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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

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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).

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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).