

Evaluation of Commonly Used Serological Tests for Detection of *Coxiella burnetii* Antibodies in Well-Defined Acute and Follow-Up Sera

M. C. A. Wegdam-Blans,^a C. C. H. Wielders,^{b,c} J. Meekelenkamp,^b J. M. Korbeek,^a T. Herremans,^c H. T. Tjhie,^a H. A. Bijlmer,^c M. P. G. Koopmans,^c and P. M. Schneeberger^b

Department of Medical Microbiology, Laboratory for Pathology and Medical Microbiology (PAMM), Veldhoven, The Netherlands^a; Department of Medical Microbiology and Infection Control, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands^b; and Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands^c

In this study, we compared *Coxiella burnetii* IgG phase I, IgG phase II, and IgM phase II detection among a commercially available enzyme-linked immunosorbent assay (ELISA) (Virion/Serion), an indirect fluorescent antibody test (IFAT) (Focus Diagnostics), and a complement fixation test (CFT) (Virion/Serion). For this, we used a unique collection of acute- and convalescent-phase sera from 126 patients with acute Q fever diagnosed by positive *Coxiella burnetii* PCR of blood. We were able to establish a reliable date of onset of disease, since DNA is detectable within 2 weeks after the start of symptoms. In acute samples, at $t = 0$, IFAT demonstrated IgM phase II antibodies in significantly more sera than did ELISA (31.8% versus 19.7%), although the portion of solitary IgM phase II was equal for IFAT and for ELISA (18.2% and 16.7%, respectively). Twelve months after the diagnosis of acute Q fever, 83.5% and 62.2% of the sera were still positive for IgM phase II with IFAT and ELISA, respectively. At 12 months IFAT IgG phase II showed the slowest decline. Therefore, definitive serological evidence of acute Q fever cannot be based on a single serum sample in areas of epidemicity and should involve measurement of both IgM and IgG antibodies in paired serum. Based on IgG phase II antibody detection in paired samples (at 0 and 3 months) from 62 patients, IFAT confirmed more cases than ELISA and CFT, but the differences were not statically significant (100% for IFAT, 95.2% for ELISA, and 96.8% for CFT). This study demonstrated that the three serological tests are equally effective in diagnosing acute Q fever within 3 months of start of symptoms. In follow-up sera, more IgG antibodies were detected by IFAT than by ELISA or CFT, making IFAT more suitable for prevaccination screening programs.

Q fever is a zoonotic disease, and human infections result mainly from inhalation of *Coxiella burnetii*-contaminated aerosols (14). A major goat-related epidemic in The Netherlands, starting in 2007, resulted in more than 4,000 acute Q fever cases notified in the national infectious disease notification system (3, 6, 13). The epidemic was brought under control through targeted vaccination and culling of goats, resulting in a sharp decline of cases in 2010 (3). Clinical manifestations occur in about half of the acute Q fever cases after an incubation period of 2 to 4 weeks (14). Moreover, clinical symptoms may be indiscriminative and vary from flu-like illness to a more severe syndrome with high fever, severe headache, gastrointestinal complaints, pneumonia, and hepatitis (14).

Laboratory diagnosis of acute Q fever is ideally based on a combination of PCR and serology in blood (16). PCR has been shown to be positive for almost all early acute Q fever patients that have not yet mounted an antibody response and in almost all of those that have just mounted an IgM phase II antibody response (16). Conversely, PCR becomes negative in patients that have developed IgG antibodies (16). However, the laboratory diagnosis of an acute *C. burnetii* infection in routine practice is mainly based on serology, because the majority of samples will not be submitted to the laboratory within 2 weeks after the onset of the disease. IgM phase II is the first antibody to be detected in blood, followed by IgG phase II (5). The serologic diagnosis of acute Q fever based on a single serum sample can be inaccurate, since positive IgM phase II may persist for a longer period and solitary IgM can be false positive (15, 16). Therefore, seroconversion or a 4-fold increase in

the IgG phase II titer is used to confirm the diagnosis of acute Q fever (5).

There are different serological tests available for Q fever, including Indirect Fluorescent Antibody Tests (IFAT), Enzyme-linked Immunosorbent Assays (ELISA) and Complement Fixation Tests (CFT). In The Netherlands, stringent criteria were developed to support clinical decision making, based on our observations and the different serological test outcomes during the epidemic, including an algorithm that includes confirmatory testing (15, 18). Briefly, this algorithm comprises the use of PCR and serology tests, where the choice of a first-line assay depends on the time between the first day of illness and the serum collection: for patients sampled within the first 2 weeks of illness, PCR is recommended. For patients with first contact with a physician later than 2 weeks post-illness onset or for patients for whom the date of illness onset was not known, serology is recommended as the initial test. The aim of the present study was to compare the three serological assays using a large number of acute- and sequential convalescent-phase serum samples from a patient group with

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Address correspondence to M. C. A. Wegdam-Blans, m.wegdam@pamm.nl.

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acute Q fever, from which the onset of disease could be estimated within 2 weeks, since all of them had been diagnosed with Q fever through positive PCR. We compared the diagnostic performances of different tests for acute disease, as well as the kinetics in sequential serum samples.

MATERIALS AND METHODS

Case definition. Acute Q fever was diagnosed based on a positive *C. burnetii* PCR result (NucliSENS easyMAG; bioMérieux, Boxtel, The Netherlands) in peripheral blood combined with clinical symptoms consistent with acute Q fever syndrome and in the absence of signs and symptoms of chronic Q fever.

Serum samples. Patients diagnosed with acute Q fever during the Q fever epidemic were routinely monitored at 3, 6, and 12 months after diagnosis ($t = 3$, $t = 6$, and $t = 12$) using IFAT. For the purpose of this study, all sera were retested with ELISA and CFT. A total of 126 patients, diagnosed between March and December 2009, were included (433 serum samples), with the following distribution of samples per time point: 66 at time zero ($t = 0$) (time of diagnosis) and 121 at $t = 3$, 121 at $t = 6$, and 125 at $t = 12$ months after diagnosis. Paired sera at time points 0 and 3 months were available for 62 acute Q fever patients. Age, sex, and laboratory results for all patients were extracted from the hospital database, and the data were made anonymous for use in further analysis.

IFAT (Focus Diagnostics, Cypress, CA). IgG phase I and II and IgM phase I and II were measured according to the manufacturer's instructions, with the exception of the dilution scheme: serum samples were diluted 2-fold starting with 1:32 by adding 5 μ l of serum to 155 μ l of phosphate-buffered saline. First, single dilutions of 1:32 were viewed using a fluorescence microscope (at a magnification of $\times 400$). Whenever sera were still positive after eight dilutions (1:4,096), titers were set at 8,192.

CFT (Virion/Serion, Würzburg, Germany). Phase I and II were measured according to the manufacturer's instructions. After the procedure steps, a ready-to-use hemolytic system (Virion/Serion, Germany) was used before measuring the titers by eye. Dilutions with 100% of hemolysis were defined as positive. A positive result was defined as having an endpoint dilution of $\geq 1:8$. Titers were set at 1,024 when sera were still positive after eight dilutions (1:512).

ELISA (Virion/Serion, Würzburg, Germany). IgG phase I and II and IgM phase II were processed on a fully automated 4-plate ELISA processing system (DSX). Different dilution protocols were used according to the manufacturer's instructions, using a 1:100 dilution for IgG phase I and IgM phase II and a 1:500 dilution for the IgG phase II assay. Data were analyzed according to the Virion/Serion protocol, reporting IgG phase I and IgM phase II qualitatively and IgG phase II quantitatively. IgG phase I and IgM phase II were positive whenever the measured absorbance was more than 10% above the extinction of the cutoff control. Ambiguous results were added to negative results. IgG phase I extinctions were expressed in optical density (OD) values. IgG phase II extinctions were expressed in U/ml titer using a logistic-log-model calculation and were defined as positive when the titer was > 30 U/ml.

Data analysis. Statistical analysis included computations of frequencies and analysis of agreement between ELISA, IFAT, and CFT. McNemar exact tests with a binomial distribution were used to test for significant differences between the frequencies obtained with the three diagnostic tests. Spearman's rank correlation was used to investigate the agreement between the tests at time point 3. To investigate the kinetics of IgG phase II using the different methods, ratios of the titers between successive time points was examined. Ratios were calculated by dividing the value at 6 months by that at 3 months (6/3) and that at 12 months by that at 6 months (12/6) for each patient. This was carried out for each method, and the average ratio was calculated afterwards. A ratio above 1 was in accordance with a rise in titer, and a ratio below 1 was in accordance with a decline in titer. Significances of ratio differences between the methods and time points were computed using a dependent t test for paired samples.

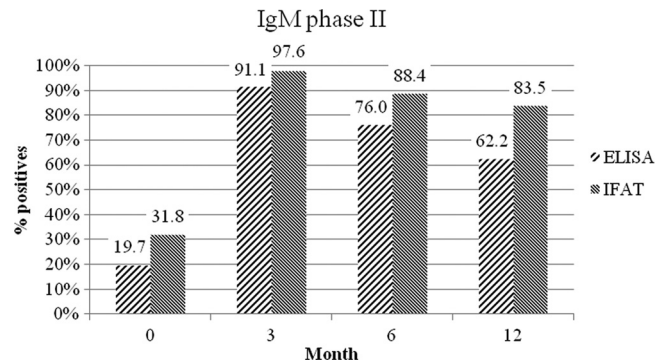


FIG 1 Performance of Q fever IgM phase II testing using ELISA and IFAT at different time points (percentages are indicated above the bars). Significant differences between IFAT and ELISA were measured at time points 0, 3, 6, and 12 months ($P = 0.008$, $P = 0.021$, $P = 0.001$, and $P = 0.000$, respectively).

RESULTS

Patients' characteristics. The mean age (\pm standard deviation [SD]) of the 126 patients was 51.2 ± 15.3 years, and 83 patients (65.9%) were male. For the 62 acute Q fever patients with paired sera available at time points 0 (all PCR-positive samples) and 3 months, mean age, 53.3 ± 15.0 years and 40 patients (64.6%).

Early serology results (0 and 3 months). At $t = 0$, when all samples were PCR positive, IgM phase II antibodies were more frequently detected by IFAT than by ELISA (31.8% [21/66] and 19.7% [13/66] of the patients, respectively [$P = 0.008$]) (Fig. 1). IgG phase II was positive in 13.6% (9/66) and 3.0% (2/66) for IFAT and ELISA, respectively ($P = 0.016$). This means that the solitary IgM phase II antigen response was equal in IFAT and in ELISA (18.2% and 16.7% for IFAT and ELISA, respectively; difference not significant). Based on IgG phase II antibody detection in paired samples (at 0 and 3 months) from 62 patients, IFAT confirmed more cases than ELISA and CFT, but the differences were not statistically significant: 100% using IFAT, 95.2% using ELISA, and 96.8% using CFT (IFAT versus ELISA, $P = 0.250$; IFAT versus CFT, $P = 0.500$; ELISA versus CFT, $P = 1.000$) (Table 1).

Figure 2A to C show the comparison of ELISA and IFAT IgG phase II and CFT phase II at $t = 3$ months. Agreement between IFAT and CFT, expressed in the Spearman's rank correlation coefficient, was better compared to agreements between IFAT and ELISA or CFT and ELISA at $t = 3$ months (CFT versus IFAT, $r = 0.85$; IFAT versus ELISA, $r = 0.45$; CFT versus ELISA, $r = 0.34$). At $t = 3$ months, almost all 121 samples tested highly positive for IgG phase II: the median titer was 2,048, 128, and 72.7 for IFAT, CFT, and ELISA respectively (Table 2). The percentage IgG phase II test results were higher using IFAT or CFT than ELISA, but this difference was not statistically significant.

Late serology results (6 and 12 months). At $t = 6$ and $t = 12$ months, IgM antibodies persisted in a high proportion of patients, but this differed depending on the test used (83.5% versus 62.2% of patients who were IgM phase II positive at $t = 12$ by IFAT and ELISA, respectively ($P = 0.000$) [Fig. 1]). Although declining, a substantial part of the IgM phase II titers measured with IFAT remained highly positive even at 12 months (Table 3). The time kinetics of IgG antibodies are shown in Fig. 3 and 4. IgG phase II and IgG phase I were both detected significantly more often with IFAT in follow-up sera (Fig. 3). IFAT showed no decline (ratio $>$

TABLE 1 Numbers and percentages of confirmed cases using paired samples from time points 0 (time of diagnosis) and 3 months based on IFAT IgG phase II, ELISA IgG phase II, and CFT phase II ($n = 62$)

Assay	No. (%) testing positive ^a at month 0	<i>P</i> value ^b at month 0	No. (%) testing positive ^a at month 3	<i>P</i> value ^b at month 3	No. (%) of confirmed cases ^c at month 3	<i>P</i> value ^b of confirmed cases ^c at month 3
IFAT, IgG phase II	9 (14.5)	0.020 (IFAT vs ELISA)	62 (100)	0.250 (IFAT vs ELISA)	62 (100)	0.025 (IFAT vs ELISA)
ELISA, IgG phase II	2 (3.2)	1.000 (ELISA vs CFT)	59 (95.2)	0.250 (ELISA vs CFT)	59 (95.2)	1.000 (ELISA vs CFT)
CFT, phase II	3 (4.8)	0.030 (CFT vs IFAT)	62 (100)	NA ^d (CFT vs IFAT)	60 (96.8)	0.500 (CFT vs IFAT)

^a "Positive" is defined as an IgG phase II titer of ≥ 32 (IFAT), a positive extinction (ELISA), or a phase II titer of ≥ 8 (CFT).

^b McNemar exact test (binomial distribution).

^c Based on IgG phase II seroconversion between month 0 and 3 (negative IgG phase I at month 0 and positive IgG phase I at month 3) or a 4-fold rise in the IgG phase II titer.

^d NA, not applicable (no discordant pairs).

1) during the 12 months of follow-up compared to results by ELISA and CFT. The IFAT 6/3 ratio is significant different compared to those of CFT and ELISA ($P = 0.002$ and $P = 0.023$, respectively), as well as the IFAT 12/6 ratio ($P = 0.000$ and $P = 0.005$). This means that IgG phase II titers measured with IFAT

faded less rapidly after acute infection than ELISA- and CFT-measured titers. (Fig. 4).

Differences in IgG phase I detection were remarkable in follow-up sera. Significant differences between tests were observed at all of the time points except $t = 0$. At $t = 3$ months, IgG phase I measured with

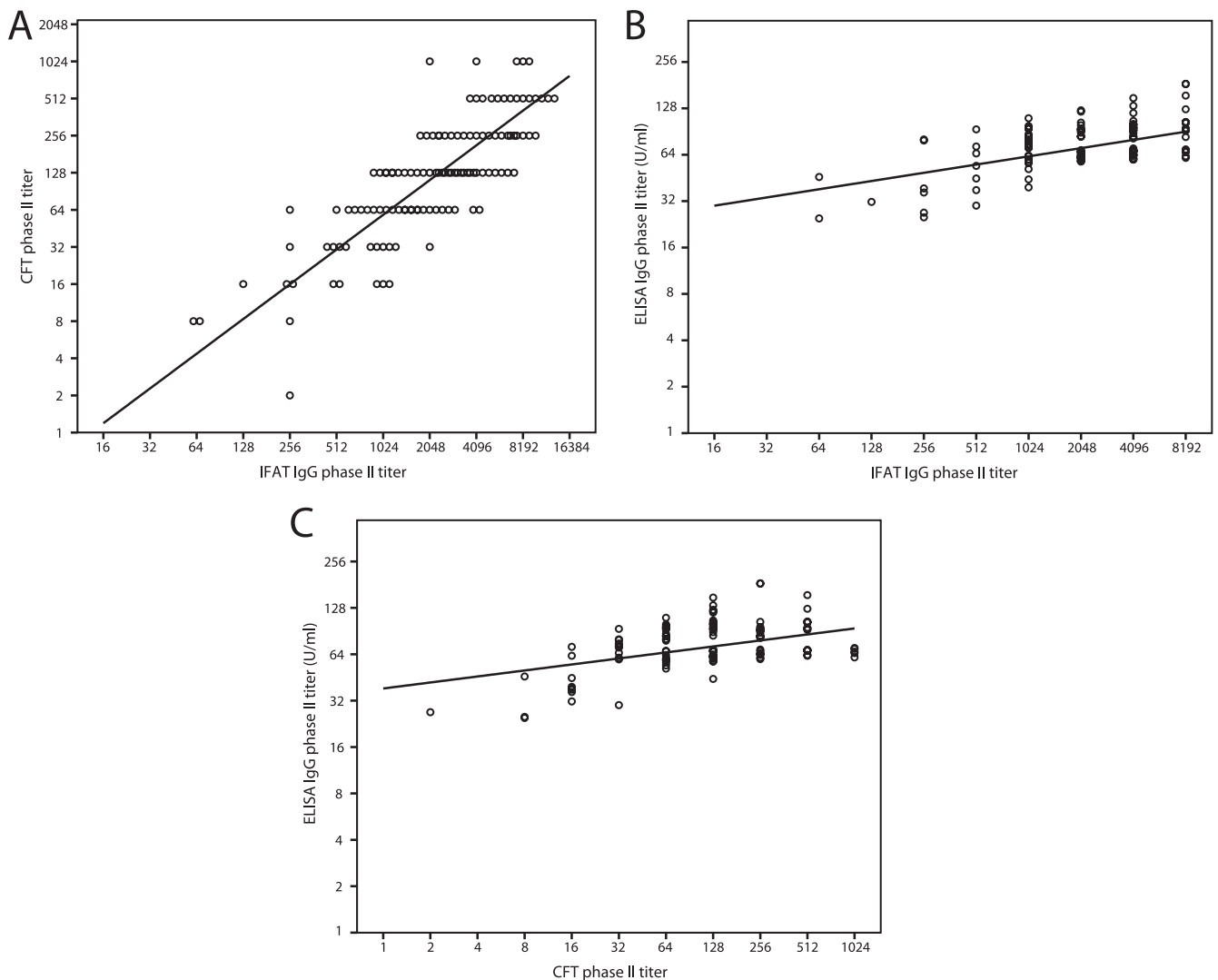


FIG 2 (A to C) Logarithmic (2-log) relation between IgG phase II tests at time point 3 months. (A) IFAT and CFT; Spearman's rank correlation $r = 0.85$. (B) IFAT and ELISA; $r = 0.45$. (C) CFT and ELISA; $r = 0.34$. (IFA IgG phase II titer of 16 equals IFA IgG phase II titer of <32 ; IFA IgG phase II titer of 8,192 equals IFA IgG phase II titer of $>4,096$; CFT phase II titer of 2 equals CFT phase II titer of <4 ; CFT phase II titer of 1,024 equals CFT phase II titer of >512 .)

TABLE 2 Description of IgG phase II dilutions measured in 121 samples at month 3 for the three serological assays

Parameter	Result by:		
	IFAT	CFT	ELISA ^a
Minimum	1:64	1:8	31.6
Maximum	1:8,192	1:1,024	184.6
Median	1:2,048	1:128	72.7
Percentage (%) ^b	100	98.4	94.3

^a IgG phase II results are expressed in units per milliliter (U/ml) using a logistic-log-model calculation.

^b Percentage of positive results. Differences are not significant: IFAT versus ELISA, $P = 0.063$; IFAT versus CFT, $P = 1.000$; ELISA versus CFT, $P = 0.125$.

IFAT was significantly more positive than ELISA or CFT: IFAT, 80.2% (97/121); ELISA, 8.3% (10/121); CFT, 7.4% (9/121) (IFAT versus ELISA and IFAT versus CFT, $P = 0.000$; ELISA versus CFT, $P = 1.000$). At $t = 6$ months, the same difference was observed, except that in addition ELISA and CFT differences were statistically significant: IFAT, 86.8% (105/121); ELISA, 33.9% (41/121); CFT, 24.0% (29/121) (IFAT versus ELISA and IFAT versus CFT, $P = 0.000$; ELISA versus CFT, $P = 0.008$). Results at $t = 12$ months showed differences comparable to those at $t = 6$ months: IFAT, 76.0% (95/125); ELISA, 28.0% (35/125); and CFT, 13.6% (14/125) ($P = 0.000$ for all three combinations of tests) (Fig. 3).

DISCUSSION

In the present study, we used a unique, very well-defined, acute Q fever patient group, based on positive PCR results for acute-phase sera. As detection of *C. burnetii* DNA in serum with PCR is highly time dependent, being almost negligible approximately 2 weeks after the onset of acute Q fever symptoms, the date of onset of disease in this serum collection could be calculated within 2 weeks. Due to this short period of DNA detection time, serological tests will always be necessary in diagnostic algorithms. Besides, not all diagnostic laboratories have Q fever PCR facilities available.

Based on the comparison of three serological tests, IFAT (Focus Diagnostics), ELISA (Virion/Serion), and CFT (Virion/Serion) at onset of disease and at 3, 6, and 12 months after positive PCR, conclusions can be drawn on the concordance and the values of these assays in the diagnosis of acute and past Q fever infections.

IgM phase II antibodies are the first to be detected in acute Q fever, after detection of *C. burnetii* DNA in serum (5). There is an overlap between the disappearance of DNA and the detection of IgM phase II antibodies (11, 16). In our study this overlap is more pronounced with IFAT than with ELISA: IgM phase II results measured with IFAT and ELISA were 31.8% and 19.7%, respectively. At 12 months IgM antibodies persisted both with IFAT (83.5%) and with ELISA (62.2%). In earlier reports IFAT IgM phase II dilution cutoff of $\geq 1:50$ was proposed as a sole diagnostic marker of acute Q fever, and it was shown that IgM titers declined to undetectable levels within 4 months (5, 8, 14). However, in our study, IgM phase II antibody was still detectable in a major part of the 12 months' sera with significant titers, which is consistent with a previous study showing persisting IgM antibodies in late follow-up serum samples (7). We showed that IgM phase II and IgG phase II can persist for up to a year and probably longer and that also the decline in IgG phase II titers is slow, especially with IFAT. This means that serum samples with isolated IgM phase II or samples with low IgM and IgG phase II titers, diagnosis of acute Q

TABLE 3 Percentages of samples with negative IFAT IgM phase II, low-positive IgM phase II, and high-positive IgM phase II results

Time point	% of samples with IgM phase II result (endpoint dilution)		
	Negative (<1:32)	Low positive (1:32–1:256)	High positive (>1:256)
Month 0 ^a	71.4	23.8	4.8
Month 3	1.7	15.7	82.6
Month 6	11.6	34.7	53.7
Month 12	16.0	64.0	20.0

^a Two positive samples were not diluted further because sera were lost. Therefore, percentages are based on 120 sera.

fever should be confirmed with a second serum sample yielding significant high titers (12, 16). In contrast, if a first serum sample already shows high titers of both IgM and IgG phase II no definitive conclusions can be drawn, as this may be compatible with both acute infection and past infection. The predictive values of single serological results could be altered by changing cutoff values but this highly depends on the incidence and prevalence of Q fever in a certain area in a certain period. For instance in the south of The Netherlands in 2007 and 2008, when there was a low prevalence and a moderate incidence (6), low titers would most likely be associated with acute Q fever (or with an aspecific result). In 2009 when there was an incredibly high incidence, most low titers would be associated with acute Q fever (12). Then from 2010 and onwards, again a period of low incidence, low titers would most likely be associated with past infections, because of the high prevalence of antibodies due to the epidemic (6). In our opinion, definitive serological evidence of acute Q fever cannot be based on a single serum sample only but should involve measurement of both IgM and IgG antibodies in paired serum samples.

In the present study, no significant differences were found between IFAT, CFT, and ELISA in confirming acute Q fever in 62 paired sera within 3 months, based on IgG phase II. There was a moderate-to-good agreement between the tests regarding the levels of titers, with IFAT and CFT showing the closest agreement. Hence, choosing one serological technique above another could be based on practical preferences, but the differences in test performances showed in this study should be taken into account, too. IFAT and CFT are more laborious techniques, and interpretation (especially with regard to IFAT) can be subjective. IFAT is the reference method for serological diagnosis, since it is able to distinguish between acute, past, and chronic infection (14). Finally, ELISA is easy to perform and is adapted for automation. Our results are in agreement with those of previous studies, but those were all limited by small sample sizes and variability in the definition of acute Q fever (2, 4, 9, 17).

Significant differences were demonstrated in follow-up sera in IgG phase I and phase II antibody detection. The decline of IgG phase II antibody titers was slower with IFAT (Fig. 4), and IgG phase II antibodies were detected significantly more often with IFAT after 1 year (Fig. 3) than was the case with ELISA and CFT. It is likely that these phenomena continue longer than 12 months. This makes IFAT the most preferable method in studies where high sensitivity is essential, such as vaccination strategies, where false-negative results should be avoided. Alternatively, IFAT could be used in combination with ELISA, for example in cases with a large number of samples: ELISA as the primary screening method

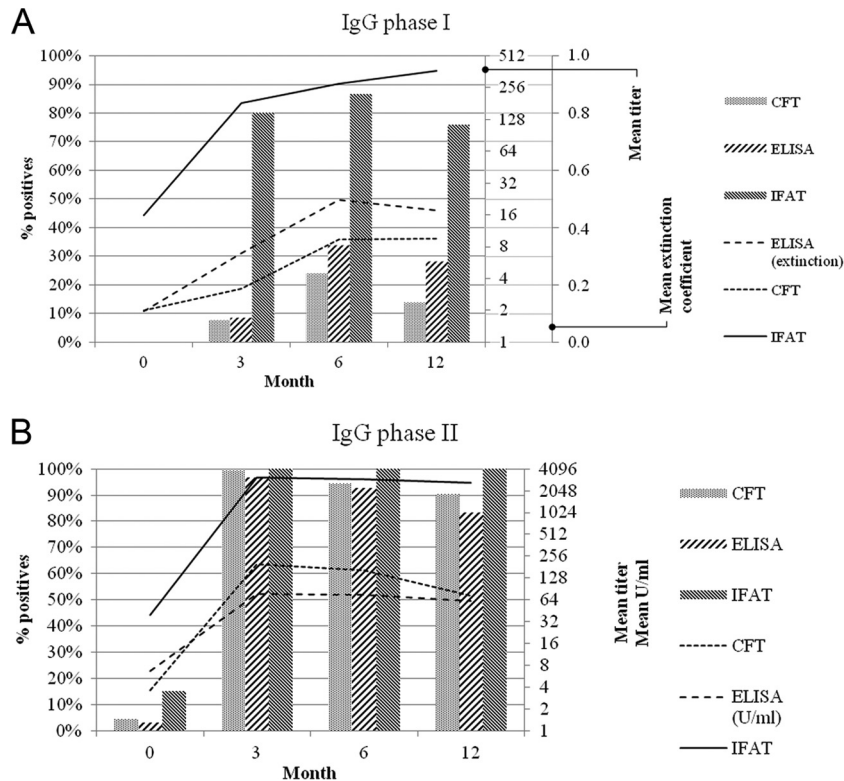


FIG 3 (A) Overview of IgG phase I detection with IFAT, ELISA, and CFT at time points 0, 3, 6, and 12 months. Bars represent percentages of positive results (left axis), and lines represent mean titers or mean extinction coefficients (both right axis). (B) Overview of IgG phase II detection with IFAT, ELISA, and CFT at the same time points. Bars represent percentages of positive results (left axis), and lines represent mean titers or mean U/ml. IgG phase II ELISA results are expressed in U/ml using a 2-log calculation model and therefore can be plotted at the same axis as those of IFAT.

and IFAT for retesting of ELISA-negative results (1, 10). Differences in IgG phase I antibodies were most interesting, with significantly more positive titers using IFAT, although low-range dilutions (1:32 to 1:128) were overrepresented in our IFAT results.

As far as we know, this is the first report of the comparison of IgG phase I testing using different serological methods with follow-up sera. Unfortunately we were unable to address the issue of chronic Q fever diagnosis because of the sample size and inclusion criteria of this study.

Due to the selected dataset, no conclusions can be drawn concerning the sensitivity and specificity of the assays in this study. Moreover, because the interval of the first convalescent sample was set at month 3, this study does not contain data on the intermediate period. If this intermediate period analysis of cut-off values were possible: lower cutoff values could be used to screen for positives that still need to be confirmed with a titer rise to exclude past infections or aspecific reactions. Another drawback of this study is the limited follow-up period. Differences in decline rates of IgG phase II could only be analyzed for a period of 1 year. Further studies are needed to confirm whether these differences will persist over a longer period.

In conclusion, in this clearly defined acute Q fever patient cohort, we demonstrated that confirmation of acute Q fever within 3 months is reliable, independent of the test method used. IgM phase II is not often solitary positive early in the disease but is still detectable in a major part of the samples at 12 months, especially with IFAT. This complicates the diagnosis in epidemics and in regions of endemicity, making paired sera necessary to confirm the diagnosis by a significant (fourfold) titer rise in IgG phase II. IgG phase I and II antibodies in follow-up sera were detected significantly more often with IFAT than with CFT and ELISA, making IFAT more suitable for vaccination strategies.

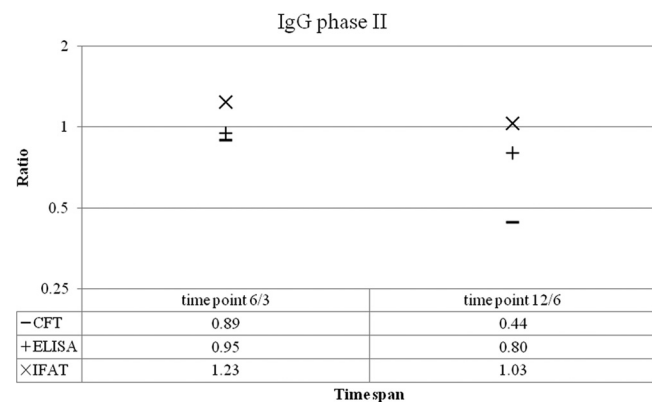


FIG 4 Average IgG phase II ratios at different time points for IFAT, ELISA, and CFT. A ratio above 1 is in accordance with a rise, and a ratio below 1 is in accordance with a decline, in titer.

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