 16
		7 months	32	< 16
22	NA	16 days	2048	2048
		7 months	512	512
23	NA	12 days	⩾ 512	< 16
24	NA	10 days	32	< 16
25	NA	7 days	⩾ 512	< 16
26	NT	13 days	⩾ 512	64

* NT, not typable; † NA, not available.

this patient. This patient was an 18-year-old female with no history of past or recent pneumonia who had had acute diarrhoea and vomiting prior to isolation of campylobacter from her stool culture.

Effect of absorption on genuine legionella sera

Absorption with campylobacter blocking fluid (Penner type 11) had no effect on the legionella titres from 8 patients with culture-proven legionnaires' disease and 2 patients with positive legionella urinary antigen (Table 4). Of the 12 patients with suspected legionnaires' disease there was no absorption of antibody from 10 patients and only a twofold reduction (not significant) from one other.

Absorption with this blocking fluid did however reduce the titres from patient LD15 (Table 4). This was a 22-year-old man with pneumonia who had a legionella titre of 64/128 on admission to hospital. His convalescent sample (8 days later) had the same titre. Absorption with blocking fluid reduced the legionella titres of both sera to < 16. He presented to hospital with a 1-day history of chest pain and haemoptysis that followed several days of a productive cough. He was otherwise fit and healthy and worked at a foundry. Interestingly he gave a history of diarrhoea and vomiting which had occurred in the week before admission and which had been attributed to eating beef-burgers. His chest infection responded promptly to erythromycin. Sputum and blood cultures were negative as was

Table 4. *Effect of absorption with campylobacter blocking fluid on legionella titres from confirmed (LD1–LD10) or suspected (LD11–LD22) cases of legionnaires' disease*

		Legionella IFAT		
Patient	Clinical details	Acute	Convalescent	Convalescent after absorption
LD1	Pneumonia, following renal transplant	< 16	32	32
LD2	Pneumonia	< 16	512	512
LD3	Pneumonia	NA*	512	512
LD4	Pneumonia	< 16	64	64
LD5	Pneumonia	< 16	32	32
LD6	Pneumonia	NA	32	32
LD7	Pneumonia	NA	32	32
LD8	Pneumonia	NA	128	128
LD9	Pneumonia	32	128	128
LD10	Pneumonia; returned from Greece	< 16	4096	4096
LD11	Pneumonia; returned from Greece	< 16	128	128
LD12	Pneumonia	NA	512	512
LD13	Pneumonia	512	512	512
LD14	Pneumonia; returned from Caribbean	< 16	256	128
LD15	Pneumonia	64/128	64/128	< 16
LD16	Pneumonia	< 16	64	64
LD17	Pneumonia; returned from Spain	< 16	512	512
LD18	Pneumonia; returned from Portugal	< 16	512	512
LD19	Pneumonia; returned from Turkey	< 16	512	512
LD20	Pneumonia	NA	4096	4096
LD21	Pneumonia	NA	128	128
LD22	Pneumonia	16	64	64

Patients LD1–8: *L. pneumophila* serogroup 1 isolated from sputum.
Patients LD9–10: *L. pneumophila* serogroup 1 antigen detected in urine.
* NA, not available.

serology for *Mycoplasma pneumoniae*, influenza A and B, adenovirus, chlamydia and *Coxiella burnetii*. Stool cultures had not been performed.

DISCUSSION

Legionella seropositivity can occur in a large proportion of patients with campylobacter gastroenteritis. In a previous study [4] 8 of 11 patients (73%) had positive titres in early convalescent sera whereas in this study 25% of 83 patients were legionella seropositive. Although smaller it remains a significant proportion. The difference may be due to the small number of patients included in the first study. Moreover, the patients in this study were largely from the community whereas the previous study was from patients whose gastroenteritis was severe

enough to cause admission to an Infectious Diseases Unit, although we have not investigated the relationship between disease severity and legionella seropositivity.

Serological cross-reaction between legionella and campylobacter has also been described by Cheesbrough and colleagues [6]; 4 of 16 patients (25%) with campylobacter infection were legionella seropositive by IFAT.

Of the 21 patients who were seropositive in this study, 11 (52%) had legionella titres of $\geqslant 128$. Single titres of 128 or above, together with appropriate clinical features are considered a presumptive diagnosis of legionellosis. This cross-reaction therefore has the potential to cause diagnostic confusion which in turn may lead to inappropriate epidemiological investigation.

In approximately half of the patients who were followed up there were still detectable legionella-reactive antibodies 6–9 months after the campylobacter infection, although the titres had generally declined. This indicates that the serological cross-reaction between campylobacter and legionella is not necessarily short-lived despite the observation that the cross-reacting antibodies in the IFAT are mainly of the IgM class [4].

The number of reported cases of campylobacter in 1990 was 35000 [7]. However the number of cases confirmed microbiologically is probably overshadowed by the actual number of infections. One survey [8] has estimated an incidence of 1100 per 100000 population per year (approximately 500000 cases per year in England and Wales). Thus approximately 125000 patients per year may develop legionella seropositivity and this may persist for up to 6–9 months in 70000. This has significant implications for seroprevalence studies, for the serological investigation of suspected outbreaks and may account for a proportion of the background prevalence of legionella-reactive antibodies in the adult population.

There was a wide range of campylobacter serotypes isolated from the stool cultures of the patients in this study. These included the more common serotypes (Penner types 1, 2 and 4) endemic in England (E. Sutcliffe, personal communication). Legionella cross-reaction was not associated with any one particular campylobacter serotype and there was no specific correlation between serotype and the development of legionella seropositivity. However the number of patients with each serotype was small.

Depending on the campylobacter strain used, legionella-reactive antibodies were inhibited by diluting sera in the heated supernatant of a campylobacter suspension (blocking fluid). This suggests that soluble heat-stable campylobacter antigens were involved in the cross-reaction. Although the Penner typing scheme is based on heat-stable soluble antigens [5], the cross-reaction is not limited to one serotype specific epitope as infection with a range of campylobacter serotypes was associated with the cross-reaction.

We have previously found that campylobacter blocking fluid prepared from a patient's own isolate can inhibit cross-reacting antibodies in that patient's serum but that a blocking fluid prepared from a randomly selected campylobacter strain may fail to do so (unpublished), a finding confirmed by Fallon and Abraham [9] who also noted that an extract from a randomly selected strain of *Escherichia coli* [10] had no effect as a blocking fluid on any campylobacter sera. In this study we found that some campylobacter blocking fluids were efficient at inhibiting this

serological cross-reaction whereas others did not. Furthermore several blocking fluids completely inhibited the cross-reaction from some patients but not from others (Table 2). This suggests that several antigens may be responsible for the cross-reaction and that the absorption with one campylobacter serotype is dependent on the presence of the same antigen on the serotype generating the cross-reaction. It is also possible that the antigen(s) involved may not be shared between different strains of the same Penner serotype.

The blocking fluid prepared from Penner type 11 campylobacter reference strain was the most efficient at inhibiting the legionella cross-reaction irrespective of the serotype of the campylobacter isolated from the patient's stool culture. Overall the titres were significantly inhibited in 24 of 26 patients studied. One patient had low legionella titres (16/32) which were not inhibited by this blocking fluid. It is possible that these titres were in fact not due to cross-reaction with campylobacter but present in the patient's sera prior to the campylobacter infection. As the background seroprevalence of legionella antibodies at low titres is 1–3% [11, 12] then we would expect one or two patients from our sample to have pre-existing legionella antibodies.

Patient 22 (Table 3) however had a significant legionella titre of 2048 which was not inhibited by type 11 blocking fluid. Unfortunately the campylobacter isolate from this patient was not available for serotyping or for testing cross-reactivity. However this serum was not inhibited by any of the campylobacter blocking fluids which actively absorbed other sera. Furthermore there was no reduction in titre after absorption with a blocking fluid prepared from *E. coli* which may prevent non-specific cross-reactions with other gram-negative bacteria that can occur when using heat-killed antigens in immunofluorescence [10, 13], although this is not thought to occur when using formolized yolk-sac antigen in legionella IFA tests [1, 2]. These results are difficult to explain as the patient had no clinical features past or present to suggest infection with legionella. Possibly the campylobacter infection did lead to legionella seropositivity but the campylobacter strain was antigenically distinct from any of the reference strains tested. Alternatively it is possible that the legionella-reactive antibodies in this case were not related to the campylobacter infection but to some other event.

Absorption of sera with campylobacter blocking fluid Penner type 11 did not have any effect on legionella IFAT titres from ten patients with confirmed legionnaires' disease (Patients LD1–10, Table 4). Although the diagnosis of legionellosis in the other 12 patients was made on clinical and serological findings alone the results also tend to suggest that this blocking fluid does not reduce genuine legionella titres. Interestingly the legionella titres were inhibited from patient LD15 (Table 4). However we now believe that this patient was unlikely to have had legionellosis on the retrospective analysis of his illness as he was young, had no risk factors for legionellosis and had had a recent episode of food-associated gastroenteritis prior to his respiratory symptoms. That his legionella titres were inhibited by the blocking fluid suggests that the gastroenteritis was probably due to campylobacter infection and his chest symptoms to some other cause. This case demonstrates the potential for diagnostic confusion that may occur in routine legionella serology tests.

Preparation of campylobacter blocking fluids were straightforward. Using

Penner type 11 reference strain, the blocking fluid reliably inhibited false-positive legionella titres in the majority of patients without affecting legionella-specific antibody. This blocking fluid was stable for at least 4 months if stored at 4 °C. The absorption step was a simple procedure and we recommend its incorporation into the legionella IFAT. We suggest that any serum with a positive legionella IFAT titre ($\geqslant 16$) is retested after overnight absorption with this blocking fluid. However caution should still be maintained in interpreting positive legionella serology even after absorption as this procedure has only been validated for patients with a limited range of campylobacter serotypes. This will particularly apply to countries other than England and Wales where the predominant campylobacter serotypes may be different. Blocking fluids prepared from other campylobacter strains may not have reliable absorbing activity. This may include clinical isolates of Penner type 11, although our preliminary data (not shown) suggest that these may be as efficient as the Reference type strain.

We recommend that every effort be made to confirm suspected legionellosis by culture in order to prevent diagnostic confusion. This aids epidemiological investigation as clinical and environmental isolates of legionella can be compared. Where the diagnosis is based on serology alone the test should be repeated after absorption with the appropriate campylobacter blocking fluid.

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