Comparative Immunology, Microbiology and Infectious Diseases 58 (2018) 17–25

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Identification of rickettsial immunoreactive proteins using a proximity ligation assay Western blotting and the traditional immunoproteomic approach

Malgorzata Kowalczewska[a,*], Aymar N'Djatchi[b], Claude Nappez[b], Sulaf Alwassouf[c], Philippe Decloquement[b], Nicholas Armstrong[b], Khalid El Karkouri[a], Sophie Edouard[b], Didier Raoult[b]

[a] VITROME, IRD, AP-HM, SSA, Université Aix-Marseille, Institut Hospitalier Universitaire-Méditerranée Infection, 19-21, Bd Jean Moulin, 13005, Marseille, France
[b] МЕФИ, IRD, AP-HM, Université Aix-Marseille, Institut Hospitalier Universitaire-Méditerranée Infection, 19-21, Bd Jean Moulin, 13005, Marseille, France
[c] Emergence des Pathologies Virales, EPV UMR_D 190, IRD, EHESP, Faculté de Médecine, 27, Bd Jean Moulin, Marseille, 13385, France

ARTICLE INFO

Keywords:
Rickettsia africae
Rickettsia conorii
Serodiagnosis
Immunoproteomics
Proximity ligation assay

ABSTRACT

The closely related species *Rickettsia conorii* and *R. africae* are both etiological agents of rickettsiosis, a tick-borne serious infective disease. The laboratory diagnosis is based on serology, but remains not enough specific to provide the diagnosis at the species level. Here, we attempted to identify specific proteins that would enable the discrimination of *R. africae* sp from *R. conorii* sp infections.

We screened 22 *R. africae*- and 24 *R. conorii*-infected sera at different course of infection using a traditional immunoproteomic approach. In parallel, we focused on the technical development of a "relatively new technique" named a proximity ligation assay coupled to two-dimensional Western blotting.

The top range markers of *R. africae* early infection were rpoA, atpD, and acnA, ORF0029, *R. africae* active infection were rOmpB β-peptide, OmpA, groEL and ORF1174, early *R. conorii* infection was prsA, RC0031, pepA, *R. conorii* active infection were ftsZ, cycM and rpoA.

They are candidates for serodiagnosis of rickettsioses.

1. Introduction

The spotted fever group (SFG) *Rickettsia* are obligate intracellular bacteria associated with arthropods [1]. The SFG group is composed of several closely related *Rickettsia* species [2,3], such as, *R. conorii*, *R. africae*, *R. massiliae, R. slovaca* and others.

Rickettsia africae is responsible for African tick bite fever (ATBF), which was initially considered to be caused by *R. conorii* [4–7], the etiological agent of Mediterranean spotted fever (MSF). These two distinct tick-transmitted diseases, ATBF and MSF, respectively, co-exist in sub-Saharan Africa [8] are transmitted by two distinct ticks. *R. africae* is transmitted by non-host-specific hard ticks of the genus *Amblyomma,* frequently infesting wild ungulates and cattle, but also feeds readily on humans, often with multiple inoculation eschars [8]. *R. conorii* is transmitted by the brown dog tick *Rhipicephalus sanguineus*, relatively host-specific, exceptionally feeding on people with a characteristic inoculation eschar namely, a blackspot, rarely multiple [9,10]. Tick- borne diseases were long considered as an endemic in tropical zones. However, climate changes, as well, as human activities, modified geographical distribution of tick-borne diseases [11,12]. At present, *R. africae* is probably the most frequent in sub-Saharan Africa with *ca.* 50% of seroprevalence against *Rickettsia* spp. in healthy rural populations, the French West Indies [13,14] Oceania [15], and has also been recently reported in Union of Comores [16]. *R. conorii* is mainly endemic in Mediterranean countries of North Africa and Southern Europe [12,17]. Both *R. africae* and *R. conorii* were detected in ticks removed from humans in Turkey [18], also in ticks from Kenya [19] and should be considered as potential pathogens [20]. However, the

Abbreviations: ATBF, African tick bite fever; MSF, Mediterranean spotted fever; IFA, immunofluorescence assay; PCR, polymerase chain reaction; PLA WB, proximity ligation assay-based Western blotting; RCA, rolling cycle amplification; HRP, horseradish peroxidase; HBD, healthy blood donors; PCR, polymerase chain reaction; PLS-EDA, partial least squares-enhanced discriminate analysis

* Corresponding author.
E-mail addresses: m.kowalczewska@univ-amu.fr (K. Malgorzata), ndjatchiaymar@yahoo.fr (N. Aymar), claude.nappez@univ-amu.fr (N. Claude), sulaf_alwassouf@yahoo.com (A. Sulaf), Philippe.DECLOQUEMENT@univ-amu.fr (D. Philippe), nicholas.armstrong@univ-amu.fr (A. Nicholas), sophie.edouard@univ-amu.fr (E. Sophie), didier.raoult@gmail.com (R. Didier).

https://doi.org/10.1016/j.cimid.2018.06.004
Received 27 October 2017; Received in revised form 16 March 2018; Accepted 10 June 2018

cases with *R. africae* in Europe are all imported by travelers from endemic zones [21–23,7]. ATBF is usually a benign disease, but perhaps more severe for the elderly population [24]. Indeed, MSF is usually a mild disease, can lead in about 10% of cases to severe multiorgan dysfunction, often resulting from delayed diagnosis [10]. Flu-like symptoms are also common to both diseases [10].

A human case of rickettsiosis is not always easy to diagnose from its clinical picture as the typical symptoms may not always be present, instead of the presence of fever, which is only one apparent clinical sign [20,25]. A definitive diagnosis is made by serology (IFA, immunofluorescence assay) and molecular tools like different polymerase chain reaction (PCR) systems are used [26,27] or cell culture [28]. In all cases, isolation and identification of causative agent from clinical sample, gives a definitive diagnosis, but is limited to the laboratories with BSL3 facilities, which is always fastidious. Consequently, isolates available from clinical samples are rare. Indeed, serological cross-reactions among different species hamper a correct identification of the specific rickettsiosis by serology [3].

Our laboratory, the French National Reference Center for *Rickettsia*, *Coxiella* and *Bartonella*, treats the serum samples received from different regions of World. A gold diagnostic standard for rickettsioses is immunofluorescence assay (IFA) followed by real-time PCR [29,30]. The diagnosis by the IFA is confirmed by seroconversion or by a fourfold rise in titers between acute and convalescence serum samples [25,29]. The IFA faced the difficulty that a detectable level of antibodies against *Rickettsia* sp. does not appear in the blood until day 7 or 10 after the onset of the disease [20,32]. Therefore, PCR is very useful for diagnosis at early stages of infection characterized by negative serology [20,27]. Molecular detection has been simplified by directly using eschar swab samples [26,27,31]. Diagnosis by real-time PCR has gained sensitivity and specificity by using new generation primers [27,30].

Despite the progress made in diagnosis of rickettsiosis last decade, we noticed in our laboratory a recurrent problem to discriminate *R. conorii* from *R. africae* at early stage of infection.

The IFA allows the diagnosis of genus *Rickettsia* spp., but it is insufficient for identifying the etiologic agent at the species level. Furthermore, it is important to note that sufficient serological titers in ATBF appear later than in other rickettsioses [6,25,32]. Western blotting and antigen adsorption have also been used routinely and are considered to be a powerful serodiagnostic tools for seroepidemiology, especially when used in questionable cases, they allow the confirmation of serologic diagnoses obtained by conventional methods [3,29]. Notwithstanding this fact, the whole cell antigens used in this method is limited and cross-reacts with different *Rickettsia*, making it difficult to identify the definitive etiological agent [25,33].

In this context, the objective of our work was to propose new diagnostic alternatives that discriminate R. *conorii* from *R. africae* infections, possibly with a focus on the early stages. Consequently, we have undertaken a challenge to develop a method which combines in some way, serology and PCR. Here, we have applied the *in situ* proximity ligation assay (PLA) in 2-D Western blotting. *In situ* PLA is based on simultaneous recognition of individual or complexes of protein molecules by two oligonucleotide labeled antibodies (PLA probes), which then give rise to a ligation-dependent amplifiable DNA molecule. Signal amplification generated by each detected pair of probes [34–41], is possible by *e.g.* real-time PCR for detection of proteins in solution, or by isothermal rolling circle amplification (RCA) of circularized reporter DNA strands for localized detection of target proteins previously transferred onto nitrocellulose membrane [42]. These dual antibodies recognition allows the discrimination between closely similar proteins [34,35,43], which means to overcome obstacles encountered in traditional WB. The PLA WB was already documented in the early 1990 s, but was abandoned for years [44]. The rebirth of one-dimensional PLA WB was observed in 2000 and coincided with immuno-PCR (iPCR) development [34,37,42]. Indeed, iPCR allowed diagnosis in our laboratory of Q fever at the early stage [45]. *In situ* PLA WB has been successfully applied to a whole cell pathogen approach [46].

Consequently, we introduced this “relatively new”, sensitive and specific technique to identify antigens suitable for serodiagnosis of rickettsioses due to *R. conorii* and *R. africae*. We could simultaneously evaluate RCA-based detection in PLA WB with this based on horseradish peroxidase conjugated (HRP), used in classical WB. Finally, although surface exposed proteins rOmpB, rOmpA which have been already proven as suitable diagnostic antigens [47–50], we identified potential new targets that allow quite good discrimination of both *Rickettsia* spp. at different course of infection (acute and convalescent phase);

2. Material and methods

2.1. Human sera

We used for this study, sera from 22 patients (Rco: S1-S22) infected by *R. conorii* and sera from 24 patients diagnosed with *R. africae* infection (Raf: S1-S24). All patients who had participated in this study were diagnosed at the French National Reference Center (FNRC) (Marseille, France) after giving informed consent. The diagnosis was based on clinical picture, serology, and more rarely on isolation or molecular identification of the causative agent from blood or skin samples [25].

2.1.1. Indirect immunofluorescence assay

We used the reference method, indirect immunofluorescence assay (IFA) using *R. conorii* and *R. africae* antigens purified from L929 cells as previously described [3,29]. As a negative control, previously tested serum from a healthy blood donor was used and as a positive control, a serum from a patient with proven IgG and IgM end-point titres of 1/128 and 1/256, respectively, to *R. conorii* or/and *R. africae* [25,29].

2.1.2. PCR assay

DNA was extracted from sera, skin biopsy or skin swab [26,31], using QiAMP DNA Mini Kit (Qiagen) according to manufacturer’s instructions. The quality of DNA extraction was checked by using quantitative real-time PCR (qPCR) (Light Cycler 2.0, Roche) for a housekeeping gene encoding beta-actin [52]. All DNA samples were screened by qPCR using the 1029 system based on the RC0338 gene (referenced by *R. conorii* genome AE006914) encoding a hypothetical protein that is present in all tick-borne *rickettsiae* [31]. For the positive case with $Ct < 35$, a second species-specific qPCR was performed and targetRC0743 for *R. conorii* or a fragment of the RAF ORF0659 gene encoding adenine methylase for *R. africae* [52]. Any sample with CT value ≤ 35 is considered as positive [52].

2.1.3. Cases definition

Case was defined by the association of clinical symptoms (fever, eschar, lympho-adenopathy) with the serologic criteria IgM titres $\geq$1:64 and/or IgG titres $\geq$1:128, and/or a fourfold increase in two sera within a 2–4 week interval, seroconversion, and/or a positive PCR on sera or skin sample. We classified the patients into 2 groups:

(i) Patients with early infection due to *R. conorii* (eRco) (S1–4, S6, S8, S16, S22) or to *R. africae* (eRaf)(S1-15, S17, S19, S23) had a negative serology at the time of experience and had received final diagnosis based on clinical picture and/or laboratory diagnosis. (ii) *R. conorii* (Rco) (S5, S7, S9–15, S17–21) and *R. africae* (Raf) (S16, S18, S20, S21, S22, S24) convalescent patients with positive serological titers. All cases are detailed in Supplementary material 1.Ten pooled sera from anonymous healthy blood donors were included as control group. They were probed either on *R. conorii* (HBD Rco) or *R. africae* membrane (HBD Raf).

2.2. Growth of Rickettsiae

Rickettsia africae (strain ESF-5) and *Rickettsia conorii* (strain Seven Malish, ATCC VR-630) were propagated in a confluent monolayer of murine fibroblast L929 cells [51] and purified on a discontinuous renografin gradient as previously reported [53]. Purified bacteria were washed in PBS (10,000 × *g*, 10 min, and 4 °C) and stored at −80 °C until further use. All purification steps were checked by Gimenez staining [54].

2.3. Preparation of crude extracts for 2-D gel electrophoresis

Purified bacteria were lysed by sonication in a rehydration solution (7 M urea, 2 M thiourea, 4% w/v CHAPS) and centrifuged (10,000 × *g*, 20 min, 4 °C) to remove cell debris and unbroken cells. The whole cell protein extract was precipitated using the PlusOne 2-D Clean-Up Kit (GE Healthcare). The final pellet was resuspended in rehydration solution, and the protein concentration was determined using a modified Bradford method [55].

All IEF (Immobiline DryStrips gels (7 cm, 13 cm or 18 cm, pH 3–10, GE Healthcare) rehydrated with 15 µg (7 cm), 30 µg (13 cm) or 200 µg (18 cm) of solubilized proteins) and 2-D electrophoresis steps were performed as previously described [56]. The proteins were resolved by electrophoresis through a 10% SDS-polyacrylamide gel (EttanTM DALT, GE Healthcare) at 5 W/gel for 30 min, followed by 17 W/gel for 4–5 h. Following electrophoresis, the gels were either silver-stained or transferred onto nitrocellulose membranes. Digital images were generated using transmission scanning (Image Scanner, GE Healthcare). Spots were excised from the gel manually and identified by peptide mass fingerprinting using a MALDI-TOF MS Bruker Ultraflex I spectrometer (Bruker Daltonics) as previously described [56]. In-gel trypsin digestion (sequencing-grade modified porcine trypsin, Promega) was performed as previously described [56]. Mass spectra were internally calibrated using autolytic peptides from trypsin. Tryptic peptide mass lists were computed using a Mascot 2.4 internal server with the following parameters: peptide tolerance (monoisotopic mass) equal to 90 ppm, one missed cleavage allowed, fixed carbamidomethyl (C), and variable oxidation (M). Searches were performed against non-redundant *R. africae* ESF-5, *R. conorii* Malish 7 and *Mus musculus* sequences (NCBI RefSeq). Proteins were validated with a Mascot score superior to the calculated significance threshold ($p < 0.05$). The protein spots were identified in at least two replicates. The identified immunogenic proteins are shown in Supplementary material 1.

2.4. Western blotting

Rickettsial proteins (either *R. conorii* or *R. africae*) resolved by 2D gel electrophoresis (13 cm, pH 3–10) were transferred onto nitrocellulose membranes (Trans-blot Transfer Medium, pure Nitrocellulose Membrane 0.45 µm, Bio-Rad). Membranes were then blocked in PBS supplemented with 0.2% Tween 20 and 5% non-fat dry milk (PBS-Tween-milk) for 1.5 h at room temperature before incubation with sera from infected patients (diluted 1:100 in the blocking buffer). We used a dilution of 1:100 for Western blotting performed with a pool of 5 sera (we pooled 10 µl of each individual serum) from healthy blood donors. After 1 h of incubation, the membranes were washed three times with PBS-Tween and probed with horseradish peroxidase-conjugated goat anti-human IgG (1:5000; GE Healthcare). After treating with the secondary antibody, each membrane was washed three times as indicated above. Immunostained spots were visualized using a commercially available chemiluminescence kit (ECL™ Western Blotting Analysis System, GE Healthcare). Then, the membranes were exposed to Hyperfilm™ ECL and subsequently developed using an automated film processor (Hyperprocessor™, GE Healthcare).

2.5. Proximity ligation assay-based Western blotting (PLA WB)

The whole rickettsial proteins (15 µg) were resolved by 2D gel electrophoresis (7 cm, pH 3–10) and transferred onto nitrocellulose membranes (Bio-Rad) using a semidry transfer unit (Hoeffer Scientific) as described previously [56]. Membranes were then blocked in PBS supplemented with 0.2% Tween 20 (PBST) and 5% non-fat dry milk (PBST-milk) for 1.5 h at room temperature before incubation with sera from infected patients (diluted 1:100 in PBST-milk). After washing, the membrane was incubated for 1 h at RT with gentle rotation with Duolink® II PLA Plus probes (OLINK Bioscience) as described by the manufacturer. The detection probes (0.2–1 µg/ml) were diluted in PLA probe diluent buffer (0.5 mg/ml BSA, 5 µg/ml salmon sperm DNA, 5 mM EDTA, 0.05% Tween 20 in TBS buffer). The mem/brane was then briefly rinsed, washed twice for 10 min and incubated with a gentle orbital rotation for 40 min at 37 °C with T4 DNA ligase (Fermentas) (0.01–0.03 U/µl) and backbone and splint oligonucleotides (1–90 nM) supplied by the manufacturer (OLINK Bioscience) and diluted in oligo ligation buffer. The membrane was then washed for 5 min in PBST and then incubated with phi29 DNA polymerase (GE Healthcare) (0.01–0.125 U/µl) in a rolling circle amplification buffer for 1 h at 37 °C (OLINK Bioscience). After two rinses, the membrane was incubated with Cy5 dye (5–45 nM) (Integrated DNA Technologies) in a detection buffer (2x SSC, 0.5 mg/ml BSA, 2 µg/ml salmon sperm DNA, 0–5% formamide, 0.05% Tween 20) for 30 min at 37 °C. Finally, the membrane was rinsed twice, washed 3 times for 10 min in PBST and then rinsed quickly in PBS to remove Tween 20. Fluorescence signals were captured using a Typhoon™ FLA 9000 imager (PMT, 450 V) (GE Healthcare).

2.6. Statistical analysis

To visualize the discriminant proteins from different groups of sera used in the present study (as shown above), we performed a partial least squares-enhanced discriminant analysis (PLS-EDA) using Multibase 2015, which is an Excel add-in tool (Numerical Dynamics) [57]. PLS regression is a statistical method akin to principal components (PC) regression; it creates a linear regression model by projecting the predicted variables and the observed variables into a new space [57]. In addition to the groups established as described in Section 2.1.3., we included for statistical analysis, the groups with sera tested by PLA WB: iRco ($S16^{i}$–19^{i}), iRaf ($S16^{i}$–20^{i}) as well as, sera screened for cross-reaction: cRco ($S20^{c}$–$S22^{c}$), *R. conorii* sera probed on *R. africae* membrane and cRaf ($S21^{c}$–24^{c}), *R. africae* sera probed on *R. conorii* membrane, respectively. The statistical analysis takes into account the individual partitioning.

3. Results

3.1. Immunoreactivity patterns revealed by traditional 2-D WB

Protein profiles of silver-stained 2-D gels (18 cm, p*I* 3–10) were very similar for both *Rickettsiae, R. conorii* (Fig. 1A) and *R. africae* (Fig. 1B). In total, 1100 spots /1374 ORFs [58] and 920 spots/1030 ORFs [58] were detected on *R. conorii* and *R. africae* 2-D gels, respectively. The immunoproteomic profiles obtained for both *Rickettsia* spp. were similar and homogeneous. The majority of immunoreactive spots were clustered around a narrow pH range of 3.5–4.5 distributed over MWs of 30–195 kDa. The major dominant spot in this zone corresponded mainly to the rOmpB isoforms and clusters of *R. conorii* spots (Fig. 2A–C; fusA, def3, sdhA, dnaK, atpA, RC0185) and *R. africae* spots (Fig. 2D–F; thrS, dnaK, fusA, ftsZ, ORF0226, ORF1113, htrA, atpD). Other major immunoreactive spots were groEL, tuf, sucC, pepA, and lpxD for *R. conorii* and groEL, rpsA, pepA, sucB, tuf, and lpxD for *R. africae*. The most basic identified immunoreactive proteins were (pH 8–10) lpDx, frr, RC1281, prsA, and RC1282 (*R. conorii*) and rOmpB β-

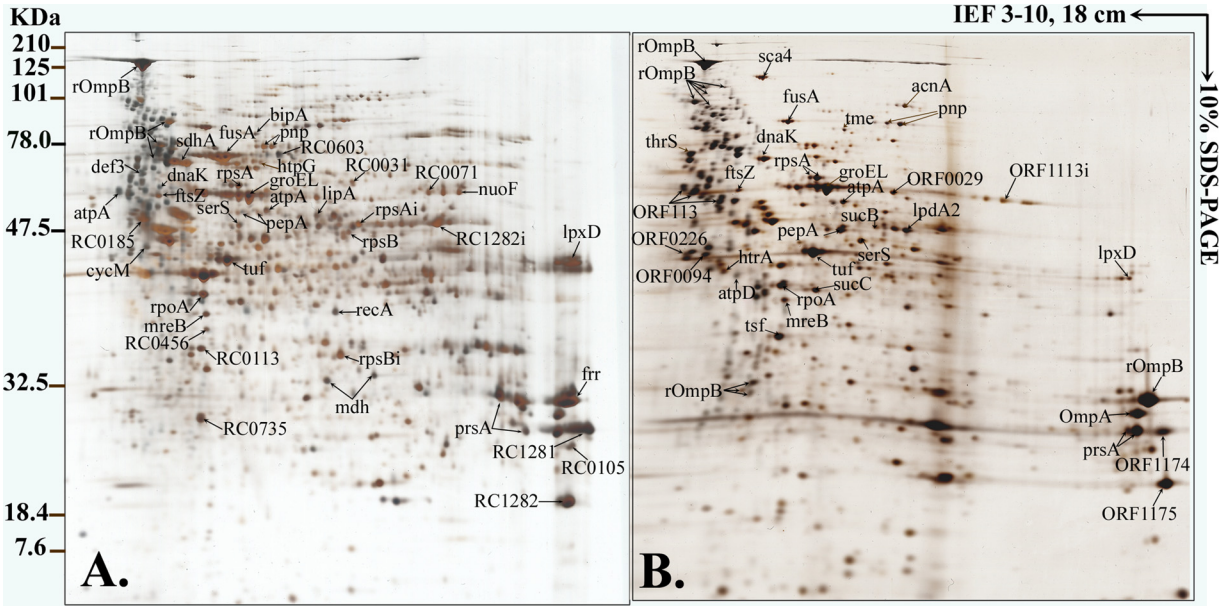

Fig. 1. A silver-stained 2-D map of whole-protein extracts from A) *R. conorii* and B) *R. africae*. The proteins (200 µg) were resolved on a 10% polyacrylamide gel. The major protein spots identified in this study, most of which reacted with the patient sera, are annotated using either the gene name or the name of the locus tag.

peptide, rOmpA, ORF1174, and ORF1175 (*R. africae*). Some of them have previously been described as immunogenic [55,59,60]. Thus, we probed 3 *R. conorii* and 4 *R. africae* sera against *R. africae* and *R. conorii* proteomes, respectively, with the aim of improving specificity (Table 1, Fig. 2). Here again, the zone of high reactivity was the cluster of spots in pH range 3–4.5 with the major rOmpB spot.

3.2. Immunoreactivity patterns revealed by 2-D PLA WB

Here, we adapted *in situ* PLA technique to detect rickettsial proteins. To determine whether *in situ* PLA can provide enhanced WB sensitivity, we compare 2D PLA WB to traditional 2D WB. The immunoreactivity profiles obtained by 2D PLA WB were very similar to those obtained with traditional WB. Mainly, the zone in the pH range 3–4.5 was highly

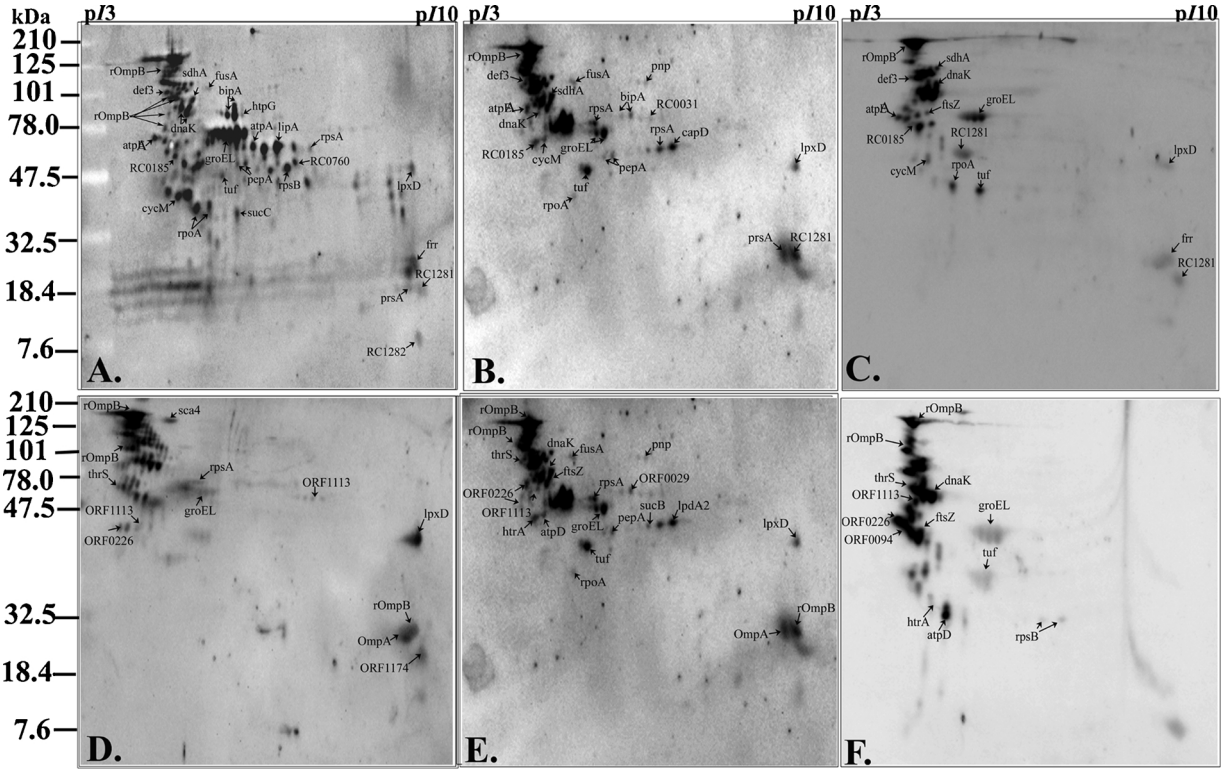

Fig. 2. Example of immunoreactivity profiles obtained with traditional 2D WB (p*I* 3–10, 13 cm): (A) *R. conorii* IFA-positive serum (1:100e) on *R. conorii*-resolved 2D proteome (30 µg). (B) *R. conorii* IFA-negative serum (1:100e) on *R. conorii*-resolved 2D proteome (30 µg). (C) *R. africae* IFA-negative serum (1:100e) on *R. conorii*-resolved 2D proteome (30 µg) (cross-reaction). (D) *R. africae* IFA-positive serum (1:100e) on *R. africae*-resolved 2D proteome (30 µg). (E) *R. africae* IFA-negative serum (1:100e) on *R. africae*-resolved 2D proteome (30 µg). (F) *R. conorii* IFA-positive serum (1:100e) on *R. africae*-resolved 2D proteome (30 µg) (cross-reaction).

Table 1
The best protein markers for *R. conorii* and *R. africae* infections.

category of sera/technique	Serological markers[a]	Statistical markers[b]	Narrow-selected markers[c]
R. conorii (all sera) (n = 22)	**ftsZ**, **cycM**, rpoA, RC0071, prsA	**ftsZ**, **cycM**, htpG, mdh, lipA, RC1221, RC0760	ftsZ, cycM, rpoA
early stage *R. conorii* infection (n = 7)	**prsA**, RC0071, nuoF, **RC0031, pepA,** lpxD	mdh, htpG, lipA, lpxD, **pepA**, **prsA**, **RC0031**	prsA, RC0031, pepA, lpxD
IFI positive *R. conorii* infection (n = 12)	**rpsA**, rpsAi, pnp, cycM, rpoA	**rpsA**, rpsB, RC0113, RC0603	rpsA
R. africae (all sera) (n = 24)	rOmpB β-peptide, OmpA, groEL, ORF1174, thrS, ORF1113, dnaK, **lpxD**	tsf, ppa, grpE, ORF0785, pepA, ORF0094, **lpxD**	rOmpB β-peptide, OmpA, groEL, ORF1174, lpxD
early stage *R. africae* infection (n = 17)	rpoA, htrA, **ORF0029**, atpD, **acnA**	ORF1113i, sca4, **ORF0029**, rpsB, **acnA**, rpsA	rpoA, htrA, ORF0029, atpD, acnA,
IFI positive *R. africae* infection (n = 3)	RC1282, lpxD, frr, cycM RC0185, rpsA, RC1282i, sdhA, RC1281, atpA, def3	tsf, ppa, grpE, ORF0785	RC1282, lpxD

Selection of the best protein markers according to [a] the value of the serological parameters and [b] the statistical analysis by PLS-EDA.
Based on [a] and [b], we selected the best markers with narrow specificity [c]. The selected markers common to both approaches are highlighted in bold.

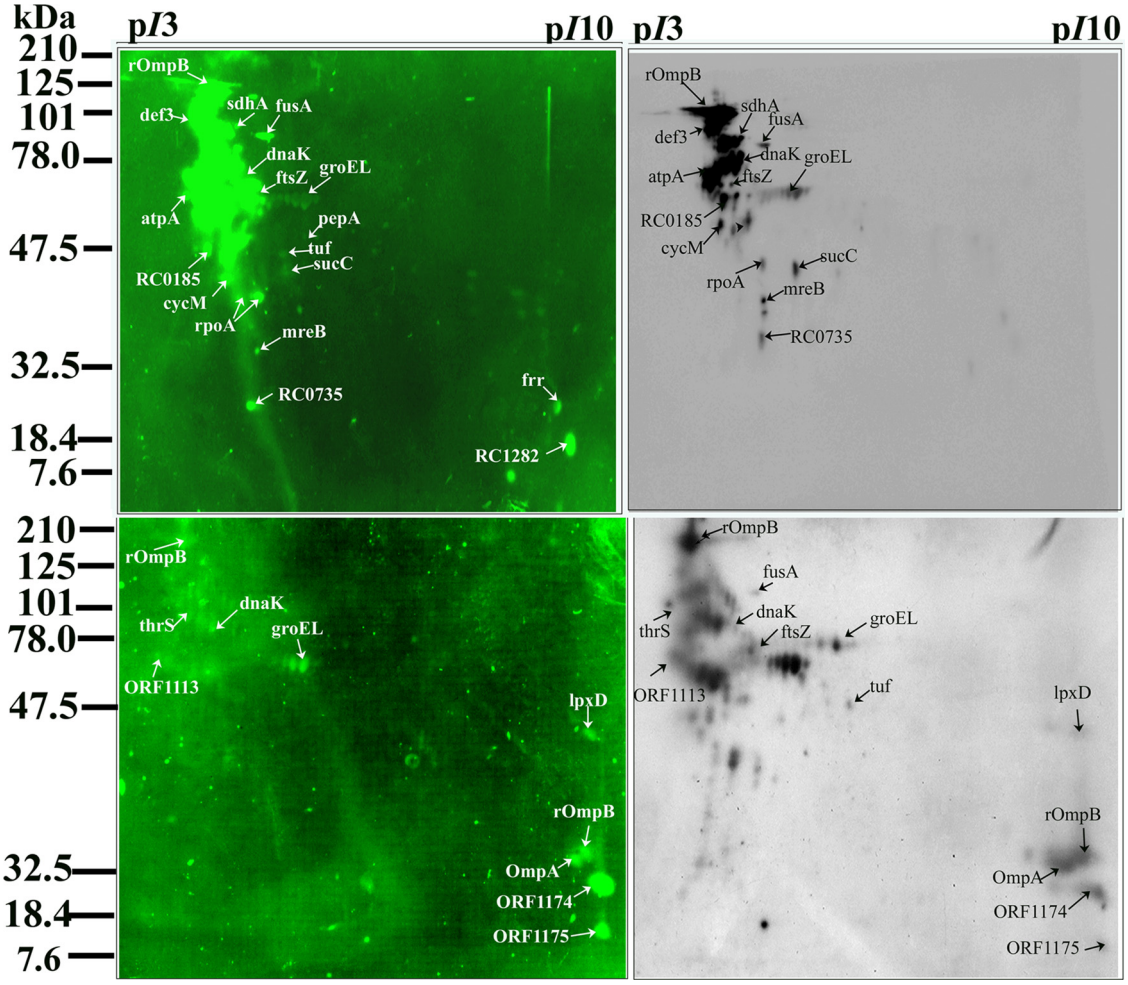

Fig. 3. Example of immunoreactivity profiles obtained with PLA 2D WB (p*I* 3–10, 7 cm): (A) PLA 2-D WB of *R. conorii* (15 μg) probed with *R. conorii* IFA-positive serum (1:100e). (B) Traditional 2-D WB of *R. conorii* (15 μg) probed with *R. conorii* IFA-positive serum (1:100e). (C) PLA 2-D WB of *R. africae* (15 μg) probed with *R. africae* (1:100e) IFA-positive serum (1:100e). (D) Traditional 2-D WB of *R. africae* (15 μg) probed with *R. conorii* IFA-positive serum (1:100e). (A, B) the same *R. conorii* serum; C), D) the same *R. africae* serum. Some of the immunoreactive proteins are annotated with the name of the locus tag as compared to the silver-stained gel.

reactive to the major rOmpB spot. In general, we obtained better spot detection sensitivity and specificity in PLA WB when the proteins were probed with IFA *R. conorii*- or *R. africae*-positive sera (Fig. 3).

3.3. Sensitivity of screening early-stage infection sera by 2-D classical WB

The sensitivity of spot detection on 2-D WB performed with early-stage *R. conorii* infection was better than with IFA-positive *R. conorii*-infected sera (Tables 2, multimedia component 3). Thus, the mean value of detected spots for early sera was 19 compared to 14 for IFA-positive sera.

With *R. africae* early-stage infection, the mean value of detected spots was 13 compared with 16 for IFA-positive *R. africae*-infected sera.

Table 2
The best protein markers for early and confirmed infections with *R. conorii* and *R. africae* according to the values of their serological parameters.

	Se[a]	Sp[b]	PPV[c]	NPV[d]	L[e]	Λ[f]
The best *R. conorii* early infection (IFA -) (n = 7) protein markers						
prsA	67	93	67	–	10	0,36
RC0071	50	93	60	–	7,5	0,53
nuoF	50	100	100	–	–	0,50
RC0031	50	90	50	–	5	0,55
pepA	67	83	44	–	4	0,4
The best *R. conorii* active infection (IFA+) (n = 12) protein markers						
rpsA	38	95	83	–	8,8	0,64
rpsAi	38	95	83	–	8,8	0,64
pnp	46	91	75	–	5,3	0,59
lpxD	54	83	64	–	3,0	0,55
cycM	69	69	56	–	2,3	0,44
rpoA	38	83	55	–	2,2	0,74
The best *R. africae* early infection (IFA -) (n = 17) protein markers						
rpoA	41	94	87	–	7	0,62
htrA	35	94	85	–	6	0,69
ORF0029	23	94	80	–	4	0,81
atpD	41	88	78	–	3,5	0,67
acnA	41	88	77	–	3,5	0,67
The best *R. africae* active infection (IFA+) (n = 3) protein markers						
RC1282	50	100	100	–	15	0,50
rpsA	50	100	100	–	15	0,50
RC1282i	50	100	100	–	15	0,50
lpxD	50	97	67	–	15	0,52
frr	50	97	67	–	15	0,52
cycM	50	97	67	–	15	0,52
RC0185	50	97	67	–	15	0,52
sdhA	50	93	50	–	7,5	0,53
RC1281	50	97	50	–	7,5	0,77
atpA	50	93	50	–	7,5	0,53
def3	50	93	50	–	7,5	0,53

The parameters of calculation: [a]Sensitivity (Se), positive result with patients/total number of patients; [b]Specificity (Sp), negative result with the control group/total number of control group individuals; [c]Positive predictive value (PPV), TP/(TP + FP); true positive (TP), false positive (FP); [d]Negative predictive value (NPV), TN/(TN + FN); true negative (TN), false negative (FN); [e]L = Se/(1-Sp) = (TP/patients) (FP/control group individuals); [f]Λ = (1-Se)/Sp = (FN/patients) (TN/control group individuals).

3.4. Comparison of the sensitivity of early-stage infection sera screened by 2D WB and 2D PLA WB

Among *R. conorii* patients with early infection, only one serum was screened in parallel with classical WB and PLA WB. Clearly, we obtained better spot detection with PLA WB (43%) than with traditional WB (33%), indicating slightly better sensitivity of PLA WB. It is important to highlight that due to the costly reagents used for PLA WB, we were limited to screening only the sera of *R. conorii*- and *R. africae*-infected patients. Therefore, we used traditional WB performed with healthy blood donors as the control group. The best markers of early *R. conorii* infection were prsA, RC0031, and pepA (Table 2, Supplementary material 2).

In the case of *R. africae* early infection, the sensitivity was comparable for both techniques (in range of 35%). Based on the lack of reactivity among pooled sera from healthy blood donors used with classical WB, the best markers of early *R. africae* infection were rpoA htrA, ORF0029, atpD and acnA (Table 2, Supplementary material 2).

3.5. Contribution of cross-reacted WB in the present study

Considering the high rate of cross-reactivity among closely genetically related rickettsial species, our aim was to determine the most-conserved highly cross-reacting *R. conorii* and *R. africae* proteins. We probed 3 *R. conorii*-infected sera (S20, S21 (IFA positive) and S22 (IFA negative) against the 2-D resolved proteome of *R. africae*, and conversely, we probed 4 *R. africae*-infected sera (S21, S22, S24 (IFA positive)), ((S23, (IFA negative)) against the 2-D resolved proteome of *R. conorii*. The cross-reactivity was noteworthy, indicating that the most prominent spots were situated in the rOmpB zone, including the best markers for *R. conorii* (ftsZ and cycM) (Fig. 2C). The cross-reactivity against ftsZ (1/4), cycM (1/4), rpoA (1/4) was noticeable. No reactivity against the early *R. conorii* infection markers (RC0031, pepA) was observed with exception of prsA cross-reacting with unique *R. africae* IFI negative serum. Two sera were cross-reacted with the active *R. conorii* infection marker rpsA. The reactivity was similar for *R. africae* and was concentrated in the zone of rOmpB spots (thrS, ORF0094, ORF1113, ftsZ, dnaK) (Fig. 2F). Only one *R. conorii* IFA-positive serum cross-reacted with the best *R. africae* protein markers: rOmpB β-peptide, OmpA and ORF1174. groEL was cross-reacted with IFI negative serum. No reaction was observed for either for rpoA, ORF0029 or acnA, all markers of early *R. africae* infection. Unique *R. conorii* IFI negative serum was cross-reacted with atpD and htrA, both markers of early *R. africae* infection.

3.6. The best discriminant proteins revealed by serological parameters

When taking into account the value of the test operating serological parameters (Tables 2, 3, and Supplementary material 2), the best serological markers for *R. conorii* were ftsZ and cycM, with sensitivity values greater than 70% and a specificity of 92%. An L value indicates protein contribution to the diagnosis. The value greater than 10 indicates its strong contribution. Indeed, for these antigens the L value was greater than 10. The L value for rpoA ($5 < L < 10$) shows its lower contribution to diagnosis; this was also observed for the remaining "top range" antigens: lpxD, pepA, tufA, and pnp ($0 < L < 5$). They showed relatively weak sensitivity (*ca.* 40%) and specificity in the range of 70%. Other immunoreactive spots detected in *R. conorii* showed both a very good sensitivity (90–100%) and a very low specificity (< 10%), for example rOmpB, showed the highest rate of cross-reactivity. *A contrario*, some spots were specific enough (85–100%) (*i.e.*, RC1282i, RC0760, serS, RC0603, RC0031) but had very low sensitivity, meaning that only one or two sera reacted with them.

For *R. africae*, the top range antigens were selected: pnp, acnA, atpD, rpoA and htrA.

They showed an excellent specificity (80–100%), but lower sensitivity (35–47%)(Table 2, Supplementary material 2).Some spots showed a high rate of cross-reactivity (*e.g.*, rOmpB, groEL, dnaK), as was the case for *R. conorii*.

3.7. The best discriminant proteins revealed by statistical analysis

The partial least squares-enhanced discriminant analysis (PLS-EDA) was an interesting tool that enabled us to show the common and different features of the reactivity profiles in the patient groups included in the present study (Supplementary material 4). The values of the principal components were as follows: PC1, 19%; PC2, 12%; PC3, 7%.

The immunoreactivity profiles of all groups included in this study were similar if we consider the most prominent results, meaning PC1 *versus* PC2. We observe the clusters of mainly *R. conorii* infected and closely co localized together sera: Rco, cRaf, eRco, iRco, in opposite to the cluster of *R. africae* infected sera regrouping categories: Raf, iRaf, eRaf, cRco. WB performed with 10 pooled sera from healthy blood donors and probed against the *R. conorii* and *R. africae* proteomes did not co-localize with patient sera, which is highlighted in the graphical representations of samples (scores) PC1 *versus* PC2 and PC2 *versus* PC3.

When we analyzed the corresponding graphics of variables (spots), we found the same projection of spots. If we consider these two major groups of sera (cluster *R. conorii versus* cluster *R. africae*), the best contributory spots to discriminate these two groups were cycM, ftsZ, prsA, pepA, tufA for cluster *R. conorii* and tsf, ppa, grpE, ORF0785, pepA, ORF0091 and lpxD for cluster *R. africae*. The best contributory

spots are shown in Table 2 and Supplementary material 4.

4. Discussion

Currently, we attempted to identify the best protein values for discriminating the serodiagnosis of *R. conorii* and *R. africae* infections. Considering the frequent difficulties in accurately diagnosing early-stage *R. africae* infection in our laboratory [20,29], we undertook a challenge to discover rickettsial markers using two approaches: traditional 2D-immunoproteomics and *in situ* PLA-2D WB. The first step for both approaches used here was traditional 2D technology. The spots detected by both methods were matched with a silver-stained gel and excised for MALDI-TOF identification. Thus, the reference 2-D gels were common for both approaches. We decided to work in the pH range 3–10 to cover a global proteome; thus, the identification and detection of spots clustered in this zone was our major limitation. Therefore, feature studies should be performed with a wide pH range rickettsial proteome (pH 3–5) to better resolve the majority of interesting spots.

Considering that the *in situ* PLA-2D WB, offers increased detection sensitivity (20–50-fold) over traditional WB, along with the ability to identify the interacting proteins [61,62], our first goal was to adapt this approach for *Rickettsiae* species-specific discrimination. Secondly, by using two different detection systems, RCA *versus* classical HRP-based, respectively, we raised an opportunity to evaluate their sensitivity [63]. Thus, the specificity is conferred by a first antibody from serum. However, we obtained the enhanced sensitivity only in cases of *R. conorii* early infection. Thus, the limit of detection was reached in the traditional WB, resulting in missing frr and RC1281 spots. We also observe a type of “bleaching” of the signal in the strongly reactive zone (cluster of spots with rOmpB) in PLA WB.

Indeed, due to the relatively high fluorescence background in 2D PLA WB, we missed some spots with a weak signal. In summary, excepting encouraging results obtained with *R. conorii* early infection, our results showed that within the limits of patient sera used, the HRP based detection system was comparable to R *ca.* In general, *in situ* PLA-based detection method is considered as much more sensitive than classical methods [42] except for Warford et al., who reports un-usefulness of *in situ* PLA in immunohistocytochemistry (IHC) [63]. The kit-based *in situ* PLA is constraining and costly to use. Thus, gains of sensitivity of 10% are rather questionable and far from 20 to 50 fold change [42,61,64]. Although, *in situ* PLA-based methods are described as highly sensitive, not fortuitous, we did not obtain the results that we had been expected. Thus, the future use of *in situ* PLA technology seemed to be compromised for *Rickettsia* spp.

We considered both techniques, PLA 2D WB and traditional WB, together to select the best diagnostic values. Moreover, to enhance the stringent choice for diagnostic value, we first calculated the test operating serological parameters and performed a statistical analysis to resolve different patient groups and corresponding spots.

Interestingly enough, we did not obtain exactly the same results when calculating the test operating serological parameters (Table 2). This is expected considering the fact that the best statistical contributors were highly specific but had lower sensitivity. When taking into account the test operating serological parameters for cases of *R. africae* infection, all immunoreactive proteins showed either good specificity (> 75%) and a weak value for sensitivity (range of 15–30%) or, inversely, a good value for specificity (> 75%) and a weak value for sensitivity. The top range *R. africae* markers were rOmpB β-peptide, OmpA, groEL, and ORF1174. They had sensitivities of 80–100% and specificities of 92–100%. They are useful for diagnosis considering that most of the commercial bacterial serodiagnostic kits reach the value of 75% for both sensitivity and specificity [29,65].The spots indicated by the statistical analysis characterized only 3 *R. africae* IFA-positive sera (S16, S18, S20). Thus, the results for IFA-positive sera are most probably underestimated.

The best markers of early-stage *R. africae* infection were rpoA, atpD, and acnA with sensitivities of 40% and excellent specificities of 88–94%. All of the best markers had an excellent specificity (in the range of 90%) and variable sensitivity (40–70 %).

Considering late apparition of serological titers in ATFB [6,25,32] and difficult species specific diagnosis of rickettsiosis, these 3 proteins remain promising for an early stage *R. africae* diagnosis and they might be a future target for serodiagnosis development. The best markers of *R. conorii* infection were cycM and ftsZ spots, detected in both early-stage sera (5/7 eRco) and IFA-positive sera (10/12 Rco). Indeed, cycM did not cross-react with healthy blood donors (HBD) sera, but cross-reacted with one *R. africae* serum. ftsZ cross-reacted with 8/24 *R. africae* sera. Moreover, these 2 proteins were also been shown to be strong contributors in PLS-EDA. However, we should carefully consider them, because they are located in high density and poorly resolved zone, which potentially may be a source of error. The remaining best *R. conorii* marker was lpxD (5/7 eRco; 6/12 Rco) and rpoA (5/7 eRco; 4/12 Rco), respectively. The *R. conorii* lpxD did not crossed reacted with *R. africae* sera.

In this study, we evaluated a new technique named *in situ* PLA 2D WB for rickettsial protein targets screening. It enabled us to select several species-specific markers of rickettsial disease at different stages of infection. The most efficient *R. africae* early infection markers were rpoA, htrA, atpD, acnA and ORF0029 described here for the first time. The best *R. africae* active infection markers were: rOmpB β-peptide, OmpA, groEL and newly described here hypothetical protein ORF1174. The most promisisng markers of early *R. conorii* infection was prsA, lpxD, pepA,RC0031 and *R. conorii* active infection markers (ftsZ and cycM) which were all selected for the first time. The newly reported rickettsial diagnostic markers deserve further consideration for serodiagnosis improvements. ELISA performed with recombinant proteins is a valuable tool to enable the evaluation of the best targets [47]. We could include larger cohort of patients and controls.

Ethical statement

Experiments were performed at the FNCR (French Nationnal Centre de Reference pour *Rickettsia*, *Coxiella* et *Bartonella*) after the clinical protocol was approved by the Institutional Review Board of Ethics Committee of The Institut Federatif de Recherche 48 (n° 11-013). All subjects provided written informed consent for participation in the study, sample collection and analyses. None minor was involved in this study. The present study does not contain any individual participant's data in the form of images, videos, voice recordings *etc.*

Competing interests

The authors declare that they have no competing interests.

Authors’contributions

AN,CN, PD, NA,SE performed experiments. MK analysed results and performed the statistical analysis. DR, CN, MK designed experiments. KE commented on the work.

MK and DR wrote the manuscript. All authors approved the manuscript.

Acknowledgements

The authors are indebted to the individuals who participated in the study.

We are grateful to Laura Doyen for her language assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2018.06.004.

References

[1] D. Raoult, V. Roux, Rickettsioses as paradigms of new or emerging infectious diseases, Clin. Microbiol. Rev. 10 (1997) 694–719.
[2] D. Raoult, G.A. Dasch, Line blot and western blot immunoassays for diagnosis of Mediterranean spotted fever, J. Clin. Microbiol. 27 (1989) 2073–2079.
[3] D. Raoult, G.A. Dasch, Immunoblot cross-reactions among *Rickettsia*, *Proteus* spp. and *Legionella* spp. in patients with Mediterranean spotted fever, FEMS Immunol. Med. Microbiol. 11 (1995) 13–18.
[4] P. Kelly, L. Matthewman, L. Beati, D. Raoult, P. Mason, M. Dreary, R. Makombe, African tick-bite fever: a new spotted fever group rickettsiosis under an old name, Lancet 340 (1992) 982–983.
[5] M. Jensenius, P. Gerlyng, G. Hasle, G. Hopen, S. Vene, A.L. Bruu, Spotted fever imported into Norway in 1997, Tidsskr Nor Laegeforen 118 (1998) 2627–2629.
[6] M. Jensenius, P.E. Fournier, P. Kelly, B. Myrvang, D. Raoult, African tick bite fever, Lancet Infect. Dis. 3 (2003) 557–564.
[7] K. Nilsson, K. Wallmenius, P. Rundlof-Nygren, S. Stromdahl, C. Pahlson, African tick bite fever in returning Swedish travellers. Report of two cases and aspects of diagnostics, Infect. Ecol. Epidemiol. 7 (2017).
[8] D. Raoult, P.E. Fournier, F. Fenollar, M. Jensenius, T. Prioe, J.J. de Pina, G. Caruso, N. Jones, H. Laferl, J.E. Rosenblatt, T.J. Marrie, *Rickettsia africae*, a tick-borne pathogen in travelers to sub-Saharan Africa, N. Engl. J. Med. 344 (2001) 1504–1510.
[9] P. Parola, F. Fenollar, S. Badiaga, P. Brouqui, D. Raoult, First documentation of *Rickettsia conorii* infection (strain Indian tick typhus) in a Traveler, Emerg. Infect. Dis. 7 (2001) 909–910.
[10] C. Rovery, D. Raoult, Mediterranean spotted fever, Infect. Dis. Clin. North Am. 22 (2008) 515–530.
[11] F. Beugnet, K. Chalvet-Monfray, Impact of climate change in the epidemiology of vector-borne diseases in domestic carnivores, Comp. Immunol. Microbiol. Infect. Dis. 36 (2013) 559–566.
[12] P. Parola, C.D. Paddock, C. Socolovschi, M.B. Labruna, O. Mediannikov, T. Kernif, M.Y. Abdad, J. Stenos, I. Bitam, P.E. Fournier, D. Raoult, Update on tick-borne rickettsioses around the world: a geographic approach, Clin. Microbiol. Rev. 26 (2013) 657–702.
[13] P. Parola, J. Jourdan, D. Raoult, Tick-borne infection caused by *Rickettsia africae* in the West Indies, N. Engl. J. Med. 338 (1998).
[14] P. Parola, C.D. Paddock, Ticks and tick-borne diseases, editorial, Ticks Tick Borne Dis. 3 (2012) 269.
[15] C. Eldin, O. Mediannikov, B. Davoust, O. Cabre, N. Barre, D. Raoult, P. Parola, Emergence of *Rickettsia africae*, Oceania, Emerg. Infect. Dis. 17 (2011) 100–102.
[16] A. Yssouf, C. Socolovschi, T. Kernif, S. Temmam, E. Lagadec, P. Tortosa, P. Parola, First molecular detection of *Rickettsia africae* in ticks from the Union of the Comoros, Parasit. Vectors 7 (2014) 444.
[17] Z. Herrador, A. Fernandez-Martinez, D. Gomez-Barroso, I. Leon, C. Vieira, A. Muro, A. Benito, Mediterranean spotted fever in Spain, 1997–2014: epidemiological situation based on hospitalization records, PLoS One 12 (2017) e0174745.
[18] A. Gargili, A.M. Palomar, K. Midilli, A. Portillo, S. Kar, J.A. Oteo, *Rickettsia* species in ticks removed from humans in Istanbul, Turkey, Vector Borne Zoonotic Dis. 12 (2012) 938–941.
[19] H. Koka, R. Sang, H.L. Kutima, L. Musila, The detection of spotted fever group *Rickettsia* DNA in tick samples from pastoral communities in Kenya, J. Med. Entomol. 54 (2017) 774–780.
[20] A. Renvoise, O. Mediannikov, D. Raoult, Old and new tick-borne rickettsioses, Int. Health 1 (2009) 17–25.
[21] A. Beltrame, A. Angheben, S. Casolari, F. Castelli, G. Magnani, G. Gaiera, F. Brillo, G. Cattani, M. Anselmi, L. Tomasoni, F. Prati, C. Norberto, C. Socolovschi, Z. Bisoffi, D. Raoult, P. Parola, Imported rickettsioses in Italy, Travel Med. Infect. Dis. 10 (2012) 201–204.
[22] B. Dzelalija, V. Punda-Polic, A. Medic, M. Dobec, *Rickettsiae* and rickettsial diseases in Croatia: implications for travel medicine, Travel Med. Infect. Dis. 14 (2016) 436–443.
[23] P.F. Albizuri, A. Sanchez, M. Feito, A. Mayor, A. Rodriguez, R. de Lucas, Fever and multiple eschars after an African Safari: report of three cases, Pediatr. Dermatol. 34 (2017) e179–e181.
[24] N. Roch, O. Epaulard, I. Pelloux, P. Pavese, J.P. Brion, D. Raoult, M. Maurin, African tick bite fever in elderly patients: 8 cases in French tourists returning from South Africa, Clin. Infect. Dis. 47 (2008) e28–e35.
[25] P. Brouqui, F. Bacellar, G. Baranton, R.J. Birtles, A. Bjoersdorff, J.R. Blanco, G. Caruso, M. Cinco, P.E. Fournier, E. Francavilla, M. Jensenius, J. Kazar, H. Laferl, A. Lakos, F.S. Lotric, M. Maurin, J.A. Oteo, P. Parola, C. Perez-Eid, O. Peter, D. Postic, D. Raoult, A. Tellez, Y. Tselentis, B. Wilske, Guidelines for the diagnosis of tick-borne bacterial diseases in Europe, Clin. Microbiol. Infect. 10 (2004) 1108–1132.
[26] C. Socolovschi, A. Renvoise, P. Brouqui, P. Parola, D. Raoult, The use of eschar swabs for the diagnosis of African tick-bite fever, Ticks Tick Borne Dis. 3 (2012) 361–363.
[27] A. Renvoise, J.M. Rolain, C. Socolovschi, D. Raoult, Widespread use of real-time PCR for rickettsial diagnosis, FEMS Immunol. Med. Microbiol. 64 (2012) 126–129.
[28] F. Gouriet, F. Fenollar, J.Y. Patrice, M. Drancourt, D. Raoult, Use of shell-vial cell culture assay for isolation of bacteria from clinical specimens: 13 years of experience, J. Clin. Microbiol. 43 (2005) 4993–5002.
[29] B. La Scola, D. Raoult, Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases, J. Clin. Microbiol. 35 (1997) 2715–2727.
[30] E.C. Guillemi, L. Tomassone, M.D. Farber, Tick-borne Rickettsiales: molecular tools for the study of an emergent group of pathogens, J. Microbiol. Methods 119 (2015) 87–97.
[31] N. Mouffok, C. Socolovschi, A. Benabdellah, A. Renvoise, P. Parola, D. Raoult, Diagnosis of rickettsioses from eschar swab samples, Algeria, Emerg. Infect. Dis. 17 (2011) 1968–1969.
[32] M. Jensenius, P.E. Fournier, K.B. Hellum, L. Wesslen, G. Caruso, T. Prio, K. Lohne, S. Vene, D. Raoult, B. Myrvang, Sequential changes in hematologic and biochemical parameters in African tick bite fever, Clin. Microbiol. Infect. 9 (2003) 678–683.
[33] P. Crespo, D. Seixas, N. Marques, J. Oliveira, S. da Cunha, A. Melico-Silvestre, Mediterranean spotted fever: case series of 24 years (1989–2012), Springerplus 4 (2015) 272.
[34] S. Fredriksson, M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S.M. Gustafsdottir, A. Ostman, U. Landegren, Protein detection using proximity-dependent DNA ligation assays, Nat. Biotechnol. 20 (2002) 473–477.
[35] S.M. Gustafsdottir, E. Schallmeiner, S. Fredriksson, M. Gullberg, O. Soderberg, M. Jarvius, J. Jarvius, M. Howell, U. Landegren, Proximity ligation assays for sensitive and specific protein analyses, Anal. Biochem. 345 (2005) 2–9.
[36] U. Landegren, F. Dahl, M. Nilsson, S. Fredriksson, J. Baner, M. Gullberg, J. Jarvius, S. Gustafsdottir, O. Soderberg, O. Ericsson, J. Stenberg, E. Schallmeiner, Padlock and proximity probes for in situ and array-based analyses: tools for the post-genomic era, Comp. Funct. Genomics 4 (2003) 525–530.
[37] U. Landegren, E. Schallmeiner, M. Nilsson, S. Fredriksson, J. Baner, M. Gullberg, J. Jarvius, S. Gustafsdottir, F. Dahl, O. Soderberg, O. Ericsson, J. Stenberg, Molecular tools for a molecular medicine: analyzing genes, transcripts and proteins using padlock and proximity probes, J. Mol. Recognit. 17 (2004) 194–197.
[38] M. Lundberg, S.B. Thorsen, E. Assarsson, A. Villablanca, B. Tran, N. Gee, M. Knowles, B.S. Nielsen, C.E. Gonzalez, R. Martin, O. Nilsson, C. Fermer, J. Schlingemann, I.J. Christensen, H.J. Nielsen, B. Ekstrom, C. Andersson, M. Gustafsson, N. Brunner, J. Stenvang, S. Fredriksson, Multiplexed homogeneous proximity ligation assays for high-throughput protein biomarker research in serological material, Mol. Cell. Proteom. 10 (2011) M110.
[39] L. Zhu, H. Koistinen, P. Wu, A. Narvanen, E. Schallmeiner, S. Fredriksson, U. Landegren, U.H. Stenman, A sensitive proximity ligation assay for active PSA, Biol. Chem. 387 (2006) 769–772.
[40] R. Ke, R.Y. Nong, S. Fredriksson, U. Landegren, M. Nilsson, Improving precision of proximity ligation assay by amplified single molecule detection, PLoS One 8 (2013) e69813.
[41] L. Ghanipour, S. Darmanis, U. Landegren, B. Glimelius, L. Pahlman, H. Birgisson, Detection of biomarkers with solid-phase proximity ligation assay in patients with colorectal cancer, Transl. Oncol. 9 (2016) 251–255.
[42] Y. Liu, J. Gu, A. Hagner-McWhirter, P. Sathiyanarayanan, M. Gullberg, O. Soderberg, J. Johansson, M. Hammond, D. Ivansson, U. Landegren, Western blotting via proximity ligation for high performance protein analysis, Mol. Cell. Proteom. 10 (2011) O111.
[43] M. Jarvius, J. Paulsson, I. Weibrecht, K.J. Leuchowius, A.C. Andersson, C. Wahlby, M. Gullberg, J. Botling, T. Sjoblom, B. Markova, A. Ostman, U. Landegren, O. Soderberg, *In situ* detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method, Mol. Cell. Proteom. 6 (2007) 1500–1509.
[44] W.N. Burnette, Western blotting : remembrance of past things, Methods Mol. Biol. 536 (2009) 5–8.
[45] N. Malou, D. Raoult, Immuno-PCR: a promising ultrasensitive diagnostic method to detect antigens and antibodies, Trends Microbiol. 19 (2011) 295–302.
[46] S.M. Gustafsdottir, A. Nordengrahn, S. Fredriksson, P. Wallgren, E. Rivera, E. Schallmeiner, M. Merza, U. Landegren, Detection of individual microbial pathogens by proximity ligation, Clin. Chem. 52 (2006) 1152–1160.
[47] M. Kowalczewska, M. Vellaiswamy, C. Nappez, R. Vincentelli, B. La Scola, D. Raoult, Protein candidates for the serodiagnosis of rickettsioses, FEMS Immunol. Med. Microbiol. 64 (2012) 130–133.
[48] W. Gong, X. Xiong, Y. Qi, J. Jiao, C. Duan, B. Wen, Identification of novel surface-exposed proteins of *Rickettsia rickettsii* by affinity purification and proteomics, PLoS One 9 (2014) e100253.
[49] Y. Qi, W. Gong, X. Xiong, J. Jiang, Y. Wang, J. Jiao, C. Duan, B. Wen, Microarray of surface-exposed proteins of *Rickettsia heilongjiangensis* for serodiagnosis of far-eastern spotted fever, BMC Infect. Dis. 14 (2014) 332.
[50] Y. Qi, X. Xiong, X. Wang, C. Duan, Y. Jia, J. Jiao, W. Gong, B. Wen, Proteome analysis and serological characterization of surface-exposed proteins of *Rickettsia heilongjiangensis*, PLoS One 8 (2013) e70440.
[51] G. Vestris, J.M. Rolain, P.E. Fournier, M.L. Birg, M. Enea, J.Y. Patrice, D. Raoult, Seven years' experience of isolation of *Rickettsia* spp. from clinical specimens using the shell vial cell culture assay, Ann. N. Y. Acad. Sci. 990 (2003) 371–374.
[52] Y. Bechah, C. Socolovschi, D. Raoult, Identification of rickettsial infections by using cutaneous swab specimens and PCR, Emerg. Infect. Dis. 17 (2011) 83–86.
[53] M.E. Eremeeva, N.M. Balayeva, D. Raoult, Purification of rickettsial cultures contaminated by mycoplasmas, Acta Virol. 38 (1994) 231–233.
[54] D.F. Gimenez, Staining *Rickettsiae* in yolk-sac cultures, Stain Technol. 39 (1964) 135–140.
[55] P. Renesto, S. Azza, A. Dolla, P. Fourquet, G. Vestris, J.P. Gorvel, D. Raoult, *Rickettsia conorii* and *R. prowazekii* proteome analysis by 2DE-MS: a step toward functional analysis of rickettsial genomes, Ann. N. Y. Acad. Sci. 1063 (2005) 90–93.
[56] M. Kowalczewska, F. Fenollar, C. Villard, S. Azza, M. Roux, D. Raoult, An immunoproteomic approach for identification of clinical biomarkers of Whipple's disease, Proteom. Clin. Appl. 2 (2008) 504–516.
[57] V. Kuzina, C.T. Ekstrom, S.B. Andersen, J.K. Nielsen, C.E. Olsen, S. Bak, Identification of defense compounds in *Barbarea vulgaris* against the herbivore *Phyllotreta nemorum* by an ecometabolomic approach, Plant Physiol. 151 (2009)

1977–1990.
[58] P.E. Fournier, K. El Karkouri, Q. Leroy, C. Robert, B. Giumelli, P. Renesto, C. Socolovschi, P. Parola, S. Audic, D. Raoult, Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in *Rickettsia* species may be explained by genome reduction, BMC Genomics Stain Technol. 10 (2009) 166.
[59] P. Renesto, S. Azza, A. Dolla, P. Fourquet, G. Vestris, J.P. Gorvel, D. Raoult, Proteome analysis of *Rickettsia conorii* by two-dimensional gel electrophoresis coupled with mass spectrometry, FEMS Microbiol. Lett. 245 (2005) 231–238.
[60] P. Renesto, L. Samson, H. Ogata, S. Azza, P. Fourquet, J.P. Gorvel, R.A. Heinzen, D. Raoult, Identification of two putative rickettsial adhesins by proteomic analysis, Res. Microbiol. 157 (2006) 605–612.
[61] C.M. Clausson, A. Allalou, I. Weibrecht, S. Mahmoudi, M. Farnebo, U. Landegren, C. Wahlby, O. Soderberg, Increasing the dynamic range of in situ PLA, Nat. Methods 8 (2011) 892–893.
[62] C.M. Clausson, L. Arngarden, O. Ishaq, A. Klaesson, M. Kuhnemund, K. Grannas, B. Koos, X. Qian, P. Ranefall, T. Krzywkowski, H. Brismar, M. Nilsson, C. Wahlby, O. Soderberg, Compaction of rolling circle amplification products increases signal integrity and signal-to-noise ratio, Sci. Rep. 5 (2015) 12317.
[63] A. Warford, H. Akbar, D. Riberio, Antigen retrieval, blocking, detection and visualisation systems in immunohistochemistry: a review and practical evaluation of tyramide and rolling circle amplification systems, Methods 70 (2014) 28–33.
[64] S. Darmanis, C.J. Gallant, V.D. Marinescu, M. Niklasson, A. Segerman, G. Flamourakis, S. Fredriksson, E. Assarsson, M. Lundberg, S. Nelander, B. Westermark, U. Landegren, Simultaneous multiplexed measurement of RNA and proteins in single cells, Cell Rep. 14 (2016) 380–389.
[65] M.E. Eremeeva, N.M. Balayeva, V.F. Ignatovich, D. Raoult, Proteinic and genomic identification of spotted fever group *rickettsiae* isolated in the former USSR, J. Clin. Microbiol. 31 (1993) 2625–2633.