ORIGINAL ARTICLE 10.1111/j.1469-0691.2008.02075.x

Chlamydophila pneumoniae diagnostics: importance of methodology in relation to timing of sampling

D. Hvidsten[1], D. S. Halvorsen[1], B. P. Berdal[2] and T. J. Gutteberg[1]

1) *Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø and* 2) *Institute of Microbiology, Armed Forces Medical Services, Oslo, Norway*

Abstract

The diagnostic impact of PCR-based detection was compared to single-serum IgM antibody measurement and IgG antibody seroconversion during an outbreak of *Chlamydophila pneumoniae* in a military community. Nasopharyngeal swabs for PCR-based detection, and serum, were obtained from 127 conscripts during the outbreak. Serum, drawn many months before the outbreak, provided the baseline antibody status. *C. pneumoniae* IgM and IgG antibodies were assayed using microimmunofluorescence (MIF), enzyme immunoassay (EIA) and recombinant ELISA (rELISA). Two reference standard tests were applied: (i) *C. pneumoniae* PCR; and (ii) assay of *C. pneumoniae* IgM antibodies, defined as positive if ≥2 IgM antibody assays (i.e. rELISA with MIF and/or EIA) were positive. In 33 subjects, of whom two tested negative according to IgM antibody assays and IgG seroconversion, *C. pneumoniae* DNA was detected by PCR. The sensitivities were 79%, 85%, 88% and 68%, respectively, and the specificities were 86%, 84%, 78% and 93%, respectively, for MIF IgM, EIA IgM, rELISA IgM and PCR. In two subjects, acute infection was diagnosed on the basis of IgG antibody seroconversion alone. The sensitivity of PCR detection was lower than that of any IgM antibody assay. This may be explained by the late sampling, or clearance of the organism following antibiotic treatment. The results of assay evaluation studies are affected not only by the choice of reference standard tests, but also by the timing of sampling for the different test principles used. On the basis of these findings, a combination of nasopharyngeal swabbing for PCR detection and specific single-serum IgM measurement is recommended in cases of acute respiratory *C. pneumoniae* infection.

Keywords: disease outbreaks, immunoglobulin M, polymerase chain reaction, seroconversion, serologic tests, time factors
Original Submission: 22 May 2007; **Revised Submission:** 24 January 2008; **Accepted:** 28 May 2008
Associate Editor: M. Ieven
Clin Microbiol Infect 2009; **15:** 42–49

Corresponding author and reprint requests: D. Hvidsten, Department of Microbiology and Infection Control, Box 56, University Hospital of North Norway, N-9038 Tromsø, Norway
E-mail: dag.hvidsten@unn.no

Introduction

Diagnosing acute respiratory *Chlamydophila pneumoniae* infection is a challenge. Although some patients present with a chronic cough or atypical pneumonia [1], most *C. pneumoniae* infections are asymptomatic or mild, and remain unrecognized [2–4]. Establishing a diagnosis of *C. pneumoniae* is important because of its impact on antibiotic treatment of symptomatic individuals, and in order to contain outbreaks. With regard to the detection of *C. pneumoniae*, many studies have shown poor agreement between the results obtained by culture, PCR and serology [3–6], whereas others have found good agreement [7,8].

The diagnostic accuracy of the microimmunofluorescence (MIF) test has also been questioned [4,5,9–13]. Following primary infection, IgM antibodies detectable by MIF may not appear before 3 weeks after onset of illness, and IgG antibodies may not reach levels detectable by MIF for 6–8 weeks [14].

In contrast, recombinant ELISA (rELISA) measures *Chlamydia* genus-specific lipopolysaccharide antibodies, starting at the onset of *C. pneumoniae* symptoms [8,13,14]. Thus, rELISA has been reported to be very sensitive but also less specific, giving rise to false-positive results [11,15]. The recently introduced *C. pneumoniae*-specific enzyme immunoassay (EIA) has been evaluated, with promising results [15].

Generally, the detection of a specific antibody response is regarded as evidence of infection. Likewise, the direct detection of the intracellular bacterium *C. pneumoniae* in respiratory samples is considered to be sufficient evidence for the diagnosis of *C. pneumoniae* respiratory tract infection. These two very different diagnostic principles—antibody

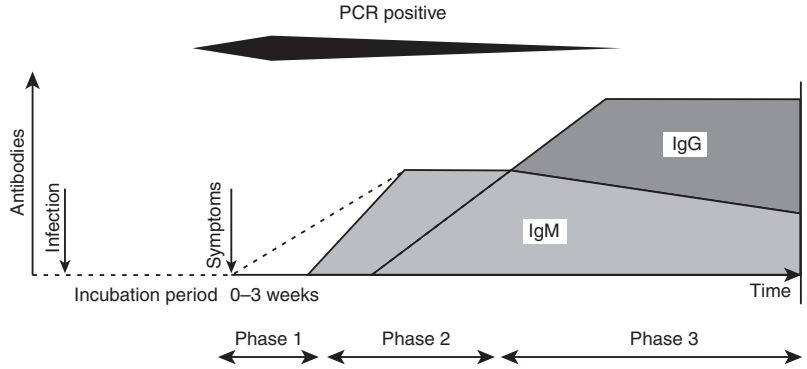

FIG. 1. The theoretical dynamics of PCR and serology results in acute *Chlamydophila pneumoniae* infection. The test outcome will depend on the choice of the reference standard. If serum IgM antibody is chosen as the reference standard, a positive PCR result is taken as 'false-positive' during the first few weeks following onset of illness before the IgM antibody test turns positive (phase 1). Normally, PCR becomes negative after some days to months, considered as 'false-negative' (phase 3). If PCR is applied as the reference standard, the IgM antibody is 'false-negative' in phase 1 and appears 'false-positive' in phase 3. Only in phase 2 will the results agree. *Chlamydia* genus-specific IgM antibodies is first and may be present in the acute-phase serum, whereas microimmunofluorescence (specific) IgM antibodies may not appear for 3 weeks; IgG, serum IgG antibodies may not reach diagnostic titre levels for 6–8 weeks [14].

measurement and detection of DNA—may yield different time-dependent results during infection, as observed in pneumonia caused by other atypical community-acquired pathogens [16–18]. We postulate that during *C. pneumoniae* infection, PCR assays detect DNA in clinical samples earlier than serological assays detect an IgM antibody response. Consequently, in assay evaluation studies, the choice of reference standards and the timing of sampling will influence the results (Fig. 1).

The aim of this study was to compare PCR-based detection of *C. pneumoniae* DNA with three different *C. pneumoniae* antibody assays. Access to stored frozen sera, drawn 8–11 months before the outbreak, provided an individual baseline or pre-outbreak antibody status, which was of great advantage when differentiating between the IgM and IgG antibody responses during the outbreak and the baseline seroreactivity.

Materials and Methods

Subjects and study design

The outbreak, lasting from April to the end of June 2000, occurred among approximately 2000 conscripts stationed in two military camps in Troms county of northern Norway. A serum sample ('pre-outbreak serum') was drawn routinely from each conscript upon enrolment in August 1999. One mL was frozen in polystyrene containers, which were sealed with paraffin wax and stored at −20°C at the Norwegian Armed Forces Institute of Microbiology, as reported previously [2]. A second blood sample ('outbreak serum') was drawn from the eligible conscripts during the outbreak. The sera were analysed 1.5–2 years after the outbreak. Shortly before analysis, the pre-outbreak sera were thawed and shipped overnight to the laboratory in charge of the analyses.

Subjects were eligible for the study if a pre-outbreak serum, an outbreak serum and a nasopharyngeal swab sample were available. The nasopharyngeal samples were taken only once, at the same time as the outbreak sera. Clinical illness and respiratory tract symptoms were the reasons for nasopharyngeal sampling for PCR-based detection and serological testing, initially for several respiratory pathogens, in order to find the causative infectious agent. Conscripts from the same unit with mild symptoms or without symptoms were also asked to submit specimens. Information regarding age, gender, antimicrobial treatment and respiratory symptoms were obtained in interviews and/or from medical records. The conscripts gave verbal informed consent to participate in the study, which was approved and recommended by the Norwegian armed forces.

Definitions

The case definition of acute respiratory *C. pneumoniae* infection was that of a conscript living in a closed military community during the outbreak with: (i) a positive test result according to the definitions; and (ii) a negative IgM serology finding at baseline. Irrespective of respiratory symptoms, these conscripts represent the test-positive group. Taking into account that PCR detection of *C. pneumoniae* DNA is a test principle that is different from that of the IgM and IgG antibody assays, two reference standards were applied: (i) PCR results; and (ii) IgM antibody results.

The IgM antibody reference standard was considered positive when ≥2 independent IgM assays were positive. As MIF and EIA assays are produced by the same manufacturer and employ an identical chlamydial antigen, the definition excludes the combination MIF/EIA IgM positivity.

IgG seroconversion was defined as: (i) a ≥3-fold OD increase of rELISA IgG in combination with (ii) a ≥4-fold titre increase of MIF IgG and/or (iii) a ≥1.5-fold increase of EIA IgG if the first value was <130 enzyme immunounits (EIU) *or* a ≥1.3-fold increase if the first value was >130 EIU.

A true-negative result was defined as any result that was negative according to both PCR and IgG seroconversion, and <2 IgM antibody assays yielding positive results (test-negative group). The combination of positive results according to MIF/EIA IgM and/or IgG seroconversion was also defined as negative for the reasons mentioned above. For calculation of *C. pneumoniae* antibody prevalence in the 127 conscripts before and during the outbreak, samples were defined as positive when ≥2 IgM and/or ≥2 IgG antibody assays were positive.

Nasopharyngeal swabs and PCR-based detection

A thin, flexible metallic swab with a rayon tip was inserted through one of the nostrils into the nasopharyngeal tract for 15 s before being placed in 2.5 mL of transport medium (medium essential medium (Gibco), fetal bovine serum, Hepes 1 M, gentamicin 50 mg/L, adjusted to pH 7.1–7.3).

Vials were sent to the laboratory overnight at ambient temperature, and stored at 4°C for 0–4 days until PCR analysis. A modified nested *C. pneumoniae* PCR with outer primers (CP1 and CP2) specific for the major outer membrane protein genes (*ompA*) of *C. pneumoniae* and *Chlamydia psittaci* was performed as described by Tong and Sillis [19]. The outer primers (CP1, 5′-TTACAAGCCTTGCCTGTAGG-3′; and CP2, 5′-GCGATCCCAAATGTTTAAGGC-3′) allowed amplification of a 333-bp product, and the internal primers (CPC, 5′-TTATTAATTGATGGTACAATA-3′; and CPD, 5′-ATCTACGGCAGTAGTATAGTT-3′) allowed amplification of a 207-bp product. Internal primers are considered to be highly specific for *C. pneumoniae* [20]. The sample was vortexed for 1 min, and then the rayon tip was discarded before the sample was centrifuged at 2500 g for 10 min. After removal of 1.5 mL of the supernatant, the sample was vortexed and divided into two aliquots of 200 μL each, one of which was processed as a native sample, and the other of which was spiked with 2000 copies/mL of *C. pneumoniae* DNA. The DNA extraction was performed using the manual QIAmp DNA mini kit or the automatic QIAmp 96 DNA Blood BioRobot 9604 kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. PCR was performed with one of three PCR systems: PE 9600, PE 9700 or PE 2400 (PE Applied Biosystems, Foster city, CA, USA). In the rack, each tube of native sample was preceded by a tube with PCR buffer and followed by the spiked sample and a negative sample containing ddH_2O. For the first PCR, the thermal cycling was initiated at 95°C for 5 min, and this was followed by 20 cycles, each consisting of three 1-min periods: denaturation at 94°C; annealing at 65°C; and elongation at 72°C. The annealing temperature was lowered by 0.5°C every cycle to 55°C. The next 20 cycles were carried out with cycles of 30 s at 94°C, 55°C and 72°C. The second PCR consisted of 30 cycles with incubation at 95°C for 5 min, followed by 94°C for 15 s and two periods at 50°C and 72°C, each for 30 s. The amplification products were separated by agarose (3%) gel electrophoresis for 1 h in buffer (0.75 × phosphate-buffered saline) and visualized by ethidium bromide staining. The detection limit of the second PCR was 5 fg, corresponding to approximately five genome copies. The reagents were prepared in a clean room, and the principle of unidirectional workflow was applied, with separate rooms and separate ventilation for each PCR.

Serological assays

During the *C. pneumoniae* outbreak, the MIF test was performed routinely and was repeated using paired sera, in conjunction with two other antibody assays. On the basis of preliminary MIF results, equal numbers of positive and negative samples were analysed within the same run. Paired sera from each subject within each run were randomized and blindly analysed. The sera were assayed for IgM and IgG antibodies with three commercially available tests that are based on different immunoassay principles, adhering strictly to the manufacturers' instructions concerning protocol, calculation and reporting of results (Table 1).

With the MIF test (Labsystems, Helsinki, Finland), sera were screened for IgM antibody detection at a 1 : 8 dilution. Reactive sera were further diluted 1 : 16, 1 : 32 and 1 : 64. The IgG analyses started with screening at 1 : 16 and, if reactive, were diluted two-fold from 1 : 32 to 1 : 512.

The calculation for the EIA test (Labsystems) was performed according to: $OD_{sample} - OD_{blank}/OD_{calibrator} - OD_{blank} \times k$ where k is a constant.

In order to compare EIA with MIF, the manufacturer applies a constant (k = 130) in the calculation of IgG antibody values, and recommends reporting results in enzyme immunounits (EIU). Samples with absorbance higher than that of the positive control were further diluted 1 : 200, 1 : 400 and 1 : 800 for IgG.

The rELISA test applies recombinant genus-specific *Chlamydia* antigens (Medac, Hamburg, Germany). After the blank

TABLE 1. Cut-off values for serological *Chlamydophila pneumoniae* IgM and IgG antibody assays and criteria for IgG antibody seroconversion[a]

Assay (unit)	Sample		Criteria for seroconversion
	Negative	Positive	
MIF (titre)			
IgG	<32	≥32	≥4-fold titre increase
IgM	<16	≥16	
EIA (EIU)			
IgG	<30[b]	>45	If <130, ≥1.5-fold increase If >130, ≥1.3-fold increase
IgM	<1.1[b]	>1.1	
rELISA (cut-off index)[c]			
IgG	<0.9[b]	>1.1	≥3-fold increase
IgM	<0.85[b]	>1.15	

MIF, microimmunofluorescence; EIA, enzyme immunoassay; rELISA, recombinant ELISA; EIU, enzyme immunounits.
[a]Cut-off values and criteria for seroconversion are according to the manufacturers' recommendations.
[b]For the computation, all intermediate values ('grey zone') were considered to be negative.
[c]Cut-off index defined as $OD_{sample}/OD_{cut\text{-}off}$.

was subtracted from all values, the cut-off was calculated as follows: OD of the negative control +0.37 for IgM and +0.32 for IgG. The cut-off index is defined as: $OD_{sample}/OD_{cut\text{-}off}$. Samples with a cut-off index >1.15 for IgM and >1.10 for IgG were considered positive. Samples with OD >2.0 were further diluted 1 : 800 for IgG.

Statistical methods

Sensitivity and specificity were calculated by SPSS 14.0.1 for Windows. Differences in duration of illness were calculated by the Mann–Whitney *U*-test. Cochran's *Q*-test was used to compare the detection rates of more than two assays. The strength of agreement between two tests was calculated with kappa (κ) values [21]. All values were dichotomized into positive or negative, except when testing the correlation between two IgM antibody assays by Spearman's rank correlation; p <0.05 (two-tailed) was considered to be statistically significant. The linear regression of cumulative sensitivity of the PCR and IgM antibody assays were calculated with Graph Pad Prism 3.00.

Results

Classification

Samples were obtained from 127 conscripts (123 males and four females with mean age (SD) of 20.2 years (1.03), range 18.7–27.1 years). In the test-positive group (n = 48), respiratory symptoms were reported by 44 conscripts (100%; no data, n = 4). Antibiotics were prescribed in 37 cases (88%; no data, n = 6). The corresponding figures in the test-negative group (n = 79) were 54 (77%; no data, n = 9) with respiratory symptoms and 14 (26%; no data, n = 25) who received antibiotics.

PCR and serology

Among the 127 conscripts studied, 33 were PCR-positive, 40 were IgM antibody-seropositive and 12 demonstrated IgG seroconversion, according to the study criteria (Table 2). Regarding the IgM antibody-positive and seroconversion samples, 29 cases were PCR-positive and 13 were PCR-negative. The median interval from start of symptoms until sampling was 18 days (range 3–45 days) in the PCR-positive group and 47.0 days (range 12–76 days) in the PCR-negative group. The difference between the intervals was statistically significant (p 0.01) (Fig. 2). The median for all positive samples was 24 days. The detection rate of each IgM assay differed significantly from that of the others and from those of PCR. The strength of agreement between any two of the assays (one PCR and three IgM assays) varied from 'moderate' (κ = 0.41–0.60) to 'good' (κ = 0.61–0.80) [21]: PCR vs. rELISA, κ = 0.56; PCR vs. MIF, κ = 0.61; PCR vs. EIA, κ = 0.63; MIF vs. rELISA, κ = 0.67; EIA vs. rELISA, κ = 0.71; and MIF vs. EIA, κ = 0.78.

The strength of agreement was also tested for the following combinations: PCR vs. ≥2 IgM positive, κ = 0.64; PCR vs. ≥2 IgM *and* IgG seroconversion, κ = 0.65; PCR vs. IgG seroconversion, κ = 0.25. (κ = 0.21–0.40 is interpreted as 'poor' agreement.) MIF and EIA IgM antibody assay results correlated significantly (r = 0.80, p <0.01). Two subjects tested exclusively IgM-positive using MIF, and two tested IgM-positive using EIA. Seven subjects tested exclusively IgM-positive using rELISA. Two subjects tested positive using PCR alone. Their nasopharyngeal and blood samples were taken shortly after onset of illness, at days 11 and 14. Tables 3 and 4 show sensitivity, specificity and positive predictive values.

Time-dependent cumulative sensitivities

The cumulative sensitivity of *C. pneumoniae* PCR was higher than that of the IgM antibody assay during the first weeks after onset of illness, but decreased with time. The cumulative sensitivity of the IgM antibody assays increased. The regression line of PCR was $y = -2.58x + 96$ (r = 0.76, p 0.03) and that of the IgM antibody assays was $y = 1.84x + 73$ (r = 0.90, p 0.002). The lines intersect at 5 weeks (Fig. 3).

IgG antibody seroconversion

In eight cases, IgG seroconversion was demonstrated with the combination of rELISA and EIA, and seroconversion according to all three IgG assays was seen in four cases.

TABLE 2. Outcome of different combinations of tests during an outbreak of *Chlamydophila pneumoniae* infection

No. of positive tests	Combination of tests	No. of subjects	Accumulated cases
5	PCR, IgM (MIF, EIA, rELISA), serocon version[a]	5	
No. of five positive tests		5	5
4	PCR, IgM (MIF, EIA, rELISA)	19	
4	IgM (MIF, EIA, rELISA), seroconversion	1	
4	PCR, IgM (EIA, rELISA), seroconversion	1	
No. of four positive tests		21	26
3	IgM (MIF, EIA, rELISA)	8	
3	PCR, IgM (MIF, rELISA)	1	
3	PCR, IgM (EIA, rELISA)	1	
3	IgM (EIA, rELISA), seroconversion	1	
No. of three positive tests		11	37
2	PCR, seroconversion, (rELISA IgM)[b]	2	
2	IgM (MIF, rELISA)	1	
2	IgM (EIA, rELISA)	2	
No. of two positive tests		5	42
1	PCR	2	
1	PCR, (MIF/EIA IgM)[b]	1	
1	PCR, (EIA IgM)[b]	1	
1	Seroconversion, (rELISA IgM)[b]	1	
1	Seroconversion	1	
No. of one positive test[c]		6	48
0	MIF, EIA	1	
0	MIF	2	
0	EIA	2	
0	rELISA	7	
No. of one reactive test[d]		12	60
No. of reactive[d] and negative tests			67
No. of all tests			127

MIF, microimmunofluorescence test; EIA, enzyme immunoassay; rELISA, recombinant ELISA.
[a]IgG seroconversion is considered as 'one test' in this table.
[b]Reactive assays defined as negative (in parenthesis).
[c]Positive cases according to the definition.
[d]Negative cases according to the definition.

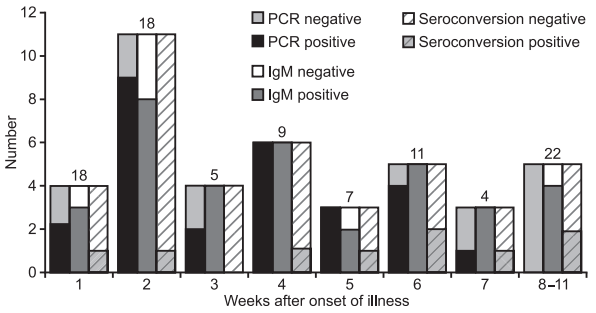

FIG. 2. Results of PCR, IgM antibody assays and IgG seroconversion in relation to timing of sampling after onset of acute respiratory *Chlamydophila pneumoniae* infection. Data from 41 positive cases (date of sampling missing, $n = 7$). A case was defined as *C. pneumoniae*-positive if (i) the nasopharyngeal sample was PCR-positive and/or the serum sample was positive (ii) in ≥2 *C. pneumoniae* IgM antibody assays and/or (iii) with respect to IgG seroconversion. The total number of specimens for each time period is presented above the bars.

Two of these cases became positive by seroconversion only. Seroconversion found with the combination of MIF and EIA IgG was observed in four cases and considered to be negative. The seroconversion-positive samples were taken with a median interval from the start of symptoms of 37 days

TABLE 3. Sensitivity, specificity and positive predictive value (PPV) of three *Chlamydophila pneumoniae* IgM antibody assays and IgG seroconversion, with the *C. pneumoniae* PCR assay as reference standard

	Sensitivity (%)	Specificity (%)	PPV (%)
MIF IgM	26/33 (79)	81/94 (86)	26/39 (67)
EIA IgM	28/33 (85)	79/94 (84)	28/43 (65)
rELISA IgM	29/33 (88)	73/94 (78)	29/50 (58)
IgM (≥2 positive)	27/33 (82)	81/94 (86)	27/40 (68)
IgG seroconversion	8/33 (24)	90/94 (96)	8/12 (67)
IgM (≥2 positive) and/or IgG seroconversion	29/33 (88)	79/94 (84)	29/44 (66)

$n = 127$; MIF, microimmunofluorescence test; EIA, enzyme immunoassay; rELISA, recombinant ELISA.

TABLE 4. Sensitivity, specificity and positive predictive value (PPV) of the *Chlamydophila pneumoniae* PCR assay and IgG seroconversion, with *C. pneumoniae* IgM assays (≥2 positive) as a reference standard

	Sensitivity (%)	Specificity (%)	PPV (%)
PCR	27/40 (68)	81/87 (93)	27/33 (81)
IgG seroconversion	8/40 (20)	83/87 (95)	8/12 (67)

$n = 127$.

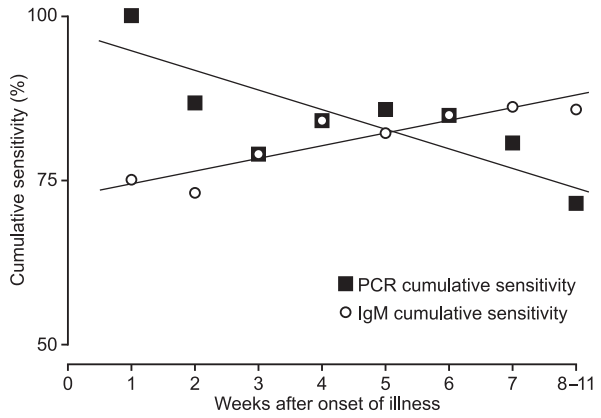

FIG. 3. Cumulative sensitivity of *Chlamydophila pneumoniae* PCR and IgM antibody assays in relation to timing of sampling after onset of acute respiratory *C pneumoniae* infection. Data from 41 positive cases (missing data, $n = 7$). A case was defined as *C. pneumoniae* positive if (i) the nasopharyngeal sample was PCR-positive and/or the serum sample was positive (ii) in ≥2 *C. pneumoniae* IgM antibody assays and/or (iii) with respect to IgG seroconversion. The lines intersect at 5 weeks.

(range 7–70 days) (Fig. 2). This accounted for a difference between IgM-positive and IgG seroconversion-positive samples, although not a significant difference. rELISA IgG alone revealed seroconversion in another 13 cases. In this group, the median time after onset of illness was only 17 days. In contrast, using MIF and EIA, IgG seroconversion was seen in one and four cases, respectively.

IgM and IgG antibody prevalence

Of the 79 conscripts who were negative according to PCR and remained negative according to the IgM antibody reference standard assay and IgG seroconversion during the outbreak, 26 (33%) seemed to be protected, exhibiting two or three positive results according to IgG antibody assays before the outbreak. In contrast, only four (8%) of the 48 conscripts who yielded positive outbreak samples according to the definition were IgG-positive before the outbreak.

Discussion

During an outbreak of acute respiratory tract infection in a military community, an in-house PCR procedure and three commercial *C. pneumoniae* IgM and IgG antibody assays were evaluated with the participation of 127 conscripts. The nasopharyngeal samples from 33 (26%) subjects were PCR-positive, and ≥2 IgM antibody assays were positive for 40 (31%) of them. IgG seroconversion was observed in only 12 subjects. The sensitivity of the PCR-based assay was significantly higher if the sampling was performed during the first weeks of illness (Figs 2 and 3). All together, 48 cases were positive on the basis of PCR and/or ≥2 IgM antibody assay and/or IgG seroconversion results; among these, 24 cases (50%) were positive on the basis of PCR and all three IgM assays.

The main finding of this study is that agreement between the IgM tests is good, and that the EIA IgM test in particular had both high sensitivity and specificity. The agreement [21] between PCR and the MIF and EIA IgM antibody assays was good, but the agreement between the IgM antibody assays was better than that between PCR and the IgM assays.

The sensitivity of PCR was lower than that of any IgM assay. Generally, PCR results are influenced by the sampling technique, transport conditions, PCR equipment, assay conditions and inhibitory substances. The lower sensitivity of PCR observed during the later stages of infection may be explained by clearance of the organism following antibiotic treatment [8], as the majority of the test-positive group had received macrolide treatment before sampling. The PCR-based detection of *C. pneumoniae* demonstrated high specificity in this study. Only two subjects, from whom samples were taken shortly after disease onset on days 11 and 14 without evidence of IgM and IgG seroreactivity, were positive according to PCR alone. One of them was IgG-positive before and during the outbreak and may have been reinfected.

The specificity of nested PCR has been questioned, and false-positive results due to contamination or amplicon carryover have been reported [22]. Great care has been taken to avoid contamination in this in-house nested PCR procedure. Few *C. pneumoniae*-positive samples were reported by the laboratory elsewhere in the region before, during or after the outbreak [20]. This study does not confirm earlier reports that demonstrated, using PCR, a high rate of *C. pneumoniae* carriage in healthy or sick subjects [3], mainly children, without serological evidence of infection [23]. Whether bacteria are detected or not may depend on the actual endemic situation.

A surprisingly large number of subjects who were IgM antibody-positive within the first weeks of illness was observed, as in other studies [24]. This is a shorter period than expected for an IgM antibody response [14]. This may be due in part to the conscripts' and the doctors' difficulties in distinguishing *C. pneumoniae* infection from the many other respiratory infections often occurring in military camps [25]. The time of onset of illness is uncertain under such circumstances. The acute *C. pneumoniae* infection often shows a biphasic pattern with a protracted course [26], which may delay the first consultation and makes determination of the real onset of illness difficult.

Only two of 48 positive cases were positive according to seroconversion alone. The duration from onset of illness to sampling may have been too short for the IgG antibody to reach seroconversion levels. Overall, IgM antibody assays and/or IgG serology have high sensitivity and specificity (Table 3).

This study showed that the MIF test is less sensitive than rELISA, which is reported to be less specific [11,15]. Detection of IgM antibodies contributed more to the diagnosis of acute *C. pneumoniae* infection than previously reported. Among PCR-positive subjects, Boman *et al.* found 39% of the subjects to be IgM antibody-positive using MIF when only one acute-phase serum sample, taken simultaneously with the upper respiratory tract sample for PCR, was analysed [7]. If a single IgM antibody test had been relied upon solely, the diagnosis of acute infection according to the study definition would have been made in 79%, 85% and 88% of the cases using MIF, EIA and rELISA, respectively. These results are unlikely to have been due to an 'overdiagnosis' of IgM antibody-positive samples, given the fact that all samples were defined as IgM antibody-negative before the outbreak.

Comparison of an assay with a reference standard with inverse time-dependent results, such as comparison of PCR and serology, is unsatisfactory. Alternatively, the sensitivity and specificity of the IgM antibody assays could have been based on comparison with each other or with the MIF test, the recognized method of choice [5,7,15]. Aside from technical aspects and the subjective MIF reading, the long delay with which seropositivity occurs is a major drawback, causing assay results obtained in the early phase to be labelled 'false-positive' (Fig. 1).

The conclusion of an assay evaluation study depends on the choice of the reference standard, and when the reference standard and the test assay demonstrate inverse dynamics, the definitive result is also determined by the timing of sampling. Surprisingly, timing of sampling is seldom reported in papers when reference standards are discussed.

In summary, PCR-based detection of nasopharyngeal *C. pneumoniae* was more specific but less sensitive than that based on specific IgM antibody analysis, and IgG seroconversion contributed less to the diagnosis than expected. This study involving military conscripts during a *C. pneumoniae* outbreak demonstrates the importance of the timing of sampling when comparing PCR and serological techniques.

Acknowledgements

We would like to express our gratitude for the cooperation of the military doctors who treated the conscripts: Ø. Eskeland, G. Indrebø and G. Sneen. We would also like to acknowledge the able technical assistance of J. T. Berg, F. E. Larsen and L. B. Heide, who performed most of the laboratory work, and T. A. Gutteberg and B. O. Eriksen for data support. We thank G. Tylden and P. A. Csángó for critical reading of the manuscript.

Transparency Declaration

The authors declare no conflict of interest in relation to this study.

References

1. Cunha BA. The atypical pneumonias: clinical diagnosis and importance. *Clin Microbiol Infect* 2006; 12 (suppl 3): 12–24.
2. Berdal BP, Scheel O, Ogaard AR, Hoel T, Gutteberg TJ, Anestad G. Spread of subclinical *Chlamydia pneumoniae* infection in a closed community. *Scand J Infect Dis* 1992; 24: 431–436.
3. Normann E, Gnarpe J, Gnarpe H, Wettergren B. *Chlamydia pneumoniae* in children attending day-care centers in Gavle, Sweden. *Pediatr Infect Dis J* 1998; 17: 474–478.
4. Hyman CL, Roblin PM, Gaydos CA, Quinn TC, Schachter J, Hammerschlag MR. Prevalence of asymptomatic nasopharyngeal carriage of *Chlamydia pneumoniae* in subjectively healthy adults: assessment by polymerase chain reaction–enzyme immunoassay and culture. *Clin Infect Dis* 1995; 20: 1174–1178.
5. Verkooyen RP, Willemse D, Hiep-van Casteren SC *et al.* Evaluation of PCR, culture, and serology for diagnosis of *Chlamydia pneumoniae* respiratory infections. *J Clin Microbiol* 1998; 36: 2301–2307.
6. Wellinghausen N, Straube E, Freidank H, von Baum H, Marre R, Essig A. Low prevalence of *Chlamydia pneumoniae* in adults with community-acquired pneumonia. *Int J Med Microbiol* 2006; 296: 485–491.
7. Boman J, Allard A, Persson K, Lundborg M, Juto P, Wadell G. Rapid diagnosis of respiratory *Chlamydia pneumoniae* infection by nested touchdown polymerase chain reaction compared with culture and antigen detection by EIA. *J Infect Dis* 1997; 175: 1523–1526.
8. Thom DH, Grayston JT, Campbell LA, Kuo CC, Diwan VK, Wang SP. Respiratory infection with *Chlamydia pneumoniae* in middle-aged and older adult outpatients. *Eur J Clin Microbiol Infect Dis* 1994; 13: 785–792.
9. Dowell SF, Peeling RW, Boman J *et al.* Standardizing *Chlamydia pneumoniae* assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clin Infect Dis* 2001; 33: 492–503.
10. Kern DG, Neill MA, Schachter J. A seroepidemiologic study of *Chlamydia pneumoniae* in Rhode Island. Evidence of serologic cross-reactivity. *Chest* 1993; 104: 208–213.
11. Persson K, Haidl S. Evaluation of a commercial test for antibodies to the chlamydial lipopolysaccharide (Medac) for serodiagnosis of acute infections by *Chlamydia pneumoniae* (TWAR) and *Chlamydia psittaci*. *APMIS* 2000; 108: 131–138.
12. Peeling RW, Wang SP, Grayston JT *et al. Chlamydia pneumoniae* serology: interlaboratory variation in microimmunofluorescence assay results. *J Infect Dis* 2000; 181 (suppl 3): S426–S429.
13. Tuuminen T, Palomaki P, Paavonen J. The use of serologic tests for the diagnosis of chlamydial infections. *J Microbiol Methods* 2000; 42: 265–279.

14. Grayston JT, Wang SP, Kuo CC, Campbell LA. Current knowledge on *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumonia and other acute respiratory diseases. *Eur J Clin Microbiol Infect Dis* 1989; 8: 191–202.
15. Persson K, Boman J. Comparison of five serologic tests for diagnosis of acute infections by *Chlamydia pneumoniae*. *Clin Diagn Lab Immunol* 2000; 7: 739–744.
16. Skakni L, Sardet A, Just J *et al.* Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. *J Clin Microbiol* 1992; 30: 2638–2643.
17. Waris ME, Toikka P, Saarinen T *et al.* Diagnosis of *Mycoplasma pneumoniae* pneumonia in children. *J Clin Microbiol* 1998; 36: 3155–3159.
18. Fournier PE, Raoult D. Comparison of PCR and serology assays for early diagnosis of acute Q fever. *J Clin Microbiol* 2003; 41: 5094–5098.
19. Tong CY, Sillis M. Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. *J Clin Pathol* 1993; 46: 313–317.
20. Haaheim H, Vorland L, Gutteberg TJ. Laboratory diagnosis of respiratory diseases: PCR versus serology. *Nucleosides Nucleotides Nucleic Acids* 2001; 20: 1255–1258.
21. Altman D. *Practical statistics for medical research*. London: Chapman & Hall, 1991.
22. Apfalter P, Reischl U, Hammerschlag MR. In-house nucleic acid amplification assays in research: how much quality control is needed before one can rely upon the results? *J Clin Microbiol* 2005; 43: 5835–5841.
23. Principi N, Esposito S, Blasi F, Allegra L. Role of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in children with community-acquired lower respiratory tract infections. *Clin Infect Dis* 2001; 32: 1281–1289.
24. Bennedsen M. *Chlamydia pneumoniae. Assessment of the microimmunofluorescence test for antibody detection and prevalence studies in adult patients with respiratory tract infection*. MD Thesis, Copenhagen: University of Copenhagen, 2003.
25. Gray GC, Schultz RG, Gackstetter GD *et al.* Prospective study of respiratory infections at the US Naval Academy. *Mil Med* 2001; 166: 759–763.
26. Ekman MR, Grayston JT, Visakorpi R, Kleemola M, Kuo CC, Saikku P. An epidemic of infections due to *Chlamydia pneumoniae* in military conscripts. *Clin Infect Dis* 1993; 17: 420–425.