# EFFICIENCY OF VARIOUS SEROLOGICAL TECHNIQUES FOR DIAGNOSING *COXIELLA BURNETII* INFECTION

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**Summary. –** An indirect immunofluorescence assay (IFA) using a recently developed commercial kit for detecting antibodies against *Coxiella burnetii* (*C.b.*), the etiological agent of Q fever, has been evaluated using human field serum samples. The IFA was compared with an ELISA and a complement fixation test (CFT). The IFA was based on the corpuscular *C.b.* phase I and phase II antigens specific to anti-*C.b.* phase I and II antibodies, respectively. Fifty sera from persons with symptoms of Q fever were examined in this study. The IFA compared with the ELISA showed the sensitivities of 97.7% and 87.2% for IgG and 66.7% and 60.0% for IgM phase II and I antibodies, respectively and the specificities of 100% and 90.0% for IgG and 75.9% and 64.7% for IgM phase II and phase I antibodies, respectively. Due to a limited number of sera positive in the IgA antibody testing, the data presented should be considered with caution. It appears that the IFA strikes a very good balance between high specificity and sensitivity with phase II and phase I IgG antibodies and a less satisfactory one with IgM antibodies. The CFT failed in one of the above aspects showing a good specificity but a poor sensitivity, especially for phase I antibodies. The study demonstrated that the IFA is suitable for diagnosing Q fever and its therapeutic follow-up and is a good candidate for screening sera in large numbers. A certain limitation, especially in testing early stages of the chronic disease, could be a low fluorescence intensity of the IgA positive control in comparison with the IgA negative one.

**Key words:** Coxiella burnetii; Q fever; immunofluorescence assay, ELISA; complement fixation test; diagnosis

## Introduction

Q fever belongs to a group of rickettsial infections caused by *C.b.* as the etiological agent. The disease is a widespread zoonosis that is endemic throughout the world (Marrie, 1990). *C.b.* can be carried by ticks, the principal vectors and reservoir, which transmit the agent to wild animals and cause wildlife coxiellosis or to domestic animals creating livestock reservoir of *C.b.* (Weiss *et al.,* 1991). Human infection is most often acquired by inhalation of contaminated aerosols, less frequently by drinking infected milk, infection through skin trauma, sexual contact and mother-to-foetus transmission (Ormsbee *et al.,* 1978; Marrie, 1990; Fournier *et al.,* 1998). Q fever in its acute stage is a potentially severe disease characterized by pneumonitis, hepatitis and neurological complications (Marrie, 1990). Persistent *C.b.* infections in humans may lead to a chronic disease in the form of endocarditis (Raoult *et al.,* 1990). Because of so many symptoms, clinical diagnosis of Q fever is difficult. Upon serial laboratory passages in embryonated hen eggs, *C.b.* undergoes a virulent (phase I) to low-virulent phase (phase II) variation, which is accompanied by modifications in both composition and structure of components of the cell wall outer membrane (Ftáček *et al.,* 2000; Domingues *et al.,* 2002). This phase variation is of utmost importance since it provides phase I and phase II

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**Abbreviations:** BSA = bovine serum albumin; *C.b.* = *Coxiella burnetii*; CFT = complement fixation test; EP = egg passage; FITC = fluorescein isothiocyanate; IFA = immunofluorescence assay; NM I = *C.b.* strain Nine Mile in phase I; NM II = *C.b.* strain Nine Mile in phase II; NM IIA = *C.b.* strain Nine Mile phase II artificial antigen; QFS = Q fever fatigue syndrome

antigens that can be used in Q fever diagnosis and its therapeutic follow-up. Various immunochemical tests are used to detect or assay antibodies against the phase II antigen (phase II antibodies) in the case of acute Q fever and antibodies against the phase I and phase II antigen (phase I and II antibodies) in the case of chronic Q fever (Péter *et al.,* 1987; Fournier *et al.,* 1998; Hussein *et al.,* 2001). Whereas phase I antibodies are present at higher titers only during the chronic form of the illness, phase II antibodies are largely predominant in primary acute Q fever (Peacock *et al.,* 1983; Fournier *et al.,* 1998).

In the past, CFT was mainly used to detect antibodies to *C.b.* Later, it has been found that the incidence of Q fever is higher than estimated, if IFA is used rather than CFT (Dupuis *et al.,* 1986). IFA is simple, very economical in the use of reagents and became a method of choice for a large number of sera to be screened.

Recently, an IFA using a kit from VIRCELL SL (Spain) for the diagnosis of Q fever in human sera has become available. In this study, we compared this IFA with two classical tests, ELISA and CFT in testing human sera for phase I and II antibodies against *C.b.*

## Materials and Methods

*Cultivation and purification of C.b. C.b.* strain Nine Mile in virulent phase I (egg passages (EP) 3 and 6) and low-virulent phase II (EP 164) were propagated in embryonated hen eggs. The phase I (EP 3 and 6) cells were purified by centrifugation and extraction with ether (Ormsbee, 1962; Škultéty *et al*., 1998). Purification of the phase II (EP 164) cells included three subsequent treatments with trypsin (Úrvölgyi, 1976; Toman and Škultéty, 1996). The phase I EP 6 cells were treated with potassium periodate (Schramek *et al.,* 1972) to obtain a so called "phase II artificial antigen". The phase I EP 3 cells, phase II EP 164 cells and phase II artificial antigen, designated as NM I, NM II, and NM IIA, respectively, were used as antigens in immunochemical studies.

*Serum samples* from 47 patients with acute Q fever originated from Australia (4), Bulgaria (16) and Slovakia (27). Diagnostic criteria for acute Q fever were a seroconversion or a twofold or higher increase of specific antibody titers in CFT and/or ELISA (Péter *et al.,* 1987). Three serum samples from patients with symptoms of Q fever fatigue syndrome (QFS) (Marmion *et al.,* 1996) were collected in Australia. Twenty-five positive and 25 negative control serum samples originated from Slovakia.

*CFT.* A standard microplate method with modifications described by Stamp *et al.* (1952) was employed. Sheep erythrocytes, hemolytic amboceptor and guinea pig complement were obtained from Bioveta, Ltd., Czech Republic. Sheep erythrocytes washed in saline, pre-titrated guinea pig complement, NM IIA and NM I antigens and sera diluted from 1:4 to 1:8,192 were used. The end-point titers were scored as wells exhibiting approximately or <50% lysis. A titer over 1:8 was considered positive.

*ELISA*. Ninety-six-well plates (Sarstedt) were coated at 4°C overnight with the antigen (2.5 µg/50 µl/well) in 50 mmol/l sodium carbonate/bicarbonate buffer pH 9.6. Non-specific binding was blocked with 2.5% casein (150 µl/well) at 37°C for 1 hr (Brade *et al*., 1994). Sera (50 µl/well) serially diluted from 1:100 in 2.5% casein solution containing 1.7% of bovine serum albumin (Lachema, Czech Republic) were added, the plates were incubated at 37°C for 1 hr and washed three times with PBS. Then swine anti-human IgG, IgM or IgA polyclonal antibody conjugated to the peroxidase (Sevapharma, Czech Republic) diluted 1:4,000 in 2.5% casein (50 µl/well) was added and incubation continued at 37°C for 1 hr. After threefold rinsing with PBS, the bound antibody was visualized by adding freshly prepared 0.05% 1,2-phenylenediamine (Sigma) in citrate buffer pH 5.0 containing 0.01% hydrogen peroxide as substrate. After incubation at 37°C for 20 mins the reaction was stopped by adding 2.3 mol/l sulphuric acid (50 µl/well). $A_{492}$ was read in a Multiscan MCC 340 ELISA reader (Labsystems, Finland). The relative value of positivity was calculated for each sample as follows:

$$\text{relative value} = (A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}}) \times 100$$

Relative values over 30%, between 20 and 30% and below 20% were considered positive, ambiguous and negative results, respectively. $A_{492}$ values of positive controls averaged at 1.2 ± 0.07 (standard deviation), 1.1 ± 0.06, and 0.9 ± 0.06 for the phase I IgG, IgM, and IgA antibodies, respectively. For the phase II IgG, IgM and IgA antibodies, these values averaged at 2.6 ± 0.07, 1.1 ± 0.05, and 0.9 ± 0.06, respectively. $A_{492}$ values of the negative controls averaged at 0.2±0.05, 0.13±0.07, and 0.08±0.06 for both phase I and II IgG, IgM, and IgA antibodies, respectively. In initial testing, we found that $A_{492}$ values of both test samples and positive controls remained largely unchanged at the dilutions 1:100, 1:200, and 1:400 indicating that non-specific binding did not take place in these cases. In contrast, the 1:200 dilution of the negative control has already resulted in about 50% drop of $A_{492}$ value. Thus, to ensure standard working conditions we decided to use the 1:100 dilution with all the serum samples.

*IFA.* An indirect IFA using the COXIELLA BURNETII PHASE I+II kit from VIRCELL, SL (Spain) was performed according to the manufacturer's instructions. The kit contained 10 slides with 10 wells with a *C.b.* strain Nine Mile phase I antigen spot on the left side of the well and an analogical but phase II spot on the right side. The antigens consisted of the respective formalin-killed and acetone-fixed *C.b.* corpuscles.

Twenty µl of two-fold dilutions of sera in PBS from 1:64 to 1:1,024 or 1:8,192 were added to appropriate wells. The slides were incubated for 30 mins. After gentle rinsing with PBS, the slides were immersed in PBS for 10 mins**,** washed in distilled water and air-dried. Twenty µl of an anti-human IgG-fluorescein isothiocyanate (FITC) conjugate was added per well; the slides were incubated for 30 mins, washed in PBS, rinsed with distilled water and air-dried. Unless otherwise stated the incubations of slides was performed at 37°C in a humid chamber. Examination was performed with a Nikon Eclipse E 400 fluorescent microscope at a magnification of 400x.

For IgM and IgA testing, sera were first diluted 1:1 in PBS and treated shortly with an anti-human IgG sorbent. Then, the two-fold dilutions (from 1:24 to 1:192 or 1:3072) of the sera in PBS were prepared and in 20 µl aliquots added per well. The sli-

des were incubated for 30 mins for IgA and for 90 mins for IgM. The slides were further processed as above but anti-human IgM- and IgA-FITC conjugates were used instead of the IgG equivalent. Control positive and negative sera from the kit were employed.

The sensitivity, specificity and positive and negative predictive values of IFA as compared to ELISA were calculated according to Buendía *et al.* (2001). In assaying the individual Ig classes, a serum was considered true positive or true negative when it was positive/negative in both tests. A false positive serum was positive in IFA but negative in ELISA and *vice versa*. An ambiguous serum was ambiguous in both tests. With total Ig, a true positive serum gave a true positivity at least in one Ig class. A true negative serum was true negative in all Ig classes examined. A false positive serum gave a false positivity and a false negative gave a false negativity at least in one Ig class, while other Ig classes were negative.

## Results and Discussion

The antibody reactivity against the phase I and phase II *C.b.* is shown in Table 1. Whereas IFA and ELISA showed a similar high positivity for phase II antibodies (86% and 90%, respectively), CFT was less efficient (58%). In contrast, the phase I antibody positivity was 84% by ELISA, 76% by IFA, and only 22% by CFT.

Results of a more detailed testing of 50 human sera for individual Ig classes of the phase I and phase II antibodies by IFA, ELISA and CFT are given in Table 2. In testing the phase II antibodies, the number of positive serum samples was the same (21) in IgM and very similar in IgG classes (42 vs. 43) for both IFA and ELISA. The same picture was seen in the number of negative and ambiguous samples. The situation was quite different with the phase I antibodies; here, 21 IgM-positive serum samples were found by IFA and only 15 by ELISA. However, most striking differences were found with IgA antibodies. The number of positive serum samples determined by ELISA represented a half or less (in the case of phase II antibodies) of those detected by IFA. A reason for this phenomenon might be the observed relatively small difference between the fluorescence intensity of IgA-positive and IgA-negative controls.

It is well known that CFT has a lower reactivity than ELISA and IFA in detecting *C.b.* antibodies (Péter *et al.*, 1987; Kováčová *et al.,* 1998). This experience is also supported by the results of this study (Table 1). Also the low reactivity of CFT in detecting the phase I antibodies is not surprising as this phenomenon has been reported earlier (Kováčová *et al.,* 1998).

Standard procedures were used to calculate sensitivity, specificity and positive and negative predictive values for IFA. ELISA served as the reference technique as it was found (Péter *et al.,* 1987) to give the best results in detecting *C.b.* antibodies in the past. The data presented in Table 3 were used for calculation of an IFA characteristics given in Table 4.

**Table 1. The antibody reactivity against phase I and phase II *C.b.* assayed by IFA, ELISA and CFT**

| Test | Result | No. of serum samples tested for phase I antibodies | No. of serum samples tested for phase II antibodies |
|---|---|---|---|
| IFA | P | 38 (76%) | 43 (86%) |
| | N | 11 | 5 |
| | A | 1 | 2 |
| ELISA | P | 42 (84%) | 45 (90%) |
| | N | 7 | 5 |
| | A | 1 | 0 |
| CFT | P | 11 (22%) | 29 (58%) |
| | N | 39 | 21 |
| | A | 0 | 0 |

P = positive; N = negative; A = ambiguous.

**Table 2. Phase I and phase II antibodies of IgM, IgG and IgA classes to *C.b.* assayed by IFA, ELISA and CFT**

| Test | Result | No. of samples tested for phase I antibodies | | | No. of samples tested for phase II antibodies | | |
|---|---|---|---|---|---|---|---|
| | | IgM | IgG | IgA | IgM | IgG | IgA |
| IFA | P | 21 | 35 | 8 | 21 | 42 | 14 |
| | N | 28 | 14 | 41 | 26 | 8 | 35 |
| | A | 1 | 1 | 1 | 3 | 0 | 1 |
| ELISA | P | 15 | 39 | 4 | 21 | 43 | 6 |
| | N | 33 | 10 | 41 | 26 | 7 | 42 |
| | A | 2 | 1 | 5 | 3 | 0 | 2 |
| CFT | P | – | 11 | – | – | 29 | – |
| | N | – | 39 | – | – | 21 | – |
| | A | – | – | – | – | – | – |

Fifty serum samples were tested. For the legend see Table 1.

**Table 3. Detection of phase I and phase II antibodies to *C.b.* in human sera by IFA and ELISA**

| Result | No. of serum samples tested for phase I antibodies | | | | No. of serum samples tested for phase II antibodies | | | |
|---|---|---|---|---|---|---|---|---|
| | IgM | IgG | IgA | Total Ig | IgM | IgG | IgA | Total Ig |
| True positive | 9 | 34 | 2 | 37 | 14 | 42 | 5 | 43 |
| True negative | 22 | 9 | 39 | 6 | 22 | 7 | 34 | 5 |
| False positive | 12 | 1 | 6 | 1 | 7 | 0 | 9 | 0 |
| False negative | 6 | 5 | 2 | 5 | 7 | 1 | 1 | 2 |
| Ambiguous | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |

Fifty serum samples were tested.

IFA showed the best sensitivity and specificity for IgG phase II antibodies followed by IgG phase I antibodies. Less satisfactory results were obtained for IgM antibodies of both phases althought one cannot exclude that the calculated data could be influenced by a lower abundance of these antibodies in the examined sera. As we had only a few sera positive for IgA antibodies, the data given in Tables 3 and 4 should be considered with caution. A great number of false positive results as compared to a low number of true positive

**Table 4. Comparison of IFA with ELISA in detection of antibodies to *C.b.***

| Characteristics | Values (%) for phase I antibodies | | | | Values (%) for phase II antibodies | | | |
|---|---|---|---|---|---|---|---|---|
| | IgM | IgG | IgA | Total Ig | IgM | IgG | IgA | Total Ig |
| Sensitivity | 60.0 | 87.2 | 50.0 | 88.1 | 66.7 | 97.7 | 83.3 | 95.6 |
| Specificity | 64.7 | 90.0 | 86.6 | 85.7 | 75.9 | 100 | 79.1 | 100 |
| Positive predictive value | 42.9 | 97.1 | 25 | 97.4 | 66.7 | 100 | 35.7 | 100 |
| Negative predictive value | 78.6 | 64.3 | 95.1 | 54.6 | 75.9 | 87.5 | 97.1 | 71.4 |

ELISA was used as a reference technique. Fifty serum samples were tested.

ones obtained with IFA (Table 3) was reflected in the low positive predictive values 25% and 35.7% for phase I and II IgA antibodies, respectively (Table 4). Nevertheless, IFA strikes a very good balance between high specificity and sensitivity for phase II antibodies and a satisfactory one for phase I antibodies, as it is seen for total Ig antibodies in Table 4. CFT failed in one of these aspects, showing a good specificity but a poor sensitivity, especially for phase I antibodies (data not shown).

Concordances between the serological assays were as follows: ELISA-IFA: 88% and 96%; IFA-CFT: 44% and 72%; ELISA-CFT: 44% and 72% for the phase I and II antibodies, respectively. The three techniques showed the same results for phase I and II antibodies in 19 and 36 of the 50 serum samples (38% and 72%), respectively.

ELISA and IFA are the methods of choice for screening Q fever antibodies and their Ig classes in sera of human populations at risk (personnel in agriculture, life stock production, slaughter houses, milk production, animal skin-processing industry, veterinarians, laboratory personnel, and human population in infected areas). The positive serum samples detected by one technique are recommended to be confirmed by another. During acute phase of Q fever, antibodies against phase II are detected by both tests earlier and at higher titers than those against phase I (Dupuis *et al.,* 1985; Kováčová *et al.*, 1998). In contrast, titers of antibodies to phase I are higher during the chronic phase (Fournier *et al.,* 1998). The IgM antibodies appear in the second week of acute disease and hence their detection is most useful for diagnosis of acute Q fever in endemic areas (Field *et al.,* 1983). IgG antibodies assayed by ELISA or IFA peak after 4–8 weeks of infection. In the chronic phase of the disease, testing of IgG and IgA antibodies against phase I is most useful, since equal or higher titers than those against phase II are usually found (Peacock *et al.*, 1983; Fournier *et al.*, 1998).

In this study, 50 sera were collected from patients suffering from acute Q fever and from those having the symptoms of QFS. The levels of phase I and II IgM, IgG, and IgA antibodies in the sera were assayed by three techniques. A new IFA using a recently developed kit from Vircell SL (Spain) was compared with classical ELISA and CFT. The results showed that the IFA used may be regarded as suitable for diagnosing Q fever and its therapeutic follow-up. The IFA exhibited a high specificity and sensitivity for the phase II and phase I IgG antibodies and to a lesser extent also for the phase II and phase I IgM antibodies. The technique is easy to perform, does not require standardization prior to each test and is quite free from subjective interpretations. Thus, it is a good candidate for screening sera in large numbers. A certain limitation, especially in testing sera from early stages of the chronic disease, could be a low fluorescence intensity of IgA positive control compared with that of IgA negative one.

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