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

3

4 Alain Bizzini¹, Olivier Péter², David Baud^{1,3}, Sophie Edouard⁴, Pascal Meylan¹, Gilbert Greub^{1*}

5

6 ¹ Institute of Microbiology, University of Lausanne and University Hospital Center, Lausanne,
7 Switzerland

8 ² Department of Infectious diseases, Central Institute of Valais, Sion, Switzerland

9 ³ Department of Obstetrics and Gynaecology, University Hospital of Lausanne, Lausanne,
10 Switzerland

11 ⁴ URMITE UMR 6236, Faculté de Médecine et de Pharmacie, Marseille, France

12

13 * To whom correspondence should be addressed:

14 Gilbert Greub

15 Institute of Microbiology

16 University of Lausanne and University Hospital Center

17 Rue du Bugnon 46

18 1011 Lausanne

19 Switzerland

20 Phone: +41 21 314 49 79

21 Fax: +41 21 314 40 60

22 E-mail: gilbert.greub@chuv.ch

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30

31 **ABSTRACT**

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

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

41 For acute Q fever, the sensitivity of phase 2 IgG was only of 30% with a cutoff of 1 arbitrary
42 unit (AU). In patients with acute Q fever with positive IF IgM, sensitivity reached 83% with
43 the same cutoff. Sensitivity for chronic Q fever was 100% whereas sensitivity for past Q fever
44 was 65%. Sensitivity for spotted Mediterranean fever and murine typhus were 91% and
45 100%, respectively. Both assays exhibited a good specificity in control groups, ranging from
46 79% in sera from patients with unrelated diseases or EBV positivity to 100% in sera from
47 healthy patients.

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

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

54

55 **INTRODUCTION**

56 *Coxiella burnetii*, *Rickettsia typhi* and *Rickettsia conorii* are the causative agents of Q fever,
57 murine typhus and Mediterranean spotted fever, respectively [18, 5, 16]. These obligate
58 intracellular bacteria are difficult to diagnose and culture-based methods are restricted to
59 specialized BSL3 laboratories. PCR-based detection methods have been described but are
60 essentially found in larger reference diagnostic laboratories [15, 17]. Therefore, screening
61 for Q fever and rickettsial diseases often relies on serologic techniques such as Enzyme-
62 linked immunosorbent assays (ELISA), Western-blot (WB) and immunofluorescence assays
63 (IF). Serology is especially useful for the diagnosis of chronic Q fever, in particular when a
64 biopsy of the affected organ (e.g. cardiac valve, liver) is not available. *Coxiella burnetii*
65 serological diagnosis is based on the presence of antibodies against phase 2 and phase 1
66 antigens, for the diagnosis of acute and chronic Q fever, respectively. The serological
67 diagnosis of rickettsial infections is more complex, because of the common occurrence of
68 cross-reactions between the different spotted fever group rickettsia. Conventional IFs are
69 labour-intensive and reading of the assay is operator-dependent. Recently, a new innovative
70 automated epifluorescence assay has been made available by InoDiag (Signes, France) for
71 the diagnosis of *C. burnetii*, *R. typhi* and *R. conorii*. This multiplexed antigen microarray uses
72 standardized quantities of antigens, spotted on glass slides with appropriate controls [9].
73 Except for the initial deposition of the serum samples, all subsequent steps (i.e. secondary
74 antibody depositions, incubations, washing, drying, reading and interpretation) are
75 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

83 MATERIALS AND METHODS

84 Patient population and controls

85 A total of 213 sera were studied. Tested sera from acute, chronic or past Q fever infections
86 as well as rickettsiosis cases that were positive for IgM and/or IgG using a reference indirect
87 micro-immunofluorescence technique (described below) were selected from the routine
88 serological laboratories of Lausanne and Sion, Switzerland (seroconversion, clinically
89 confirmed or compatible cases as well as sera from previous studies [2, 6, 13]). This included
90 20 sera from spotted fever rickettsiosis (11 spotted Mediterranean fever, 9 African tick-bite
91 fever), 6 sera from murine typhus, 63 sera from Q fever (12 acute, 22 chronic, 29 past
92 infections). Clinical cases corresponding to some of these sera have been previously
93 published [1, 4, 10, 11]. A total of 124 control samples were included. These consisted in 10
94 samples from EBV IgM+ sera, 101 sera from pregnant women and 13 sera from patients with
95 unrelated infections.

96 InoDiag

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

104 presence of rheumatoid factor, (iv) double-stranded DNA to detect antinuclear antibodies.
105 Serum samples were diluted at a ratio of 1:16 and applied on the slide. All subsequent steps
106 were performed automatically as previously described [6]. After the final drying step, slides
107 were imaged with an automatic InoDiag fluorescent camera analyzer and the data processed
108 using the software Inosoft (InoDiag). The end-point is a fluorescence index. For *C. burnetii*,
109 two-cutoffs for positivity were considered: a fluorescence index >1 arbitrary units (AU) and
110 >2 AU. For *Rickettsia*, only the cutoff of 2 AU was considered. Receiver operating
111 characteristics (ROC) curves were used to precise the diagnosis performance of the assay.

112 **Indirect micro-immunofluorescence**

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

121

122 RESULTS

123 Performance of the InoDiag assay in the Q fever group

124 The assay has been evaluated for the three categories of Q fever disease (acute, chronic and
125 past infection). The sensitivity and specificity for *C. burnetii* of the InoDiag assay for the
126 various tested groups are shown in the Table 1. In the acute Q fever group (n=12), 100%
127 (12/12) and 83% (10/12) sera were positive by IF for phase 2 IgM and phase 2 IgG,
128 respectively. The sensitivity of the InoDiag assay for acute Q fever with positive IgG detected
129 by IF (irrespective of the presence of IgM) was of 20% (2/10) and 30% (3/10) with cutoffs of
130 2 and 1 AU, respectively. Considering sera positive in IgM by IF in patients with acute Q
131 fever, the sensitivity reached 75% (9/12) and 83% (10/12) with cutoffs of 2 AU and 1 AU,
132 respectively. Two early seroconversions that were detected by IF already on the first sera
133 (one positive for both IgG and IgM ; one positive for phase 2 IgM only) were missed by the
134 InoDiag assay.

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

140 Even though the InoDiag assay is commercialized as a screening tool, we wondered whether
141 the InoDiag assay could also be used as a tool to monitor treatment efficacy. In successive
142 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 as 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

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

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

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

179

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

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

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

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

194 Six sera were obtained from 2 patients with murine typhus. With the conventional IF, 5 sera
195 were positive for IgM and IgG, 1 sera for IgG only. The InoDiag results were totally
196 concordant to IF results. Hence, the sensitivity of the InoDiag assay for murine typhus was
197 100% in our small cases series. Interestingly, in one of these patients coming from a zone of
198 known endemicity (North Africa), 4 out of 5 sera showed a positive signal for *C. burnetii* with
199 the InoDiag method (IgG: 2.21-3.29 ; IgM negative). Since conventional IF for *C. burnetii* was
200 negative for all these sera, these results might represent a cross-reaction of the InoDiag
201 assay rather than serological traces of a past infection.

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

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

211 Regarding cross-reactions with *Coxiella*, we observed a prevalence of *Coxiella* antibodies of
212 40% (8/26) among the 26 sera from patients with rickettsial diseases. Of these 8 sera, only 1
213 was confirmed by IF.

214

215 **DISCUSSION**

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

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

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

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

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

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

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

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

262

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266 Maladies Infectieuses et Tropicales Emergentes of the Université de la Méditerranée for
267 their excellent technical assistance.

268

269 **Figure legends**

270 **Figure 1**

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

274 **B)** Typical slides analysed by a fluorescent camera at different wavelength. UV illumination
275 (365 nm) allows the determination of the area of each spot and confirms the presence of all
276 antigens. Fluorescence readings at 470 nm and 594 nm allow the detection and
277 quantification of IgG and IgM, respectively, towards the various antigens.

278

279 **Figure 2**

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

282

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

287 **Table 2**

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

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

290

291

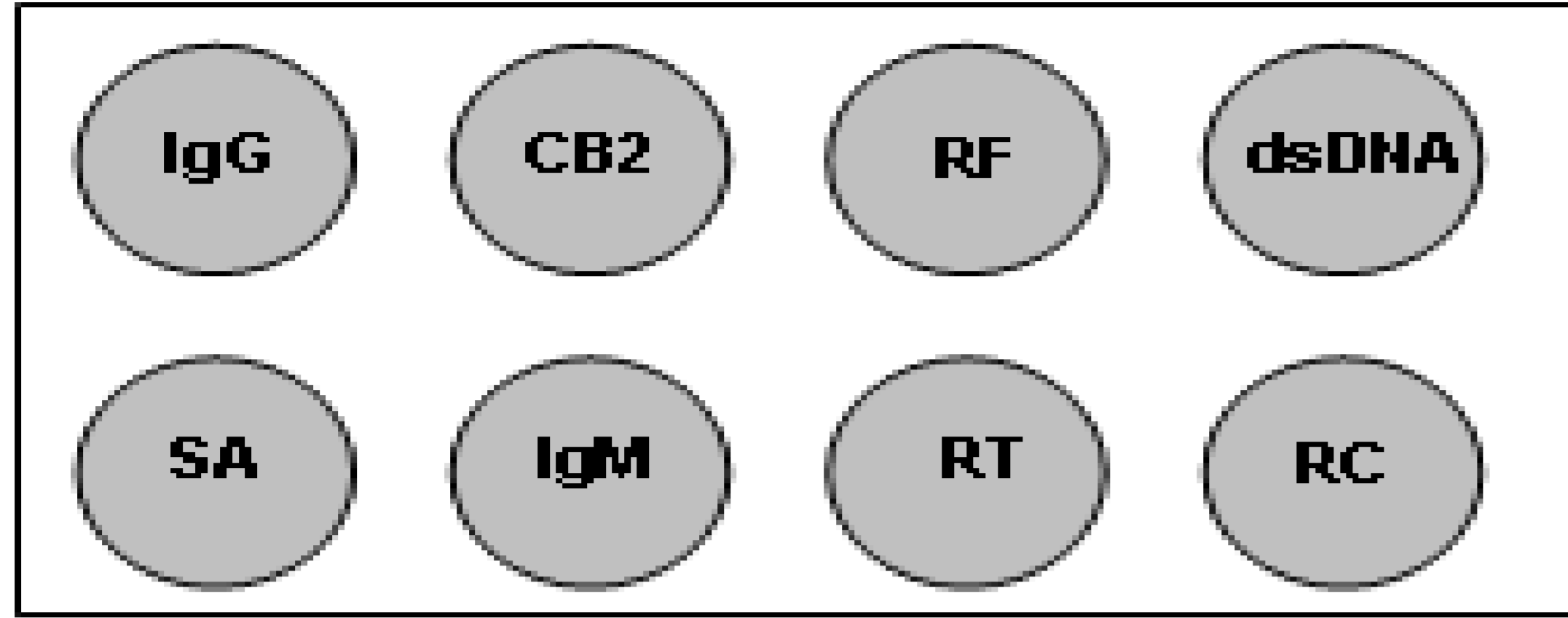
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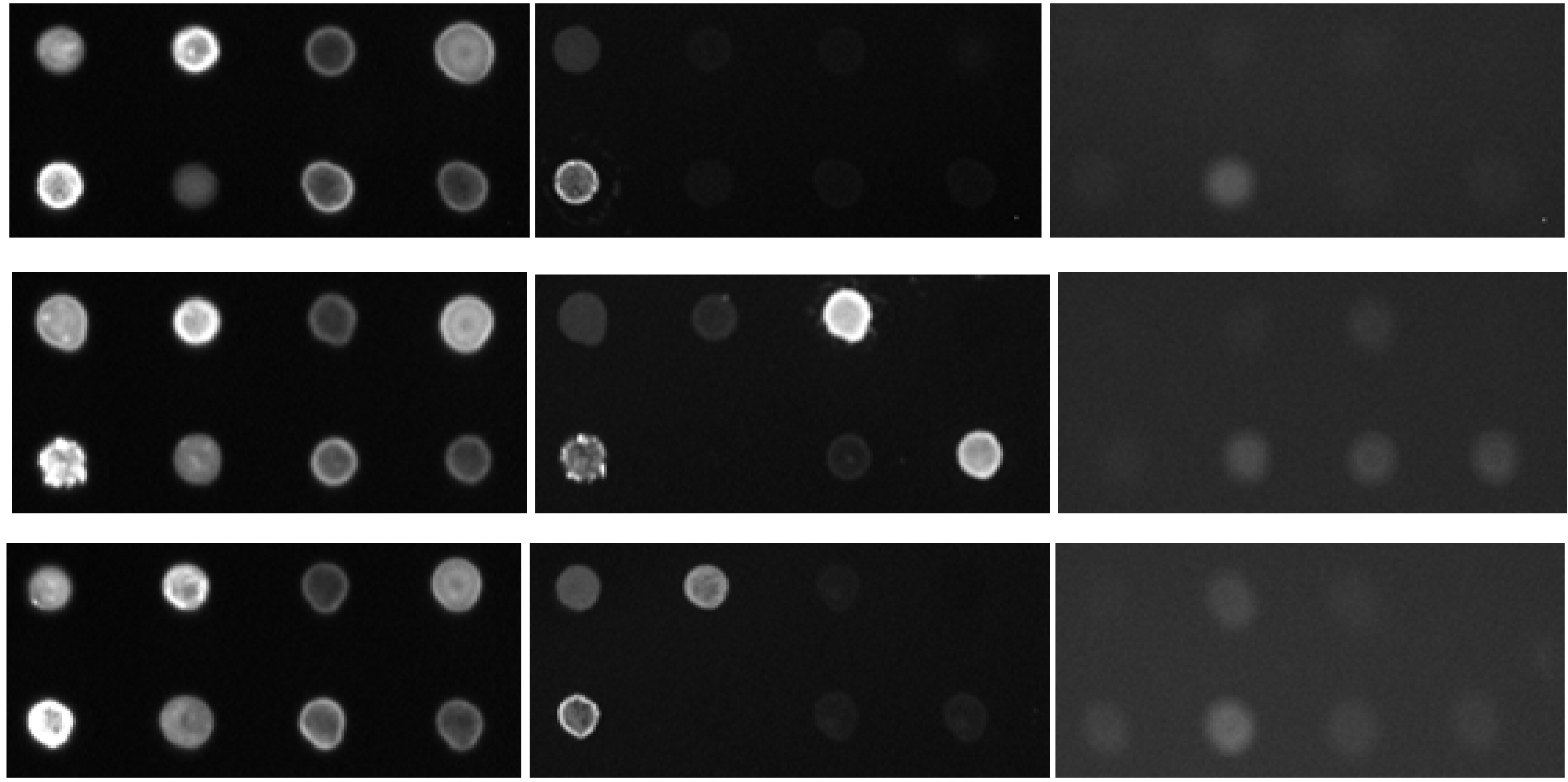
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342

A) Multiplexed antigens array



B)



Results and interpretation

Negative Sera

Rickettsia sp. positive sera

Coxiella burnetii positive sera

UV (Antigen control)

470 nm (IgG picture)

594 nm (IgM picture)

