Title: Evaluation of a new serological test for the detection of anti-Coxiella and anti-Rickettsia antibodies.
Authors: Bizzini A, Péter O, Baud D, Edouard S, Meylan P, Greub G
Journal: Microbes and infection
Year: 2015 Nov-Dec
Volume: 17
Issue: 11-12
Pages: 811-6
DOI: 10.1016/j.micinf.2015.09.015

Evaluation of a new serological test for the detection of anti-*Coxiella* and anti-*Rickettsia* antibodies

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Word count: Abstract 289. Article 2429.

Running title: multiplexed automated fluorescence assay

Keywords: multiplexed serology ; immunofluorescence ; *Coxiella burnetii* ; *Rickettsia typhi* ; *Rickettsia conorii*

ABSTRACT

Coxiella burnetii and members of the genus *Rickettsia* are obligate intracellular bacteria. Since cultivation of these organisms requires dedicated techniques, their diagnosis usually relies on serological or molecular biology methods. Immunofluorescence is considered the gold standard to detect antibody-reactivity towards these organisms. Here, we assessed the performance of a new automated epifluorescence immunoassay (InoDiag) to detect IgM and IgG against *C. burnetii, Rickettsia typhi* and *Rickettsia conorii.*

Samples were tested with the InoDiag assay. A total of 213 sera were tested, of which 63 samples from Q fever, 20 from spotted fever rickettsiosis, 6 from murine typhus and 124 controls. InoDiag results were compared to micro-immunofluorescence.

For acute Q fever, the sensitivity of phase 2 IgG was only of 30% with a cutoff of 1 arbitrary unit (AU). In patients with acute Q fever with positive IF IgM, sensitivity reached 83% with the same cutoff. Sensitivity for chronic Q fever was 100% whereas sensitivity for past Q fever was 65%. Sensitivity for spotted Mediterranean fever and murine typhus were 91% and 100%, respectively. Both assays exhibited a good specificity in control groups, ranging from 79% in sera from patients with unrelated diseases or EBV positivity to 100% in sera from healthy patients.

In conclusion, the InoDiag assay exhibits an excellent performance for the diagnosis of chronic Q fever but a very low IgG sensitivity for acute Q fever likely due to low reactivity of phase 2 antigens present on the glass slide. This defect is partially compensated by the detection of IgM. Because it exhibits a good negative predictive value, the InoDiag assay is

valuable to rule out a chronic Q fever. For the diagnosis of Rickettsial diseases, the sensitivity of the InoDiag method is similar to conventional immunofluorescence.

INTRODUCTION

Coxiella burnetii, *Rickettsia typhi* and *Rickettsia conorii* are the causative agents of Q fever, murine typhus and Mediterranean spotted fever, respectively [18, 5, 16]. These obligate intracellular bacteria are difficult to diagnose and culture-based methods are restricted to specialized BSL3 laboratories. PCR-based detection methods have been described but are essentially found in larger reference diagnostic laboratories [15, 17]. Therefore, screening for Q fever and rickettsial diseases often relies on serologic techniques such as Enzyme-linked immunosorbent assays (ELISA), Western-blots (WB) and immunofluorescence assays (IF). Serology is especially useful for the diagnosis of chronic Q fever, in particular when a biopsy of the affected organ (e.g. cardiac valve, liver) is not available. *Coxiella burnetii* serological diagnosis is based on the presence of antibodies against phase 2 and phase 1 antigens, for the diagnosis of acute and chronic Q fever, respectively. The serological diagnosis of rickettsial infections is more complex, because of the common occurrence of cross-reactions between the different spotted fever group rickettsia. Conventional IFs are labour-intensive and reading of the assay is operator-dependent. Recently, a new innovative automated epifluorescence assay has been made available by InoDiag (Signes, France) for the diagnosis of *C. burnetii*, *R. typhi* and *R. conorii*. This multiplexed antigen microarray uses standardized quantities of antigens, spotted on glass slides with appropriate controls [9]. Except for the initial deposition of the serum samples, all subsequent steps (i.e. secondary antibody depositions, incubations, washing, drying, reading and interpretation) are performed automatically. This assay has previously been shown to be a promising tool for

76 the serodiagnosis of *Chlamydia trachomatis* infection, culture-negative endocarditis and
77 atypical pneumonia [8, 9, 3].

78 In the present study, we compared the performance of the automated InoDiag serological
79 test with a gold standard indirect micro-immunofluorescence technique on sera taken from
80 patients with serologically and clinically proven Q fever or rickettsial diseases, as well as on
81 sera from pregnant women and control patients.

82

MATERIALS AND METHODS

Patient population and controls

A total of 213 sera were studied. Tested sera from acute, chronic or past Q fever infections as well as rickettsiosis cases that were positive for IgM and/or IgG using a reference indirect micro-immunofluorescence technique (described below) were selected from the routine serological laboratories of Lausanne and Sion, Switzerland (seroconversion, clinically confirmed or compatible cases as well as sera from previous studies [2, 6, 13]). This included 20 sera from spotted fever rickettsiosis (11 spotted Mediterranean fever, 9 African tick-bite fever), 6 sera from murine typhus, 63 sera from Q fever (12 acute, 22 chronic, 29 past infections). Clinical cases corresponding to some of these sera have been previously published [1, 4, 10, 11]. A total of 124 control samples were included. These consisted in 10 samples from EBV IgM+ sera, 101 sera from pregnant women and 13 sera from patients with unrelated infections.

InoDiag

The tested InoDiag assay is a fully automated multiplexed immunofluorescent assay consisting of glass slides spotted with nanolitre spots of antigens of *R. typhi*, *R. conorii*, *R. felis*, and *C. burnetii* antigens (Figure 1). The sensitivity of the assay was not evaluated for *R. felis*, the causative agent of cat-flea typhus. Slides also contain four control spots: (i) *S. aureus* ATCC 29213 to assess serum deposition, (ii) human IgG to confirm the adequate distribution of the secondary anti-human IgG antibody, (iii) human IgM to confirm the adequate distribution of the secondary anti-human IgM antibody and detect the eventual

presence of rheumatoid factor, (iv) double-stranded DNA to detect antinuclear antibodies. Serum samples were diluted at a ratio of 1:16 and applied on the slide. All subsequent steps were performed automatically as previously described [6]. After the final drying step, slides were imaged with an automatic InoDiag fluorescent camera analyzer and the data processed using the software Inosoft (InoDiag). The end-point is a fluorescence index. For *C. burnetii,* two-cutoffs for positivity were considered: a fluorescence index >1 arbitrary units (AU) and >2 AU. For *Rickettsia*, only the cutoff of 2 AU was considered. Receiver operating characteristics (ROC) curves were used to precise the diagnosis performance of the assay.

Indirect micro-immunofluorescence

Sera were tested for the presence of antibodies directed against *C. burnetii* or *Rickettsia* spp. using indirect micro-immunofluorescence (IF). Briefly, sera were screened at 1:20 to 1:80 dilution in two-fold steps using *C. burnetii* phase I and II antigens (strain Nine Miles, kindly provided by Dr W. Burgdorfer, Rocky Mountain Laboratories, Hamilton, USA), *R. conorii* and *R. typhi* (kindly provided by Dr W. Burgdorfer, Rocky Mountain Laboratories, Hamilton, USA) and *R. africae* (kindly provided by Dr D. Raoult and Dr. P-E. Fournier). We used fluorescein isothiocyanate goat anti-human specific IgG and IgM conjugates (BioMérieux, Marcy-l'Etoile, France). Positive sera were then diluted in two-fold steps from 1:20 to final dilutions.

RESULTS

Performance of the InoDiag assay in the Q fever group

The assay has been evaluated for the three categories of Q fever disease (acute, chronic and past infection). The sensitivity and specificity for *C. burnetii* of the InoDiag assay for the various tested groups are shown in the Table 1. In the acute Q fever group (n=12), 100% (12/12) and 83% (10/12) sera were positive by IF for phase 2 IgM and phase 2 IgG, respectively. The sensitivity of the InoDiag assay for acute Q fever with positive IgG detected by IF (irrespective of the presence of IgM) was of 20% (2/10) and 30% (3/10) with cutoffs of 2 and 1 AU, respectively. Considering sera positive in IgM by IF in patients with acute Q fever, the sensitivity reached 75% (9/12) and 83% (10/12) with cutoffs of 2 AU and 1 AU, respectively. Two early seroconversions that were detected by IF already on the first sera (one positive for both IgG and IgM ; one positive for phase 2 IgM only) were missed by the InoDiag assay.

In the chronic Q fever group, 22 sera were positive for IgG against both phase 1 and phase 2 antigens by IF. Among these, the InoDiag test showed a sensitivity of 100% (22/22) for IgG detection. Of note, 4 sera exhibited traces of IgM with the IF. With the InoDiag technique, 5 and 3 sera were positive in IgM with cutoff values of 1 AU and 2 AU, respectively. This might wrongly suggest the false diagnosis of an acute Q fever.

Even though the InoDiag assay is commercialized as a screening tool, we wondered whether the InoDiag assay could also be used as a tool to monitor treatment efficacy. In successive sera obtained from three patients treated for chronic Q fever, we did not observe a

143 correlation between InoDiag quantitative values and antibody titers measured by IF. Indeed,
144 over a three year follow-up of a patient with Q-fever endocarditis [11], the initial IgG
145 antibody reactivity was measured by the InoDiag assay at 5.47 whereas phase 1 and 2 IgG
146 were quantified at 1:3200 and 1:6400 by IF, respectively. In the subsequent sera, which
147 corresponds to treatment initiation, InoDiag IgG titres fell from 5.47 to 2.62 whereas IF titres
148 fell to 1:200 and 1:400. However, for a second patient with proven Q fever [10], InoDiag
149 values remained constantly highly positive (6.27-5.77) over two years under adequate
150 treatment demonstrated by decreasing IF titres, that fell from 1:800 to 1:100 and 1:1600 to
151 1:200 for phase 1 and phase 2 antibodies, respectively. Finally, for a third patient with aortic
152 prosthesis infection, titers remained elevated by IF a well as by the InoDiag assay.

153 In the past Q fever group, which included 29 sera tested positive in IgG by IF, 48% (14/29)
154 and 65% (19/29) were positive in IgG with the InoDiag assay using cutoffs of 2 AU and 1 AU,
155 respectively. IgG values obtained by the InoDiag assay for past Q fever infections ranged
156 from 2.07 to >10, which does not differ from the values obtained in the chronic Q fever
157 group. Thus, the InoDiag test cannot differentiate a past from a chronic Q fever. As well, the
158 fact that 4 sera were positive only for IgM in the InoDiag assay could lead to the incorrect
159 diagnosis of an acute recent infection whereas these sera were clearly from past Q fever
160 with initial diagnosis made 2 to 6 years before.

161 In the control group, with a cutoff of 1 AU, 6/124 sera (4.8%) were positive for *C. burnetii* IgG
162 by the InoDiag assay. Using the higher 2 AU cutoff, only one serum out of 124 (0.8%) was
163 positive for *C. burnetii* IgG. This serum originated from an EBV IgM positive patient and was
164 also tested positive by IF. When considering IF as gold standard (i.e. excluding this sera), the

specificity of the InoDiag assay would thus be of 100%. No serology was positive among pregnant women or in patients with unrelated diseases.

Regarding cross-reactions, among the 63 sera from patients with Q fever disease, we observed a prevalence of 11% (7/63) of *Rickettsia* antibodies with a cutoff of 2 AU for *Rickettsia*. Two and 1 sera were positive for *R. typhi* IgG and IgM, respectively, 2 sera were positive for either IgM or IgG against *R. conorii*, and 2 sera were positive for IgM against both *R. typhi* and *R. conorii*. Only one of these positive reactions in IgM was confirmed by IF. This apparent cross-positivity might come from the quality of the preparation of the slides, such as a non pure antigen or contamination during the spotting process.

When the different Q fever status disease (acute, chronic and past infection) are taken into consideration, the ROC curves show inferior performances to discriminate IgG and IgM anti-*C. burnetii* for acute Q fever; with areas under the curve of 0.936 and 0.9426 respectively (Figure 2). In contrast, areas under the curves are close to 1 for chronic and past infection in IgG and IgM, which testify an excellent discrimination tool (Figure 2).

Performance of the InoDiag assay in the *Rickettsia* spp. group

This group included sera from spotted Mediterranean fever, African tick-bite fever (ATBF) and murine typhus. The sensitivity and specificity for *Rickettsia* spp. of the InoDiag assay for the various tested groups are shown in the Table 2. Among 11 sera taken from patients with spotted Mediterranean fever confirmed by IF, 91% (10/11) showed antibody reactivity against *R. conorii* when tested with the InoDiag assay (Table 2). Eight were positive for both

IgM and IgG antibodies, and 2 sera were positive for either IgG or IgM antibodies. Of note, cross reactions (either IgG and/or IgM) with *R. typhi* and *R. felis* were common, being observed for 7/10 and all 10/10 cases, respectively.

Even though no specific *Rickettsia africae* antigen was spotted on the slide, we tested the InoDiag assay on 9 sera from ATBF since it is well known that spotted group *Rickettsia* exhibit numerous interspecies serological cross-reactions [14, 7]. In our study, 44% (4/9) sera reacted with the InoDiag assay, 3 being positive for IgG against *R. conorii* and 1 being positive for IgG against both *R. conorii* and *R. typhi*.

Six sera were obtained from 2 patients with murine typhus. With the conventional IF, 5 sera were positive for IgM and IgG, 1 sera for IgG only. The InoDiag results were totally concordant to IF results. Hence, the sensitivity of the InoDiag assay for murine typhus was 100% in our small cases series. Interestingly, in one of these patients coming from a zone of known endemicity (North Africa), 4 out of 5 sera showed a positive signal for *C. burnetii* with the InoDiag method (IgG: 2.21-3.29 ; IgM negative). Since conventional IF for *C. burnetii* was negative for all these sera, these results might represent a cross-reaction of the InoDiag assay rather than serological traces of a past infection.

In the control group, 7 of 124 (5.6%) sera exhibited a positive signal for *Rickettsia* spp. In the subgroup of EBV IgM positive patients, 1 sera was positive for IgG towards *R. typhi* and 1 for IgG towards *R. conorii*. None of these were positive with IF. In the subgroup of pregnant women, 1 sera was positive for IgG towards *R. typhi* (confirmed by IF), and 2 sera were positive for IgM towards either *R. typhi* or *R. conorii* (negative by IF). In the subset of

patients with unrelated diseases, the InoDiag assay determined that 1 sera was positive for IgM against *R. typhi* and 1 sera was positive for IgM against *R. typhi* and *R. conorii*. None of these sera was confirmed positive by IF. Hence in the control group 5% (6/124) of sera exhibited a positive signal by InoDiag that was not confirmed by IF.

Regarding cross-reactions with *Coxiella*, we observed a prevalence of *Coxiella* antibodies of 40% (8/26) among the 26 sera from patients with rickettsial diseases. Of these 8 sera, only 1 was confirmed by IF.

DISCUSSION

In this work, we assessed the performance of the InoDiag inoMuST slide for the diagnosis of *Coxiella* and *Rickettsia* infections.

Regarding the diagnosis of Q fever disease, the InoDiag assay has an excellent performance for the diagnosis of chronic Q fever. However, we observed a very low IgG sensitivity of the InoDiag assay in acute Q fever, that might be due to an insufficiently reactive phase 2 antigen on the glass slide. In particular, for 10 sera from acute Q fever that were positive in phase 2 IgG by conventional IF (phase 2 IgG titers ranging from 1:640 to 1: 20'480), only 2 were positive in IgG with the InoDiag assay. This might be due to the fact that the antigen spotted on the slide is rather a phase 1 antigen than a phase 2 antigen. The defect in phase 2 IgG and IgM detection for the diagnosis of acute Q fever is somehow compensated by the excellent detection of IgM. However, the InoDiag assay in its present version cannot be recommended for the diagnosis of acute Q fever.

The InoDiag assay is useful to differentiate a past Q fever from an acute Q fever, but is not able to distinguish an acute Q fever from a past Q fever with persisting IgM antibodies. As well, it cannot differentiate a past Q fever from a chronic Q fever. However, because it exhibits an excellent negative predictive value, the InoDiag assay might be used to rule out a chronic Q fever, especially in the context of *Coxiella* intravascular infection or negative blood culture endocarditis.

Regarding the potential use of the test to monitor treatment efficacy, we did not observe a correlation between the evolution of serum titers and the signal measured by the InoDiag

assay. Therefore, it is difficult to base treatment interruption on InoDiag values since it is currently recommended to treat for at least 18 months, and until a decrease of phase I IgG titer below 1:400 is observed.

For the diagnosis of Rickettsial diseases, the sensitivity of the InoDiag method is similar to conventional IF and the InoDiag assay exhibits a good specificity. Of note, the Inodiag slide provided also includes an antigen spot for *Rickettsia felis* [12], for which our study design was not targeted. Nevertheless, *R. felis* is an emerging pathogen which is likely under diagnosed and that deserves further studies. It might also be useful to add antigens of *Rickettsia africae*, which is typically the most frequently diagnosed rickettsiae in Switzerland (returning travelers) as well as antigens toward *Rickettsia slovaca*, an emerging rickettsiosis in Europe. Interestingly *R. felis*, which does not belong to either the spotted fever or the typhus group, exhibits a stronger cross-reactivity with *R. typhi* than with *R. conorii* [7]. One could therefore hypothesize that if serum titers were elevated with both *R. typhi* and *R. felis*, a serological diagnosis of typhus is more likely. In our study, 7 sera from murine typhus were examined. Of the 6 positive with the InoDiag test, all InoDiag measurements were indeed higher for *R. felis* than for *R. conorii* (data not shown). However, we observed that a higher *R. felis* signal was also present in 4/7 sera taken from patients which were in fact presenting a spotted fever due to *R. conorii* (all 4 also exhibited some antibody reactivity against *R. typhi*). Hence, the purportedly stronger InoDiag cross-reactivity with *R. felis* of murine typhus cases cannot reliably be used as a diagnostic indicator.

Because of its ease of use, the InoDiag is a promising technique for the serological diagnosis of *Coxiella* and *Rickettsia* infections. In our opinion, upgrades should nevertheless be made

by the manufacturer to improve the diagnosis of acute Q fever and provide a greater range of antigens representing the diversity of rickettsial species. As well, further prospective studies should be performed to confirm our data and to assess new versions of the *Coxiella* InoDiag assay.

ACKNOWLEDGMENTS

We thank the technicians of the serology laboratories of the University Hospital Center Lausanne, the Institut Central des Hôpitaux du Valais and the Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes of the Université de la Méditerranée for their excellent technical assistance.

Figure legends

Figure 1

A) Schematic of the of the glass slide of the multiplexed InoDiag assay. The spots correspond to the following spotted antigens : IgG, human IgG; CB2, *C. burnetii* phase 2; RF, *R. felis*; dsDNA, double-stranded DNA; SA, *S. aureus*; IgM, human IgM; RT, *R. typhi*; RC, *R. conorii*.

B) Typical slides analysed by a fluorescent camera at different wavelength. UV illumination (365 nm) allows the determination of the area of each spot and confirms the presence of all antigens. Fluorescence readings at 470 nm and 594 nm allow the detection and quantification of IgG and IgM, respectively, towards the various antigens.

Figure 2

Receiver operating characteristic curves for the performance of the Q fever InoDiag assay on sera from acute, chronic or past Q fever.

283 **Table 1**

284 **Sensitivity and specificity of the InoDiag assay for *C. burnetii***

	Setting	% (cutoff 2 AU)	% (cutoff 1 AU)
Sensitivity			
	Acute Q fever with positive IF IgM	**75%**	**83%**
	Acute Q fever with positive IF phase 2 IgG (with or without positive IgM)	**20%**	**30%**
	Past Q fever	**48%**	**65%**
	Chronic Q fever with positive IF phase 1 IgG (with or without positive IgM)	**100%**	**100%**
Specificity			
	Healthy patients	**100%**	**100%**
	Patients with other diseases or EBV + sera	**100%**	**82%**

285

286

Table 2

Sensitivity and specificity of the InoDiag assay for *Rickettsia* spp. (cutoff of 2 arbitrary units)

	Setting	%
Sensitivity		
	Spotted Meditteranean fever	**91%**
	Murine typhus	**100%**
Specificity		
	Healthy patients	**98%**
	Patients with other diseases or EBV + sera	**79%**

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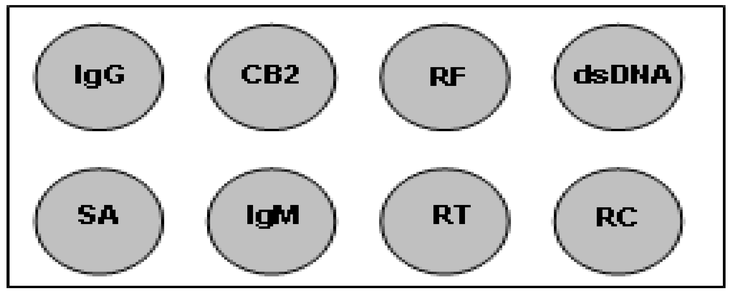
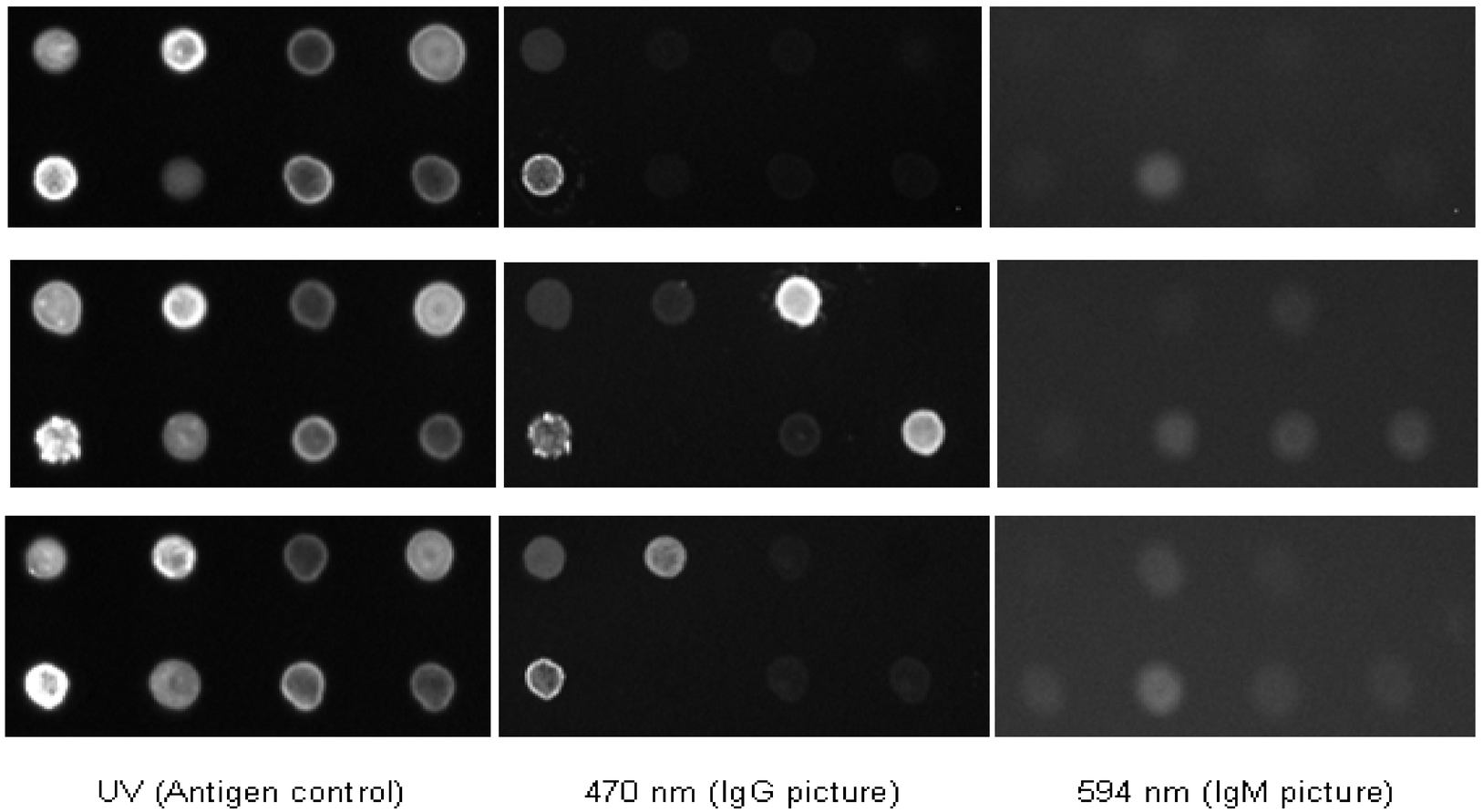
342

A) **Multiplexed antigens array**



B)

Results and interpretation

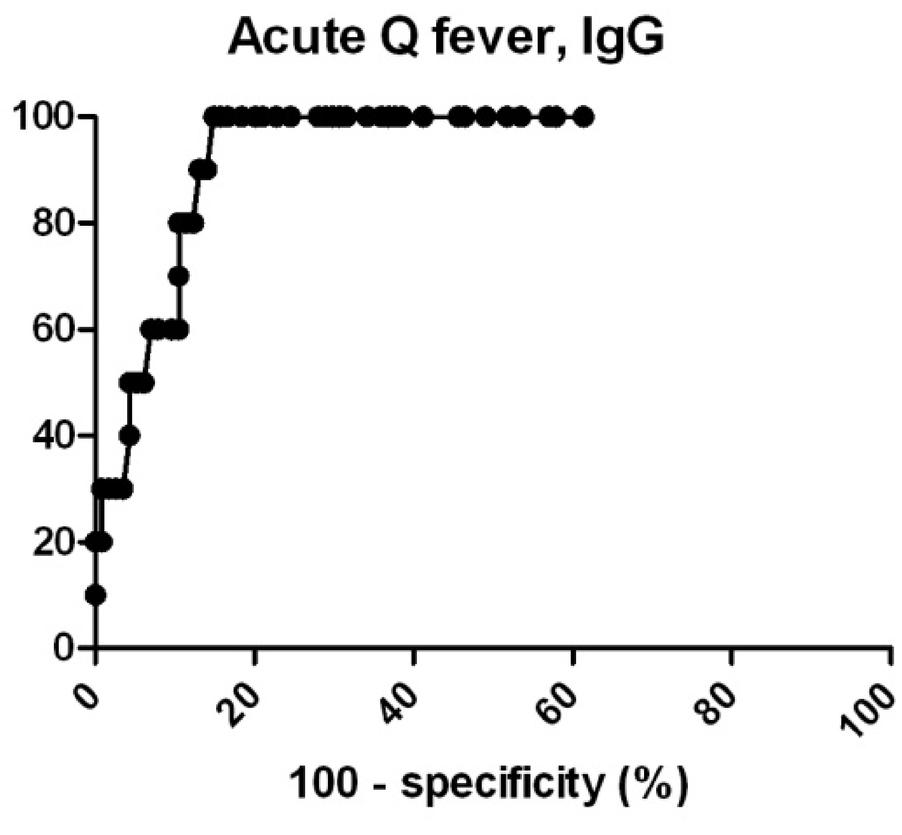


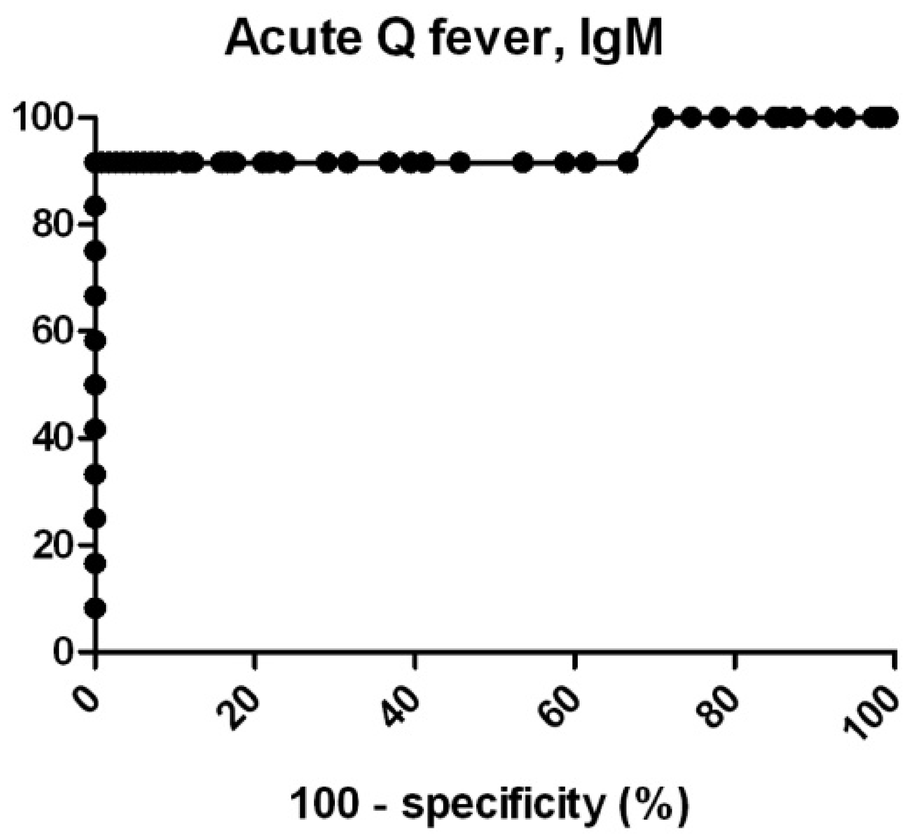
Negative Sera

Rickettsia sp. positive sera

Coxiella burnetti positive sera

UV (Antigen control) 470 nm (IgG picture) 594 nm (IgM picture)


Acute Q fever, IgG
100
80
60
40
20
0
0
20
40
60
80
100
100 - specificity (%)


Acute Q fever, IgM
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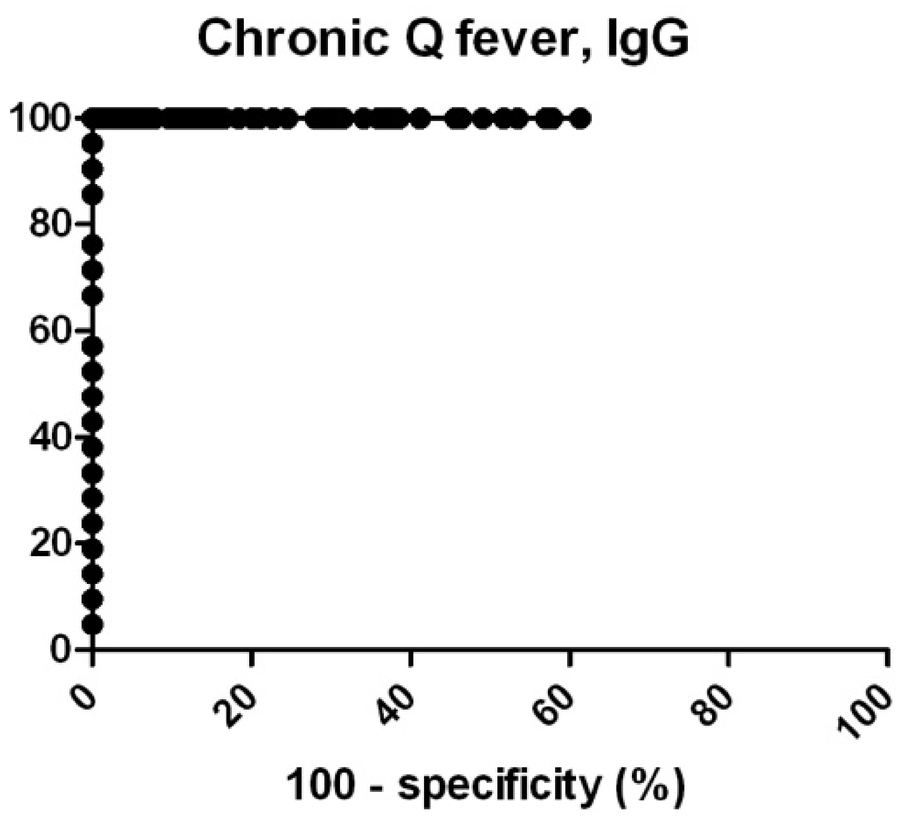
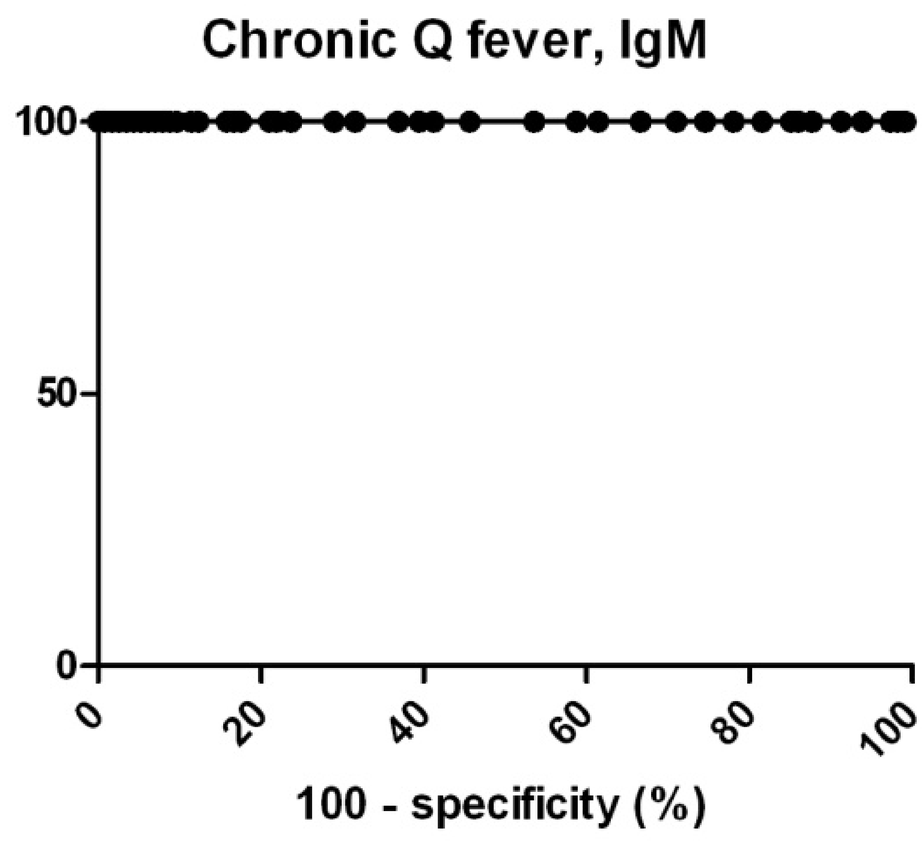
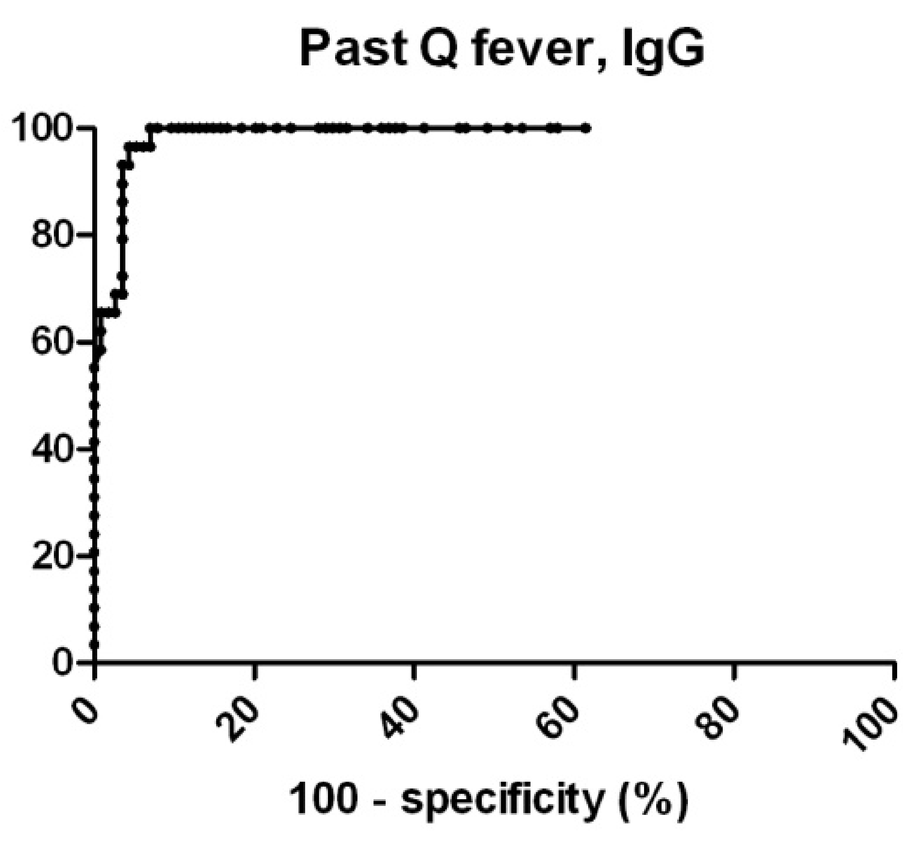
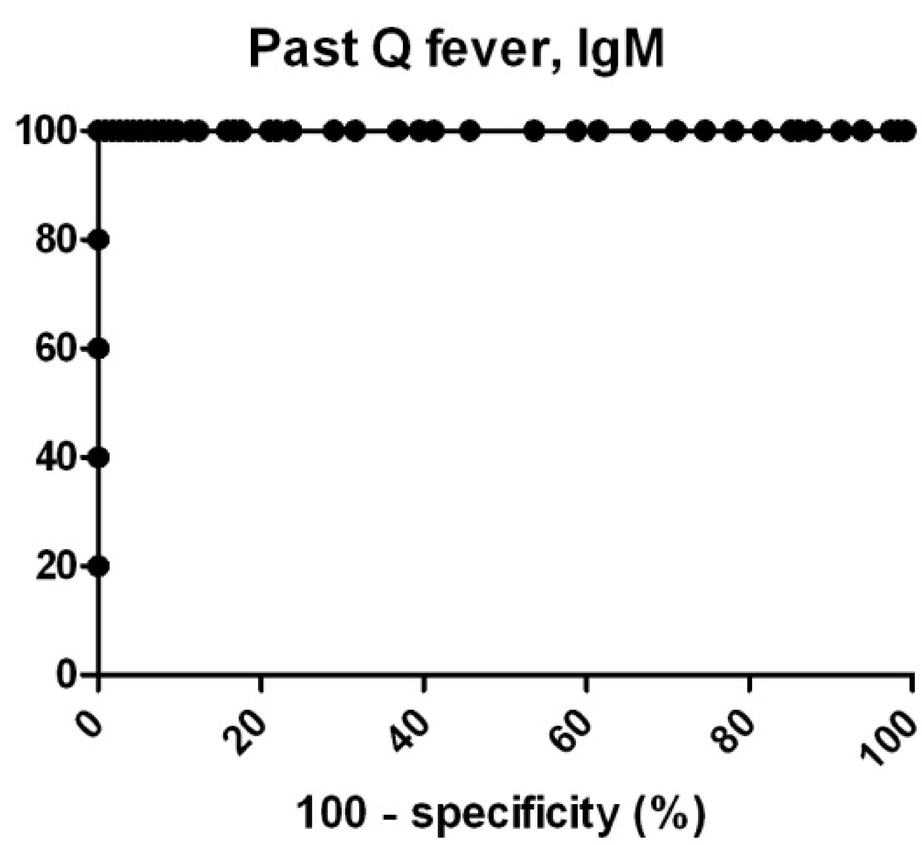
80
60
40
20
0
0
20
40
60
80
100
100 - specificity (%)


Chronic Q fever, IgG
100
80
60
40
20
0
0
20
40
60
80
100
100 - specificity (%)


Chronic Q fever, IgM
100
50
0
0
20
40
60
80
100
100 - specificity (%)


Past Q fever, IgG
100
80
60
40
20
0
0
20
40
60
80
100
100 - specificity (%)


Past Q fever, IgM
100
80
60
40
20
0
0
20
40
60
80
100
100 - specificity (%)