

Characterization of mechanisms involved in rickettsia pathogenicity

Manohari Vellaiswamy

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THESE DE DOCTORAT

présentée par

Manohari VELLAISWAMY

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Spécialité: Maladies Transmissibles et Pathologies Tropicales

Characterization of mechanisms involved in rickettsia pathogenicity

Soutenue le 23 Novembre 2011

COMPOSITION DU JURY

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Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes

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AVANT PROPOS

Le format de présentation de cette Thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'Etudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur articles publiés, acceptés ou soumis, associés d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

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LIST OF ABBREVIATIONS

Adr	Adhesin of rickettsiae
ELISA	Enzyme-linked immunosorbent assay (ELISA)
IFA	Indirect immunoflorescence assay
L929 cells	Murine fibroblastic cell line
mAb	Monoclonal antibody
MS/MS	Mass spectrometry
ORF	Open Reading Frame
PLD	Phospholipase D
rOmpA	Rickettsial outer membrane protein A
rOmpB	Rickettsial outer membrane protein B
SFG	Spotted fever group
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG	Typhus group
Vero cells	Monkey kidney epithelial cells
WB	Western blot
2DE	Bidimensional gel electrophoresis

RESUME

Les rickettsies sont de petites bactéries à Gram-négatif associées à différentes espèces d'arthropodes. Leur nature intracellulaire stricte a longtemps été un obstacle à la compréhension des mécanismes moléculaires responsables de leur pathogénicité qui restent mal connus. L'adhésion bactérienne, qui est une étape clef de l'invasion des tissus de l'hôte, met en jeu les protéines rOmpA et rOmpB (*rickettsial outer membrane proteins*), identifiées depuis longtemps comme des antigènes de surface majeurs des rickettsies. L'objectif de cette thèse a été de caractériser une autre adhésine potentielle de *Rickettsia prowazekii* récemment identifiée, soit Adr2. La stratégie mise en œuvre a été basée sur la production d'anticorps monoclonaux spécifiques de cette protéine, dont une forme recombinante a été exprimée. Cet outil a permis, non seulement de localiser Adr2 à la surface des rickettsies, mais aussi d'apporter la preuve de son rôle dans le phénomène invasif puisque les anticorps anti-Adr2 diminuent significativement la cytotoxicité des rickettsies sur les cellules épithéliales.

Un autre aspect de la pathogénicité que nous avons abordé concerne la mobilité des rickettsies du groupe boutonneux, fonction attribuée à la protéine RickA lorsque ce travail a été initié. La résolution des images obtenues par immunofluorescence, ou par microscopie électronique après marquage immunogold, montrent que l'expression de RickA est non-polarisée et répartie sur la surface entière de *Rickettsia conorii*.

Enfin, plusieurs protéines recombinantes ont été utilisées dans des tests de screening sérologiques avec des sérums de patients infectés par diverses rickettsies, avec des résultats encourageants.

L'ensemble de ces résultats contribue à une meilleure connaissance de la pathogénicité des bactéries du genre *Rickettsia*.

ABSTRACT

Rickettsiae are characterized by their strictly intracellular location, as Gram-negative bacteria growing only within the eukaryotic host cell cytoplasm. Accordingly, the molecular mechanisms responsible for invasive mechanisms are largely unknown. Adhesion is a key step for bacterial invasion of host tissues and involves the rickettsial outer membrane proteins rOmpA and rOmpB, known for a long time as major rickettsial cell surface antigens. The aim of this thesis was to gain a better understanding of another newly identified rickettsial adhesin from *Rickettsia prowazekii*, called Adr2. This task was achieved through the production of a monoclonal antibody (mAb) specific for the recombinant protein and that allowed localization of Adr2 at the bacterial cell surface. The inhibition of rickettsiae-induced cytotoxicity with this mAb confirmed the role of Adr2 in the invasion process.

Considering the putative role of the actin-based motility in the pathogenesis of the spotted fever group rickettsiae (SFG), we then focused our second part of work on the localization of RickA, a protein specific for the SFG rickettsiae and thought to be responsible for bacterial motility. Immunofluorescence assay combined with a immunogold electron microscopy yielded good-resolution images and showed a non-polarized expression of RickA that was found onto the entire bacterial surface of *Rickettsia conorii.*

Finally, twenty recombinant proteins targets were screened with sera of patients infected with various rickettsiae. We thus evidenced several putaive markers allowing to discriminate infection caused either by *Rickettsia typhi* or by *Rickettsia conorii.*

On the overall, we believe that our results improve the knowledge about the pathogenicity of bacteria from the *Rickettsia* genus.

INTRODUCTION

I. GENERALITIES

The genus *Rickettsia* includes bacterial obligate intracellular parasites associated with arthropods (tick, mite, flea, and lice) and that primarily target the microvascular endothelium. In the last two decades, new rickettsial pathogens have been associated with human illness around the world. Clinically, the common denominator in all rickettsioses is the development of increased microvascular permeability, leading to cerebral and non-cardiogenic pulmonary edema (Olano, 2005). Based on their antigenicity and intracellular actin-based motility, rickettsiae were initially classified into the typhus group (TG) including *R. prowazekii* and *R. typhi*, and the spotted fever group (SFG) which includes more than 20 different species among which *R. conorii* and *R. rickettsii* (Raoult and Roux, 1997).

Within the last decade, the availability of complete genome sequences of several rickettsial species (Table 1), allowed to gain a better knowledge not only about their evolution, but also about their metabolic capacities and the molecular mechanisms involved in their pathogenicity (Walker 2007; Balraj *et al.*, 2009). Accordingly, besides the TG and the SFG a third group including *R. bellii* and *R. canadensis* has emerged (Blanc *et al.*, 2007).

Table 1. List of rickettsial genomes sequenced

Strains	Size (Mb)	Genbank	Reference
R. prowazekii str. Madrid E	1,11	AJ235269.1	Andersson *et al* , 1998
R. conorii Malish 7	1,27	AE006914.1	Ogata *et al* , 2004
R. typhi str. Wilmington	1,11	AE017197.1	McLeod *et al* , 2004
R. sibirica 246	1,25	NZ AABW01000001	Malek *et al* , 2004
R. felis URRWXCal2	1,59	CP000053.1	Ogata *et al* , 2005
R. bellii RML 369-C	1,52	CP000087.1	Ogata *et al* , 2006
R. massiliae MTU5	1,41	CP000683.1	Blanc *et al* , 2007
R. rickettsii str. Lowa	1,27	CP000766.1	Hackstadt *et al,* 2008
R. peacockii str. Rustic	1,3	CP001227.1	Felsheim *et al,* 2009
R. africae ESF-5	1,29	CP001612.1	Fournier *et al* , 2009
R. prowazekii Rp22	1,1	CP001584	Bechah *et al* , 2010
R. heilongjiangensis 054	1,3	CP002912.1	Duan *et al* , 2011
R. rickettsii str. Sheila smith	1,26	CP000848.1	Unpublished
R. africae ESF-5	1,27	NZAAUY01000001	Unpublished
R. akaris str. Hartford	1,23	CP000847.1	Unpublished
R. bellii OSU 85-389	1,52	CP000849.1	Unpublished
R. canadensis str. McKiel	1,16	CP000409.1	Unpublished
R. japonica	1	In progress	University of Tokyo
R. prowazekii str. Madrid E vir	1,3	In progress	BCM-HGSC
R. prowazekii Nuevo Leon Amblyomma tick		In progress	BCM-HGSC
R. prowazekii Rp22		In progress	Unité des Rickettsies
R. sbvaca 13-B		In progress	Unité des Rickettsies
R. raoultii		In progress	Unité des Rickettsies
R. sibirica 246	1	AABW00000000	University of Maryland
R. grylli	2	AAQJ00000000	TIGR
R. typhi B9991CWPP		In progress	Los Alamos National laboratory
R. typhi TH1527		In progress	Los Alamos National laboratory

The advent of several complete rickettsial genome sequences also highlighted the genetic basis for metabolic differences as well as for common traits. Thus, from a careful comparative bioinformatic analysis, it was established that rickettsiae contain five autotransporters called the surface cell antigen (Sca) family (Blanc *et al.*, 2005). These proteins indeed possess 3 domains, a leader sequence that mediates transport across the cell membrane, a passenger sequence, and a transporter sequence that is inserted as a β-barrel into the outer envelope to transport the passenger sequence to the outer surface of the cell wall. In addition to the newly identified proteins Sca1, Sca2, and Sca3 that exist as split genes (interrupted into 2–4 open reading frames) in at least 1 *Rickettsia* species, this family includes Sca0, previously known as rOmpA and present only in the SFG while Sca5 (rOmpB) is present in all *Rickettsia* species. Sca4 (geneD) which shares sequence similarity, is not an autotransporter, because it lacks the transporter domain (Blanc *et al.*, 2005).

As observed for other intracellular bacteria, rickettsia pathogenicity involves sequential steps starting with recognition and adherence to the host cells. This crucial step results in the invasion of the endothelial cells through induced phagocytosis. Rickettsiae then escape from phagosome into the cytosol, where replication takes place leading to direct cell damages and death (Balraj *et al*, 2009). In addition to opening the way for bioinformatic analysis, the advances in rickettsial genome sequencing, have contributed to improve the knowledge about the molecular mechanisms involved in the pathogenicity of these bacteria. Advances in the evaluation of the pathogenesis of rickettsial disease include identification of rickettsial adhesins, a host cell receptor, signaling elements associated with entry of rickettsiae by induced phagocytosis,

rickettsial enzymes mediating phagosomal escape, and host actin-based rickettsial cell-to-cell spread.

Two putative rickettsial ligands recognized by host cell surface proteins were thus identified. One ligand corresponds to the C-terminal extremity of rOmpB called the β-peptide. Other putative ligands are proteins of unknown function encoded by the genes RC1281 and RC1282 in *R. conorii* as well as by RP827 and RP828 in *R. prowazekii* and called Adr for rickettsial adhesins (Renesto *et al*., 2006). The lysis of the phagosomal membrane that precedes rickettsia escape into the cytosolic compartment was shown to be mediated by the upregulation of genes coding for enzymes sharing a membranolytic activity, namely hemolysin C (*tly C*) and phospholipase D (*pld*) (Renesto P *et al*, 2003; Whitworth T *et al*, 2005). Historically, the actin-based motility was depicted as a major feature allowing to differentiating SFG and TG rickettsiae. Here again, it is the comparative analysis of *R. prowazekii* (TG) and *R. conorii* (SFG) genomes that allowed to identify RickA as a protein endowed for the capacity to a promote the polymerization of host cell cytoskeletal actin through the activation of Arp 2/3 (Ogata *et al*., 2001; Gouin *et al*, 2004), an hypothesis recently revisited (Balraj *et al*., 2008, Kleba *et al*., 2010).

Availability of the genome sequences and proteomic approaches also favor the development of serological tools including monoclonal antibodies (mAbs) which are useful for immunofluorescence-based localization and to demonstrate the functional activity selected targets. Here, we summarized available data concerning the use of antibodies in the field of rickettsiae, either as diagnostic tools or in more basic resaech applications.

II. AN OVERVIEW OF ANTIBODIES AS USEFUL TOOLS FOR DIAGNOSIS OF THE RICKETTSIOSIS AND THEIR CONTRIBUTION IN EXPLORATION OF RICKETTSIAL BIOLOGY

II-1. Introduction

Rickettsiae cause life-threatening rickettsioses which exist primarily in endemic and enzootic foci that occasionally give rise to sporadic or seasonal outbreaks. Rickettsial pathogens are highly specialized for obligate intracellular survival in both the vertebrate host and the invertebrate vector. While studies often focus primarily on the vertebrate host, the arthropod vector is often more important in the natural maintenance of the pathogen. The epidemiology of human diseases caused by rickettsiae is intimately related to the biology of the vector that transmits (Azad and Beard, 1998).

Tick-borne rickettsioses are caused by bacteria belonging to the SFG. These zoonoses, which are among the oldest known vector-borne diseases include the well-known Rocky Mountain spotted fever (*R. rickettsii*) and the Mediterranean spotted fever (*R. conorii*). More recently, emerging SFG rickettsiosis were identified in differents countries and are caused by various species as *R. japonica* (Japan), *R. conorii* subsp. *caspia* (Astrakhan, Africa, and Kosovo), *R. africa*e (sub-Saharan Africa and the West Indies), *R. honei* (Australia, Tasmania, Thailand), *R. slovaca* (Europe), *R. sibirica* subsp. *mongolitimonae* (China, Europe, and Africa), *R. heilongjanghensis* (China and the Russian Far East), *R.vaeschlimannii* (Africa and Europe), *R. marmionii* (Australia), and *R. parkeri* (United States). The last rickettsia is probably the best illustration, as *R. parkeri* was considered a nonpathogenic rickettsia for more than 60 years. Furthermore, the pathogenicity of *R. massiliae* has been recently demonstrated, 13 years after its isolation from ticks. Other recently

described rickettsiae, including *R. helvetica* strains (Europe and Asia) have been presented as possible pathogens (reviewed in Parola *et al*., 2005). Rickettsiae are transmitted through the tick bite, which generally implies that the rickettsiae localize to the salivary glands of the tick. Therefore, the precise molecular mechanisms responsible for the adaptation of rickettsiae to different host conditions and for reactivation of virulence are unknown.

In contrast to 15 or more validated and/or proposed tick-borne SFG species, only three named medically important rickettsial species are associated with insects. These insect-borne rickettsiae are comprised of two highly pathogenic species, *R. prowazekii* (the agent of epidemic typhus) and *R. typhi* (the agent of murine typhus), as well as *R. felis*, a species with unconfirmed pathogenicity (Gillespsie *et al*., 2009). These flea- and louse-borne rickettsiae are transmitted to humans through contamination of broken skin and mucosal surfaces by infected tick feces. Due to its survival in dried louse feces, *R. prowazekii* can also be transferred through aerosols (Bechah *et al*., 2008).

In general, and athough the clinical presentations can vary with the causative agent, the SFG rickettsiosis syndromes are similar. Among common symptoms that typically develop within 1–2 weeks of infection are fever, headache, malaise, and sometimes nausea and vomiting. Most tick-transmitted rickettsioses are accompanied by a rash or an eschar at the site of the tick bite (Parola *et al*., 2005). The flea-borne disease induced by *R. typhi* present symptoms that are shared with other infectious diseases. Most symptoms are nonspecific and require further tests to make an accurate diagnosis (Bitam *et al*., 2008). Except for epidemic louse-borne typhus (Bechah *et al*., 2008), rickettsial diseases strike mostly as isolated single cases in any particular neighborhood.

Thus, diagnosis of rickettsial infections is often difficult. An history of exposure to the appropriate vector tick, louse, flea, or mite is helpful but cannot be relied upon. While many rickettsial diseases cause mild or moderate illness, epidemic typhus and Rocky Mountain spotted fever can be severe and may be fatal in 20%–60% of untreated cases.

II-2. Antibodies as tools in diagnosis of rickettsiosis

Among the approaches developped to diagnose rickettiosis are the serologic diagnosis, the immunodetection of rickettsiae from blood or tissues and the isolation of bacteria. To date, laboratory diagnosis of rickettsioses is mainly based on various PCR assays and DNA sequencing which allows convenient and rapid identification of rickettsiae, even in non referenced laboratories (La Scola and Raoult, 1997).

Serologic diagnosis

Serological tests are the easiest methods for the diagnosis of tick-borne rickettsioses. Historically, the rickettsial diagnosis was supported by the Weil-Felix test based on the detection of antibodies to various *Proteus* species which contain antigens with cross-reacting epitopes to antigens from members of the genus *Rickettsia,* with the exception of *R. akari*. With the development of techniques for growing rickettsiae, the complement fixation test was then adapted for the detection of antibodies specific for rickettsiae. A microagglutination test based on the detection of interactions between antibodies and whole rickettsial cells was also developped. However, due to the requirement of high amounts of purified rickettsial antigens, not available commercially, this method not been widely used. Other techniques include the indirect hemagglutination and the latex agglutination tests that detect antibodies to an antigenic

erythrocyte-sensitizing substance used to coat erythrocytes or latex beads, respectively (reviewed in La Scola and Raoult, 1997).

In the early 1980th, these methods were replaced by others, easier to handle and sharing higher sensitivity and specificity, like enzyme-linked immunosorbent assay (ELISA) that was first introduced for detection of antibodies against *R. typhi* and *R. prowazekii* (Halle *et al*., 1977) and later adapted to the diagnosis of Rocky Mountain spotted fever (Clements *et al*., 1983). The rickettsial immunofluorescence assay (IFA) adapted to a micromethod format is the test of choice for the serodiagnosis of rickettsial diseases (Philip *et al*., 1976). The micro-IFA allows simultaneous detection of antibodies against several rickettsial antigens starting with a drop of serum in a single well containing multiple rickettsial antigen dots. It is considered as the "gold standard" technique and it is used as a reference technique in most laboratories. Western blot and line blot assays are also used in routine and is considered as powerful serodiagnostic tool for seroepidemiology (Raoult & Dasch, 1995).

The drawback of ELISA, IFA and western blot, is that all these methods require laboratory platforms specialized for culture and purification of rickettsiae. Moreover, and while IFA is currently the test of choice for serologic diagnosis of rickettsial infection, cross-reactive antibodies between rickettsiae species are often observed, rendering difficult the serologic identification.

Immunodetection of rickettsiae

Biopsy specimens of the skin with a rash around the lesion, preferably petechial lesions, and tache noire specimens are the most common samples used. Immunodetection methods may also be used to detect microorganisms from their arthropod vectors. Slides are air-dried and

fixed in acetone before being treated with polyclonal or monoclonal antibodies conjugated with immunofluorescent labels.

Isolation of rickettsiae

In the past, only research laboratories that had biosafety level 3 containment and personnel with extensive experience in cultivating rickettsiae were able to isolate these small and strictly intracellular Gram-negative bacilli from clinical specimens. The centrifugation shell vial, technique, first developed for cytomegalovirus culture, was adapted for the isolation of *R. conorii* (Marrero and Raoult, 1989). This method, which has led to a significant increase of laboratories suitably equipped to isolate rickettsiae, allows detection 48–72 h post-inoculation. Isolation of rickettsiae is of great importance as the ultimate diagnostic goal is recovery of the bacterial agent from a tick or a patient (La Scola and Raoult, 1996).

Molecular methods

Molecular methods based on PCR and sequencing have enabled the development of sensitive, specific and rapid tools for both the detection and identification of rickettsiae in blood, skin biopsy specimens, and even ticks. Primer sets targeting various rickettsial genes have been described and can be used in any laboratory with suitable facilities (Brouqui *et al*., 2004; Fournier *et al*., 2004).

In summary, several diagnostic methods are used for rickettsia detection. In the specialized laboratories, shell vial culture, molecular biology and serodiagnostic with IFA or adsorbed western blot are used systematically. Because it is difficult to diagnose rickettsial infection early after infection occurs, administration of antibiotic treatment before a

definitive diagnosis is still made (Pelletier and La Scola, 2010). Preventive measures are complicated because of the lack of effective and safe rickettsial vaccines (Walker, 2007). To detect efficiently bacteria in clinical samples, we need to dispose of highly sensitive, specific and available detection tests.

III-3. Antibodies as tools for physiopathological investigations

From the first description of rickettsiae as human pathogens, the rickettsiosis remained poorly understood diseases. The use of antibodies was helpful to dissect some specific aspects of pathogenesis of these obligate intracellular microorganisms. A few examples are detailed below:

-Role of rOmpA as a bacterial ligand

This 190 kDa immunodominant surface-exposed protein is thought for long to be involved in adhesion of rickettsiae to host cells, based on the protective effect against rickettsial infections in animal models afforded by the recombinant truncated rOmpA or DNA plasmid encoding this protein (Mc Donald *et al.*, 1987; Li and Walker, 1988; Vishwanath *et al.*, 1990; Sumner *et al.*, 1995; Crocquet-Valdes *et al.*, 2001). Immunoblotting and immunofluorescence assays confirmed the absence of rOmpA from *R. rickettsii* Iowa, as hypothesized from the comparative genomic analysis of *R. rickettsii* Sheila Smith (virulent) and Iowa (avirulent) strains that highlighted a deletion resulting in defect of rOmpA expression in the latter (Ellison *et al.,* 2008).

-rOmpB mediates bacterial invasion and constitutes a protective antigen for SFG rickettsiae

The mammalian receptor Ku70 was identified as involved in the rickettsial invasion process, a process where the rickettsial autotransporter protein rOmpB, intervenes as bacterial ligand (Martinez *et al.*, 2005, Chan *et al*., 2009). In a recent paper, Chan *et al.* (2011) developed mAbs which specifically recognize a conformation present in the folded, intact rOmpB passenger domain. They demonstrated that such mAbs are sufficient to confer immunity *in vivo*. Analyses *in vitro* suggest that this protection involves a mechanism of complement-mediated killing in mammalian blood, a means of rickettsial clearance that has not been previously described.

-Sca1 promotes adherence to nonphagocytic mammalian cells

Bioinformatic analysis of SFG rickettsiae allowed to identify the Sca protein family, predicted as outer surface proteins (Blanc *et al*., 2005). However, very little is known about the function(s) of these Sca proteins, with the exception of Sca0 (rOmpA) and Sca5 (rOmpB). Western-blot and immunofluorescence staining were achieved on *R. conorii* using a polyclonal antiserum directed against the N-terminal portion of the Sca1 passenger domain (amino acids 29 to 327). Data obtained demonstrated that Sca1 is present on the surface of *R. conorii* isolated from infected mammalian cells and involved in their adherence to host cells (Riley *et al*., 2010).

-Evidence for the regulation of rOmpA expression

During their life cycle, bacteria from the *Rickettsia* genus may adapt to diverse environments in the ticks and mammals. Their adaptation strategy most probably results from a selective gene expression, as depicted for

other tick-borne pathogens. Accordingly, it was observed, by RT-PCR and immunofluorescence assays, that rOmpA expression can undergo major changes. Thus, rOmpA is strongly detected when rickettsiae propagated within Vero cells while poorly expressed in bacteria collected from tick hemolymph (Rovery *et al*., 2006). Similarly, variation in rOmpA but not in rOmpB expression was also evidenced in *R. massiliae* during the *Rhipicephalus turanicus* life cycle (Ogawa et al., 2006). When inoculated from arthropod vectors to human beings, rickettsiae most probably exhibit a proteic profile different to that observed from bacteria grown in culture. *Ex-vivo* experiments aimed at characterizing this host-pathogen interaction should thus be analyzed with caution.

-The phagosomal escape involves a rickettsial phospholipase D

As several other pathogens of the genus *Listeria*, *Shigella* and *Mycobacterium*, rickettsiae rapidly gain access to the cytosol of infected cells through phagosomal vacuole escape. While the involvement for a phospholipase A_2 (PLA_2) in the entry vesicle lysis was for proposed (Winkler and Miller, 1982), the completion of rickettsial genomes revealed the absence of PLA_2-encoding gene. The first phospholipase identified within a rickettsial genome was the *R. conorii* phospholipase D (PLD). Its role as virulence factor was demonstrated through the capacity of anti-PLD antibodies to inhibit the cytotoxicity on endothelial cells (Renesto *et al*., 2003).

These data are summarized Figure 1.

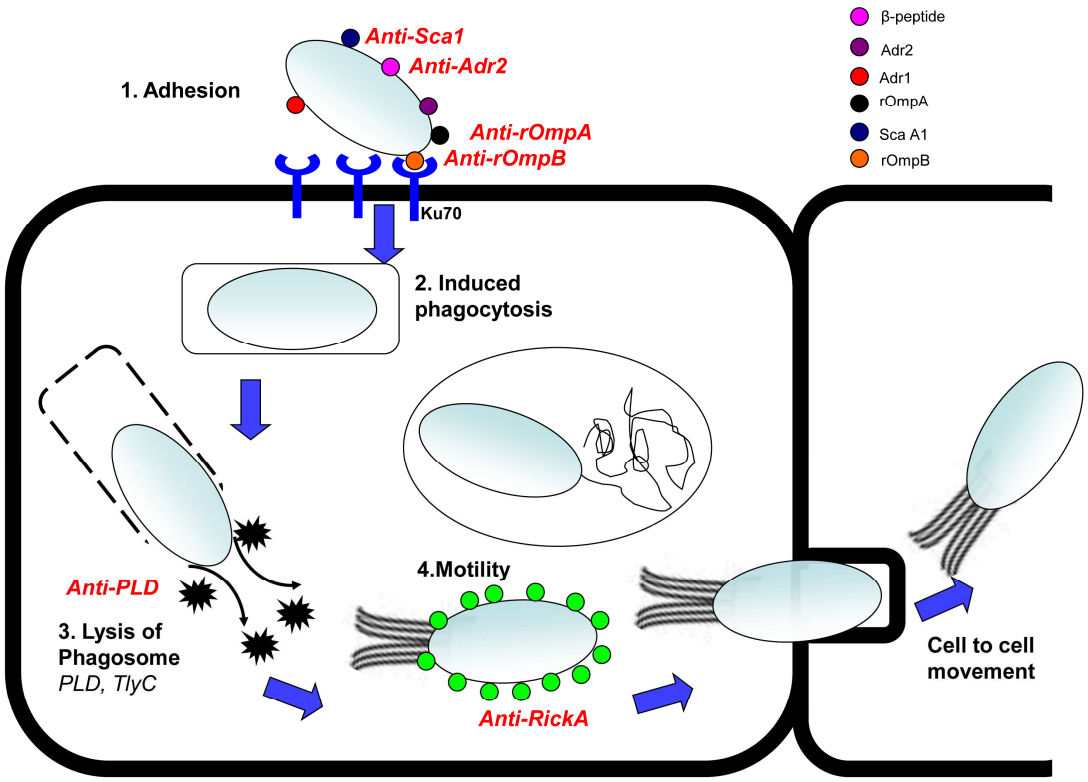


Figure 1
Schematic representation of rickettsia physiopathology

Rickettsiae express outer membrane proteins including rOmpA, rOmpB and Sca1 that are known to be involved in the binding to the host cells as it is also the case for Adr2. Their eukaryotic receptors were not yet identified, expect for rOmpB thought to interact with the membrane protein Ku70. The bacteria then invade human endothelial cells via the process of induced phagocytosis and rapidly escape from the phagosome into the host cytoplasm. Lysis of phagosome is mediated by bacterial membranolytic proteins namely phospholipase D (PLD) and hemolysin (tlyC). Thus, bacteria gain the cytosolic compartment and possibly the eukaryotic nucleus where they replicate. For rickettsiae exhibiting a motile phenotype, cell-to-cell spreading in which RickA was thought to play a role is observed while expressed over the entire bacterial surface (reviewed in Blaraj *et al.*, 2009).

Specific events for which experimental investigations were achieved using mAbs were pointed out in red.

II-4. Monoclonal antibodies – Generalities and future prospects

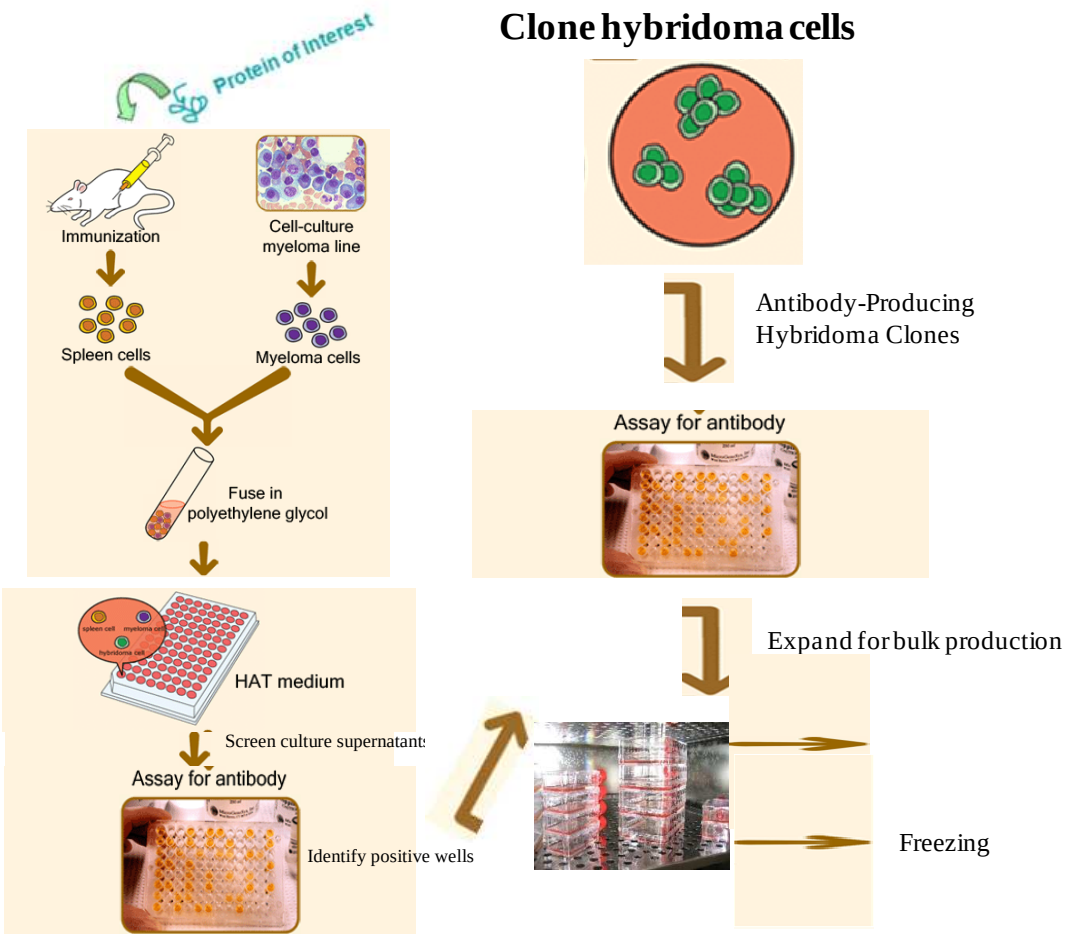
Among the techniques employed by pathologists to diagnose and study infectious diseases there is a long history of the use of mAbs. MAbs are generated *in vitro* either by hybridoma technology or recombinant DNA techniques (Kohler and Milstein, 1975). Briefly, to produce mAbs, one removes B-cells from the spleen or lymph nodes of an animal that has been challenged several times with the antigen of interest (Figure 2). These B-cells are then fused with myeloma tumor cells (hybridomas) that can grow indefinitely in culture (myeloma is a B-cell cancer). Large amounts of mAbs can thus be produced. The antibodies from the different clones are then tested for their ability to bind to the antigen (for example with a test such as ELISA) or immuno-dot blot, and the most sensitive one is picked out. MAbs can be produced in cell culture or in live animals. When the hybridoma cells are injected in the peritoneal cavity of mice, they produce tumors containing an antibody-rich fluid called ascites. Production in cell culture is usually preferred as the ascites technique may be very painful to the animal.

MAbs are homogenous immunoglobulins that, by definition, recognize one epitope and have markedly higher specific activity than polyclonal serum. Advantages of mAbs formulations are superior in homogeneity, constancy, pathogen specificity, low toxicity, enhancement of immune function. Advances in biotechnology have enabled the development of antibody-based drugs for use first in treating cancer, and recently, for treating infectious diseases. The efficacy of such antibodies has been demonstrated in various *in vitro* studies, animal models and clinical trials for a variety of both viral and bacterial pathogens.

Some concrete and efficient applications concerning the fied of rickettsiae are described below.

Figure 2

The different steps of the expression of monoclonal antibodies



OUTLINE OF THE THESIS

In this work we first focused our interest on a better characterization of *R. prowazekii* adhesins thought to play a major role in adhesion and host cell invasion process. Two distinct adhesins, called Adr1 and Adr2, and which display a high sequence homology, were initially taken in consideration. However, for unexpected reasons, we failed to express the recombinant Adr1 (RP827) as a soluble protein. Only the rickettsial Adr2 encoded by RP828 was cloned, expressed and purified in amount sufficient for immunizations. The production of mAbs was achieved through the fusion of mouse myeloma cells and spleen cells from RickA-immunized mice. Both sensitivity and specificity of the mAbs anti-Adr2 were evaluated by western blot. Their efficiency to neutralize *R. prowazekii* entry into host cell was then investigated.

In the second work, we also generated selective mAbs to gain further insights into cell-to-cell spreading, another major event of rickettsia pathogenesis. More specifically, our aim was to localize RickA in *R. conorii*. While this protein was found able to promote actin polymerisation (Gouin *et al*., 2004), its role in rickettsia motility has been the subject of debates (Balraj *et al*., 2008; Kleba *et al.*, 2010). Based on the lack of peptide signal, its localization as a membrane protein is for long questionnable. Immunofluorescence and immune electron microscopy are the strategies displayed to carefully examine this aspect.

In the last part of this work and based on the different potential rickettsial recombinant protein markers, we investigated the discrimination of infection between *R. typhi* and *R. conorii* by ELISA.

These works were described in the 3 publications presented below.

RESULTS

Article 1 – Preamble

Rickettsia prowazekii is the etiologic agent of epidemic typhus and Brill-Zinsser disease (Bechah *et al*., 2008). This is a louse-borne human pathogen which has caused large outbreaks in situations where lack of hygiene and cold weather favour louse proliferation. Humans are exposed to *R. prowazekii* through direct contact with contaminated body louse feces. Rickettsia begins its life cycle in the human host by invading the endothelial cells via the process of induced phagocytosis. Then, it rapidy escapes from the phagosome into the host cytoplasm where it replicates and eventually causes the invaded cell to burst (Walker *et al*., 2007; Balraj *et al*., 2009).

Understanding the molecular mechanisms responsible for *R. prowazekii* virulence is an important challenge. Using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) combined with high throughput matrix-assisted laser desorption/ionization time of fight (MALDI-TOF) the first proteome reference maps of both *R. conorii* and *R. prowazekii* were established (Renesto *et al*., 2005). This achievement in turn led to the identification of two putative rickettsial ligands recognized by endothelial cells and called Adr1 and Adr2 (Renesto *et al*., 2006).

Recognition of and binding to the host cell is a key step for pathogenesis. This is particularly true when considering the fact that these strictly intracellular bacteria must enter host cells to replicate and survive. Here, in order to get better knowledge about the rickettsial Adr2 adhesin, we produced mAbs directed against this protein. For this purpose, the recombinant Adr2 protein from *R. prowazekii* was cloned, expressed and purified to immunize mice. The capacity of the anti-Adr2 mAb to inhibit rickettsiae-induced cytotoxicity was also investigated.

Article 1

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Characterization of rickettsial adhesin Adr2 belonging to a new group of adhesins in α-proteobacteria

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ABSTRACT

Background: Rickettsia prowazekii is the etiological agent of epidemic typhus and is an obligate intracellular bacterium that grows as a parasite freely within the cytoplasm of a eukaryotic host cell. Previous studies have shown that rOmpA and rOmpB which belong to the family of rickettsial cell surface antigens are involved *in vitro* in the adhesion of *Rickettsiae* to epithelial cells. Recently, two putative rickettsial adhesins have been identified using high resolution 2D-PAGE coupled with mass spectrometry. In this study, we further characterize and describe the adhesin Adr2 from *R. prowazekii*.
Methodology/Principal findings: Using an overlay assay coupled with mass spectrometry two adhesins, Adr1 (RP827) and Adr2 (RP828), were identified from the *R. prowazekii* proteome Recombinant *R. prowazekii* Adr2 was expressed through fusion with Dsbc in *Escherichia coli*, purified and concentrated, thus allowing production of specific monoclonal antibodies, as confirmed by western blot assays. Finally, inhibition of rickettsiae-induced cytotoxicity with monoclonal anti-Adr2 antibody has showed a greatest impact on bacterial cell entry at 8 h post-infection (ca50%) and then decreased progressively to attempt 18% of inhibition at day 7. These, correlated to the inhibition of rickettsiae-induced cytotoxicity with monoclonal anti-rOmpB antibody. Thus, Adr2 is sufficient to mediate *R. prowazekii* entry into the cell at early stage of mammalian cell infection.
Conclusions: Our results suggest that *R. prowazekii* Adr2 could be the main actor promoting the entry of rickettsiae into the host cells. The present study opens the framework for future investigations for better understanding of the Adr2 -mediated mechanisms involved in adhesion/invasion or intracellular survival of *R. prowazekii*.

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1. Introduction

Rickettsia prowazekii is the etiological agent of epidemic typhus. This bacterium is an obligate intracellular parasite that grows freely within the cytoplasm of its eukaryotic host cell rather than in phagosomes or phagolysosome [1]. *R. prowazekii* can be isolated from shell vial cell cultures, which has replaced classic animal- and/or embryonated egg–based culture methods [2, 3]. The pathogen exhibits a slow generation time (8–12 h), undergoes steady multiplication and lyses the host cell by releasing hundreds of infectious bacteria [3]. Understanding the mechanisms involved in this unique intracytoplasmic parasitism was the goal of current study.

Bacterial cell surface proteins are involved in host-parasite interactions and are targeted by the adaptive response of the host immune system [4]. Adhesion is a key step for bacterial invasion of host tissues, and adhesins are bacterial surface proteins that recognize receptors on host cells. The expression of various genes during adhesion can activate the pathogenic process [5]. Proteins as well as structural organelles on bacterial surface mediate adhesion. The bacterial components may be capsule, lipopolysaccharide, toxins and adhesins.

The *R. japonica* rOmpB autotransporter proteins function in rickettsial adherence to and invasion of Vero cells [6]. These proteins belong to a large family of outer membrane proteins known as the surface cell antigen (Sca) family [7]. Rickettsial entry into the host cell is mediated by the rOmpB protein, which binds to the host cell receptor Ku70, a component of the DNA dependent protein kinase [8]. Cholesterol also acts as a membrane receptor for *R. prowazekii* binding [9-12]. The rOmpA protein is an immunodominant, surface-exposed autotransporter

present only in the rickettsial spotted fever group [12, 13] and may be involved in the initial adhesion of *R. rickettsii* to the host cell [14]. In the previous study, two putative rickettsial ligands recognized by host cell surface proteins were identified using high resolution 2D-PAGEcoupled with mass spectrometry [15]. The results showed that one ligand corresponds to the C terminal extremity of rOmp B called β-peptide, the second one being a protein of unknown function encoded by RC1281 in *R. conorii*. RC1281 is located downstream of its paralog, RC1282 [15]. Their orthologous genes in *R. prowazekii* are respectively RP827 and RP828 encoded proteins share striking homologies. They are respectively termed Adr1 and Adr2 for adhesion of *Rickettsiae*. Because of the presence of a signal peptide in Adr1 and Adr2 and their significant sequence homology with membrane proteins, they likely form a β barrel structure within the outer membrane, a location consistent with their putative function as adhesins. Adr1 and Adr2 are ubiquitously present within the *Rickettsia* genus and may play a critical role in their pathogenicity. However, the precise role of these proteins has not been investigated [15].

First, our attention was to characterize the role of these two adhesins Adr1 and Adr2 in rickettsial entry mechanisms to the host cell. However, we failed expression of recombinant protein Adr1 (RP827) and only the rickettsial gene Adr2 (RP828) could be cloned, expressed and purified in the amounts sufficient for mice immunizations. We produced monoclonal antibody (mAb) anti-Adr2 which was used to determine the neutralizing effect of *R. prowazekii* entry into host cell.

2. Results

2.1. Distribution of Adr1 and Adr2 within bacterial species

The sequence similarities of the putative adhesins Adr1 and Adr2 for all studied rickettsial species are shown in Suppl. M1. Adr1 and Adr2 are conserved across all rickettsial species, and the highest sequence similarity was found between *R. sibirica* and *R. africae* Adr1 (98%) and between *R. sibirica* and *R. rickettsii* Adr2 (99%). The similarity between Adr1 and Adr2 sequences was about 40% among all rickettsial species. When comparing the rickettsial ORFs (Open Reading Frame) coding for Adr1 and Adr2 against the NCBI database, using the blastP software, we found that these proteins have homologs in other bacterial species (more than 30% amino acid sequence identity) (Fig. 1). These homologs were found predominantly among the α-proteobacteria, but were also identified in γ-proteobacteria such as *Escherichia* spp. and *Salmonella* spp. (Fig. 1).

2.2. Identification of the rickettsial adhesins using the overlay assay

To identify proteins expressed on the surface of *R. prowazekii*, an overlay assay was used. As illustrated in Fig. 2, this technique allows for the localization and identification of the rickettsial adhesins. Adr1 (RP827) and Adr2 (RP828) have a theoretical molecular weight of 23 kDa and 26 kDa, respectively. To further characterize the adhesins, the separation of the protein was carried out in 2D-PAGE and detected by silver staining. Following silver staining (Fig. 2A), intensely stained protein spots were excised from the gel, and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was used for identification and analysis. A comparison of the 2D-gel/MALDI-TOF MS analysis and the overlay assay demonstrated that the spots identified in both methods were the presumed adhesions in *R. prowazekii*, RP827 and RP828. Interestingly, we have missed

identification of RP827 in *Rprowazekii* and homolog of RP828 in *R. conorii* [15]. We identified RC1281 which shares sequence homology with RP827 and sca5 (β-peptide) respectively in *R. conorii*. Only RP828 and β-peptide were identified in *R. prowazekii*. Moreover, we identified immunoreactive spot m1 which corresponds to prohibitin-2 (*Mus musculus*), but failed identification of other 2 immunoreactive spots m2, m3. Thus, this work completes and confirms previous results [15].

2.2.1. Cloning, expression and purification of rickettsial adhesins

Initially, two *R. prowazekii* genes encoding Adr1 and Adr2 were selected for cloning and expression experiments by using Gateway technology (Invitrogen, Carlsbad, CA, USA). However, despite using 2 different constructions (with N terminal Histag -DsbC and Histag-thioredoxine fusion), we have successfully attempted the expression of only one protein of *R. prowazekii.* Thus, Adr2 was purified in soluble form in sufficient yield by a Nickel affinity chromatography (suppl. M2) for further experiments. In the case of two different Adr1constructions the cloning was successful, but expression of recombinant fusion proteins (Dsbc- Adr1, trx-Adr1 and trx-Adr2) in *E. coli* Rosetta (DE3) pLysS strain failed. The migration profile of the recombinant fusion protein is shown in Fig. 3A (Coomassie staining) showing DsbC-Adr2 fusion protein about 55 kDa, which corresponds to 26 kDa Adr2 protein in fusion with DsbC (28.4 kDa). The identity of recombinant protein Adr2 was confirmed by western blot using an anti-His antibody (Fig. 3B) and by MALDI-TOF MS, respectively.

The genes encoding: groEL and RP059 were subsequently cloned according to manufacturer's instructions (Gateway Cloning Technology/Invitrogen Life Technologies). Then, expression of clones containing an N-His6 tag plus a fusion protein thioredoxin (TRX) [16]

that enhances expression of the fusion partner [17, 18] was performed as described below. The identity of these proteins was checked by MALDI-TOF as described for Adr2. Purified recombinant proteins were used to generate mAbs included as controls in 137 neutralization assay (see 4).

2.3. Production of monoclonal antibodies against Adr2

Monoclonal antibodies (mAbs) were generated against the recombinant *R. prowazekii* Adr2. The antigenic profile of the recombinant protein was analyzed using western blots and silver staining. In a western blot, the monoclonal antibody obtained from immunized mice with *R. prowazekii* Adr2, recognized proteins at the positions corresponding to the theoretical location of Adr2 (Fig. 4A). The corresponding silver-stained spot was identified by MALDI-TOF MS (Fig. 4B) as Adr2 protein.

2.4. Inhibition of R. prowazekii-induced cytotoxicity with anti-Adr2 monoclonal antibody

When *R. prowazekii* was pretreated for 20 min with increasing titers of anti-Adr2 monoclonal antibody and then added to L929 cells, cell cytotoxicity measured after 1h of incubation was 37% (dilution 1:100) and 40% (dilution 1:10), respectively. At the same time of sampling, inhibition of rickettsial entry assessed by rOmpB mAb was 53% (dilution 1:10) and 33% (dilution 1:100), respectively (Fig. 5). The % of inhibition assessed by negative specificity controls was about 5% for both mAbs: groEL and RP059. However, the greatest value of inhibition was obtained for 8h sample with the % of inhibition for Adr2 about 50% (dilution 1:10) and 43% (dilution 1:100). Indeed, the values obtained for rOmpB were 57% (dilution 1:10) and 43% (dilution 1:100). This inhibition was antibody concentration dependent for both Adr2 and rOmpB at 8h of incubation. Indeed, the % of inhibition was less than 10% for both

controls: GroEL and RP059. We observe decreased % of inhibition for both rOmpB and Adr2 at the time of sampling 24h, 120h and 168h and ranging from 38% to 18% (Adr2, dilution 1:10), from 36% to 8% (Adr2, dilution 1:100) and 48% to 36% (rOmpB, dilution 1:10), 42% to 17% (rOmpBdilution 1:100), respectively. No significant variation was observed for GroEL and RP059, except at 24h time of sampling, the inhibition was 20% for groEL. The negative control consists on uninfected cells incubated with buffer only and showed noisy background of non specific cytotoxicity which ranged about 30%. In addition, Adr2 is sufficient to mediate *R. prowazekii* entry into the cell at early stage of mammalian cell infection.

3. Discussion

In the present study, first, we selected in *R. prowazekii* genome the genes encoding for Adr1 (RP827) and Adr2 (RP828) based on previous work [15], sequenced and constructed the phylogenical tree showing the distribution of putative Adr1 and Adr2 within bacterial species including *Rickettsiae*, α- and γ-proteobacteria. Secondly, we identified in *R. prowazekii* proteome adhesins Adr1 and Adr2 and showed inhibition of *R.prowazekii* entry into the host cells by using monoclonal antibodies generated by mice immunization with recombinant fusion protein Adr2-Dsbc, rOmpB [19], as well as with recombinant proteins TRX-GroEL (RP626) and TRX-spo0J (RP059), respectively. All examined *Rickettsia* spp. share these both adhesins (Suppl. M1). Previous studies reported other adhesins differentially expressed in *Rickettsia* like the surface cell antigen (sca) family proteins and the outer membrane proteins, rOmpA and rOmpB [14, 20]. These genes have been used to study the phylogenetic relationships between *Rickettsia* spp. The *Adr1* and *Adr2* gene sequences show some heterogeneity between *Rickettsia* spp., in

accordance with the four distinct rickettsial groups (e.g., the spotted fever group, the typhus group, *R. canadensis* and *R. bellii*). A highly resolved phylogenetic tree at the group level was constructed using the *RP828* sequences (Fig. 1).We used overlay assays along with a proteomic approach to identify the adhesins [21]. From a crude extract, proteins were separated using 2D-PAGE with 6–11 strips (Fig. 2), which allowed for better resolution of the protein than the previously optimized conditions [15]. This approach allows for the localization and identification of the rickettsial adhesins using MALDI-TOF MS. Both RP827 and RP828 were detected. We observed the same pattern of results using the overlay assay, as seen in Fig. 2. Therefore, the protein identification was confirmed using both an overlay assay and western blot.

The expression and purification of recombinant Adr2 (RP828) was performed as previously described [18]. *Rickettsiae* are obligate intracellular growth requirement of the bacteria poses a challenging obstacle to their genetic manipulation [22, 23]. Numerous expression vectors are available, and the choice of a vector depends upon the protein to be expressed [22]. We have tested two different constructions in our study: protein in fusion with DsbC and Trx, respectively. Only this DsbC -RP828 could be expressed *in vivo*. We have also chosen an improved *E. coli* strain for codon usage (Rosetta pLysS). Rare codons are not only strongly associated with low yield of protein expression due to ribosome stalling and abortive translation [24, 25], but also implicated in frameshift and amino acid misincorporation [26]. Despite all these efforts to overcome technical limitations, from both selected initially adhesins (RP827 and RP828), we have successfully attempted the expression of only RP828 in fusion with DsbC. The purification of a soluble RP828 in large amounts required for mice immunization, has also revealed a

difficult task, but finally achieved by using nickel affinity chromatography.

BLAST and phylogenetic analyses demonstrated that RP827 and RP828 have homologs in other bacteria from different phyla. Some of these bacteria, such as *Brucella* spp. and *Salmonella* spp., are intracellular pathogens that bind to and enter the host cell. Adhesins have been shown to play a major role in the early steps of infection: they target a host cell receptor, allowing the bacteria to colonize or become internalized in the host cell. Thus, adhesins are mainly involved in interactions with the host cell to promote entry [27]. However, the inhibition of rickettsiae induced cytotoxicity with monoclonal anti-Adr2 antibody has showed a greatest impact on bacterial cell entry at 8h post- infection (around 50% of inhibition) and then decreased progressively to attempt 18% of inhibition at day 7. These, correlated to the inhibition of rickettsiae-induced cytotoxicity with monoclonal anti-rOmpB antibody. Thus, Adr2 is sufficient to mediate *R. prowazekii* entry into the cell at early stage of mammalian cell infection. However, the method used in this work allowed only global appreciation of this phenomenon and remains the focus on more detailed mechanisms of further studies. Thus, this result is expected if we consider rOmpA, rOmpB and RP827 are also involved in entry mechanisms of *Rickettsiae* into the host cell. Thus far, rOmpA (sca0) and rOmpB (sca5), have been shown to participate in adhesion of *Rickettsiae* to mammalian cells *in vitro* [8, 14, 20, 28]. Recently, Cardwell *et al*., [29] shown that Sca2 protein is sufficient to mediate adherence to and invasion of *R. conorii* infected cultured mammalian epithelial and endothelial cells. Inhibition (ca 30%) of these phenotypes with purified soluble Sca2 protein confirms that invasion of host cells is specifically mediated by Sca2 [29]. However, the % of protein sequence identity is about 25% for *R. typhi*, which is like *R. prowazekii* belongs to

Typhus group (TG) [29]. Its role within TG remains to be elucidated. The ability of *R. prowazekii* to induce internalization into mammalian cells is likely governed by numbered adhesin-receptor interactions which involved several partners as RP827, RP828, rOmpB proteins, Sca2 protein (Fig. 6). Indeed, the identification of mammalian receptors involved in adhesins-mediated uptake of mammalian cells is should be undertaken in ongoing studies.

Monoclonal antibodies against adhesins are an excellent tool to study these interactions between rickettsial adhesins and host mammalian receptors, may also be an efficient therapeutic agent to block binding to target cells and inhibit bacterial entry into the host cell. NadA-specific antibodies have been effective in the control of *N. meningitidis* [30]. Rickettsial surface proteins have been used to produce monoclonal antibodies that conferred protective immunity in guinea pigs and mice [14]. In addition, prophylactic vaccination with adhesins can prevent bacterial infection [31]. Despite that the monoclonal antibodies against RP828 produced in this study have not inhibited efficiently the adhesion/entry of *Rickettsia* to the host cell; however, the further orientations should focus on infectivity neutralization assays *in vivo*. Monoclonal antibodies may also be used to elucidate the *Rickettsial* physiological and pathological mechanisms. In *Orientia tsutsugamushi*, a monoclonal antibody was used to characterize its life cycle in endothelial cells [30]. Adr1 and Adr2 may act as broad-spectrum vaccine targets for all *Rickettsia* spp. since they are well conserved in the *Rickettsia* spp. examined.

4. Conclusion

Adhesion and invasion are the crucial stages of obligate intracellular infection of host cells, and adhesins are critical in bacterial virulence. We shown that Adr2 is probably one of several factors involved in adhesion/entry of *R. prowazekii* into host cell. Further investigations involving Adr2 and other adhesins may lead to the development of antimicrobials to prevent the emergence and recurrence of infections.

5. Materials and Methods

5.1. Propagation of R. prowazekii and DNA purification

R. prowazekii (URRPM22) was propagated at 32°C in monolayers of murine fibroblast L929 cell (ATCC CCL 1, European Collection of Cell Cultures 85011425) in Eagle's minimum essential medium (MEM, Invitrogen, Paisley, UK) supplemented with 2% fetal bovine serum (FCS, Gibco) and 2% L-glutamine (Gibco). Total genomic DNA was extracted from infected cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

5.2. Cloning

The *R. prowazekii RP827* , *RP828, groEl, RP059* genes were amplified using primers designed for Gateway cloning (Table 1) and the Expand High Fidelity PCR System (Roche Diagnostics, Maylan, France). Genes were amplified with 30 cycles of denaturation for 30 sec at 94°C, annealing for 45 sec at 50°C and elongation for 2 min at 68°C, followed by termination for 5 min at 68°C in a PE 9600 thermal cycler (Applied bio systems, Courtaboeuf, France). The resulting PCR products were purified through PEG precipitation and inserted into the pDONR201 vector (Gateway Cloning System, Invitrogen, USA) by the BP

recombination reaction, and according to the manufacturer' s instructions. The products of the recombination reactions were transformed into competent DH5α cells and selected on LB-agar plates containing kanamycin (50 μg/mL). Clones were confirmed using sequencing and the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Ca, USA). The second step of Gateway cloning was gene transfer into a destination vector (pDest17) by the LR reaction (Gateway Cloning System, Invitrogen, Carlsbad, CA, USA). The resulting expression plasmids were transformed into competent DH5α cells, selected on LB-agar plates with ampicillin (50 μg/mL) and confirmed by PCR.

5.3. Expression and purification

The expression and purification of recombinant proteins were performed as previously described [18, 32]. Briefly, the plasmids encoding Adr1 or Adr2, as well as groELand RP_059 were used to transform *E. coli* strain Rosetta (DE3) pLysS (Novagen, Madison, WI, USA). For expression of the recombinant proteins, bacteria were grown in the auto-induction medium ZYP5052 (1.4 liters) at 37°C for 4 h at 200 rpm [33]. Following this incubation, the temperature was lowered to 17°C, and the cells were pelleted after 18 h. The bacterial pellet was resuspended in lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole pH 8.0, 0.25 mg/ml lysozyme and 1 mM PMSF) and frozen at -80°C for at least 1 hour. After thawing the bacterial pellets and adding DNAse I (2μg/ml) and MgSO4 (20 mM), the lysed cells were centrifuged to separate the soluble fraction from the bacterial debris. The protein was purified using a nickel affinity column. For this purpose, the supernatant containing the recombinant protein DsbC-Adr2 was loaded on a 5-ml HisTrap crude nickel column (GE Healthcare, Chalfont St. Giles, UK)

equilibrated in buffer A (50 mM Tris pH 8.0, 300 mM NaCl and 10 mM imidazole) (Suppl. M2). The column was then washed with five volumes of buffer B (buffer A with 500 mM imidazole) to remove endogenous nickel-binding proteins. The protein was eluted with buffer C (buffer A + 250 mM imidazole). The protein-containing fractions were pooled and stored in 50% glycerol at -20°C. The identity of the isolated protein was confirmed using mass spectrometry.

5.4. Production of mAbs against Adr2 (RP828), rOmpB, groEL and stage sporulation protein (RP059)

The monoclonal antibody (MAb) rose against rOmpB was produced as previously described [19]. The remaining MAbs were produced by inoculation of 6- to 8-week-old immunocompetent BALB/c mice (Charles River Laboratories, St. Aubin Les Elbeuf, France) with a total of 25 μg of purified recombinant proteins Adr2, groEL, RP059 respectively, with CpG adjuvant, respectively, as described previously [34,35]. Three days after the last injection, the mice were euthanized, and the spleen was removed aseptically. Splenocytes were isolated and prepared for fusion with mouse myeloma cell line NS-1, as described [35]. Hybridoma clones were selected in RPMI medium (Invitrogen, Carlsbad, CA, USA) containing 15% FCS supplemented with HAT medium (Invitrogen, Carlsbad, CA, USA). Colonies were screened using an ELISA after 10 days. The isotypes of the MAbs were determined with an ImmunoType Mouse Monoclonal Antibody Isotyping kit with antisera to mouse immunoglobulin M (IgM), IgA, IgG1, IgG2a, IgG2b, and IgG3 (Sigma ChemicalCo.). The antiserum was affinity- purified by use of MAbTrap™ Kit (GE Healtcare) according to the manufacturer's instructions. Serum levels of of recombinant protein-specific IgG was determined by ELISA, as previously described [35]. The higher dilution

of each affinity- purified antibody recognizing the recombinant protein was estimated. In parallel, the protein content in eluted fraction was estimated by modified Bradford method (Bio-rad), as previously described [37]. The protein concentration in elution fraction was: 11.96 μg/ml (Adr2), 8.39 μg/ml (rOmpB), 8.23 μg/ml (GroEL), 9.37 μg/μl (RP059) respectively. The specificity of mAbs raised against groEl and RP059 was assessed by immunoblotting (Suppl.M3).

5.4.1 ELISA

ELISAs was performed as previously described [36] with minor modifications. Microtiter plates were coated seperatly with 40 μg of each recombinant protein from this study in 100 μl of carbonate buffer overnight at 4°C. The coated wells were washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 and blocked with 100 μl of 3% non-fat milk in PBS for 1 hr at room temperature (RT). Hybridoma supernatant (50 μl) was then added as a primary antibody and the plates were incubated for 1 hr at RT before washing with PBS supplemented with 0.1% Tween 20. Following the washes, 100 μl of goat anti-mouse biotin was added, and the plates were incubated for 1 hr at RT washed with 0.1% Tween 20 in PBS. The plates were then incubated with streptavidin for 1 hr and washed with 0.1% Tween 20 in PBS. Following this wash, 100 μl of ortho-phenylenediamine (OPD) was added, and the plates were incubated for 2–3 min at RT. After 10 min of incubation with OPD at room temperature, the reaction was stopped with 100 mL/well NaOH 1 M. Color development was assessed with a microplate reader (Multiskan EX, Labsystems, Thermo Fisher Scientific, Waltham, MA) at a wavelength of 490nm. Any samples exhibiting absorbance above or similar to the positive control was considered as positive. A positive

control consisted in polyclonal positive serum of *R. prowazekii* and a negative control consisted in pre-immune negative serum.

5.5. Sample preparation for 2D-electrophoresis

R. prowazekii RP22 was propagated in a confluent monolayer of murine fibroblast L929 cell line and purified on a renografin gradient as previously reported [37]. Purified bacteria were lysed by sonication in a solubilizing buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4% w/v CHAPS) and centrifuged (10,000×*g*, 20 min, 4°C) to remove cell debris and unbroken cells. Soluble proteins were precipitated using the PlusOne 2-D Clean-Up Kit (GE Healthcare, Chalfont St. Giles, UK). The final pellet was resuspended in solubilizing buffer, and the protein concentration was determined using the Bio-Rad DC Protein Assay.

5.5.1. 2D electrophoresis and silver staining

Immobiline DryStrips gels (13 cm, pH 6–11, GE Healthcare, Chalfont St. Giles, UK) were rehydrated overnight in 250 μl rehydration buffer (7 M urea, 4% w/v CHAPS, 12 μl/ml DeStreak, 0.5% v/v immobiline pH gradient (IPG) buffer (GE Healthcare, Chalfont St. GilesUK) containing 30 μg of solubilized proteins. IEF was carried out according to the manufacturer's protocol (IPGphor II, GE Healthcare, UK). Prior to electrophoresis in the second dimension, the strips were equilibrated for 15 min in equilibration buffer (30% v/v glycerol, 2% w/v SDS, 6 M urea, 50 mM Tris-HCl, bromophenol blue, pH 8.8) containing 65 mM DTT. This step was then repeated using equilibration buffer supplemented with 100 mM iodoacetamide. The strips were then embedded in 0.5% agarose, and the proteins were resolved by electrophoresis through a 10% SDS-polyacrylamide gel (EttanTM DALT, GE Healthcare, Chalfont St. Giles, UK) at 5 W/gel for 30 min, followed by 17 W/gel for 4–5 h. Following

electrophoresis, the gels were silver-stained, and digital images were generated using transmission scanning (ImageScanner, GE Healthcare, Chalfont St. Giles, UK) to identify the proteins. Spots excised from the gel were identified using MALDI-TOF MS and a Bruker Ultraflex spectrometer (Bruker Daltonics, Wissembourg, France) as described previously [38].

5.5.2. Overlay Assay

Overlay assays were performed as previously described [15]. *R. prowazekii* extracts (30 μg) were separated using 10% SDS-PAGE. Both silver staining and an overlay assay were then performed. Resolved 2D gels were transferred onto nitrocellulose membranes (Trans-Blot transfer medium, pure nitrocellulose membrane, Bio-Rad, Hercules, CA, USA) for 2 h using a semi-dry transfer unit (Hoefer TE 77, GE Healthcare, Chalfont St. Giles, UK). Membranes were blocked in PBS supplemented with 0.2% Tween 20 and 5% non-fat dried milk (PBS-Tween- Milk) for 1.5 h. After blocking, the membranes were incubated for 1.5 h at 4°C with biotinylated Vero cells (1:100). The reactive spots were detected using peroxidase-labeled streptavidin (1:1000; Becton-Dickinson, San Jose, CA) and chemiluminescence (ECL; GE Healthcare, Chalfont St. Giles, UK).

5.6. Western blot

Following the transfer of rickettsial proteins, the nitrocellulose membranes were blocked in PBS-Tween-Milk for 1 h before incubation with the serum of a mouse immunized with recombinant Adr2 (1:100 dilution in PBS-Tween-Milk). Following a1 h incubation, the membranes were washed three times for 10 min in 0.2% PBS-Tween-20 and probed with a 1:1000 dilution of a horseradish peroxidase–conjugated goat anti-

mouse secondary antibody (GE Healthcare, Chalfont St. Giles, UK). The blots were washed with 0.2% Tween 20 in PBS, and chemiluminescence was used to detect protein bands (ECL, GE Healthcare, Chalfont St. Giles, UK). The resulting signal was detected on Hyperfilm ECL (GE Healthcare, Chalfont St. Giles, UK) using an automated film processor (Hyperprocessor, GE Healthcare, Chalfont St. Giles, UK). We used to work with freshly transferred proteins into the nitrocellulose membrane. We have never used twice the same membrane for Western blotting experiments.

5.7. Inhibition of R. prowazekii-induced cytotoxicity on L929 cells

L929 cells grown in MEM supplemented with 4% fetal calf serum and 2 mmol/L L-glutamine, in microtiter plates, were inoculated with 3000 pfu of *R. prowazekii*/well [35, 39]. To examine whether Adr2 monoclonal antibody could inhibit the cytotoxicity of *R. prowazekii*, bacteria purified on sucrose gradient were incubated for 20 min at 4°C, with increasing dilutions of antibody, before incubation with L929 cells [35]. After 1h, 8h, 24h, 120h (5 days) and 168h (7 days) of incubation at 37°C in 5% CO2, the cell culture supernatant was removed, and cell monolayer were incubated for 1h at 37°C with 50 µl of neutral red dye (0.15% in saline [pH 5.5]). The viability of bacteria has been checked by inoculation of cell monolayer with the remaining cell culture supernatant. The same conditions were applied for specificity controls: (i) positive control performed with rOmpB mAb [19], which is known that rOmpB protein is involved in rickettsial entry [12], (ii) negative controls performed with mAbs raised against GroEL and RP059 which are most likely do not involved in cell cytotoxicity. Dye not absorbed by the viable cells was removed by 2 washes with PBS (pH 6.5). Finally, the dye absorbed by the cells was extracted by the addition of 100 µl of ethanol in

PBS (pH 4.2), and the optical density at 492nm was measured with a microplate reader (Multiskan EX, Labsystems, Thermo Fisher Scientific, Waltham, MA). At least three independent assays were performed. The results were expressed as a percentage of cytotoxicity obtained with *R. prowazekii* incubated with the buffer alone. The graphs were compiled with GraphPad Prisme software (version 3.0, GraphPad Software, San Diego, CA, USA).

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Figure 1

Distribution of Adr1 and Adr2 in bacterial species

Rickettsial Adr1 (RP827) and Adr2 (RP828) ORFs were compared against the NCBI database using the BLASTP software, and homologs in other bacterial species were identified and it was shown in phylogenetic tree.

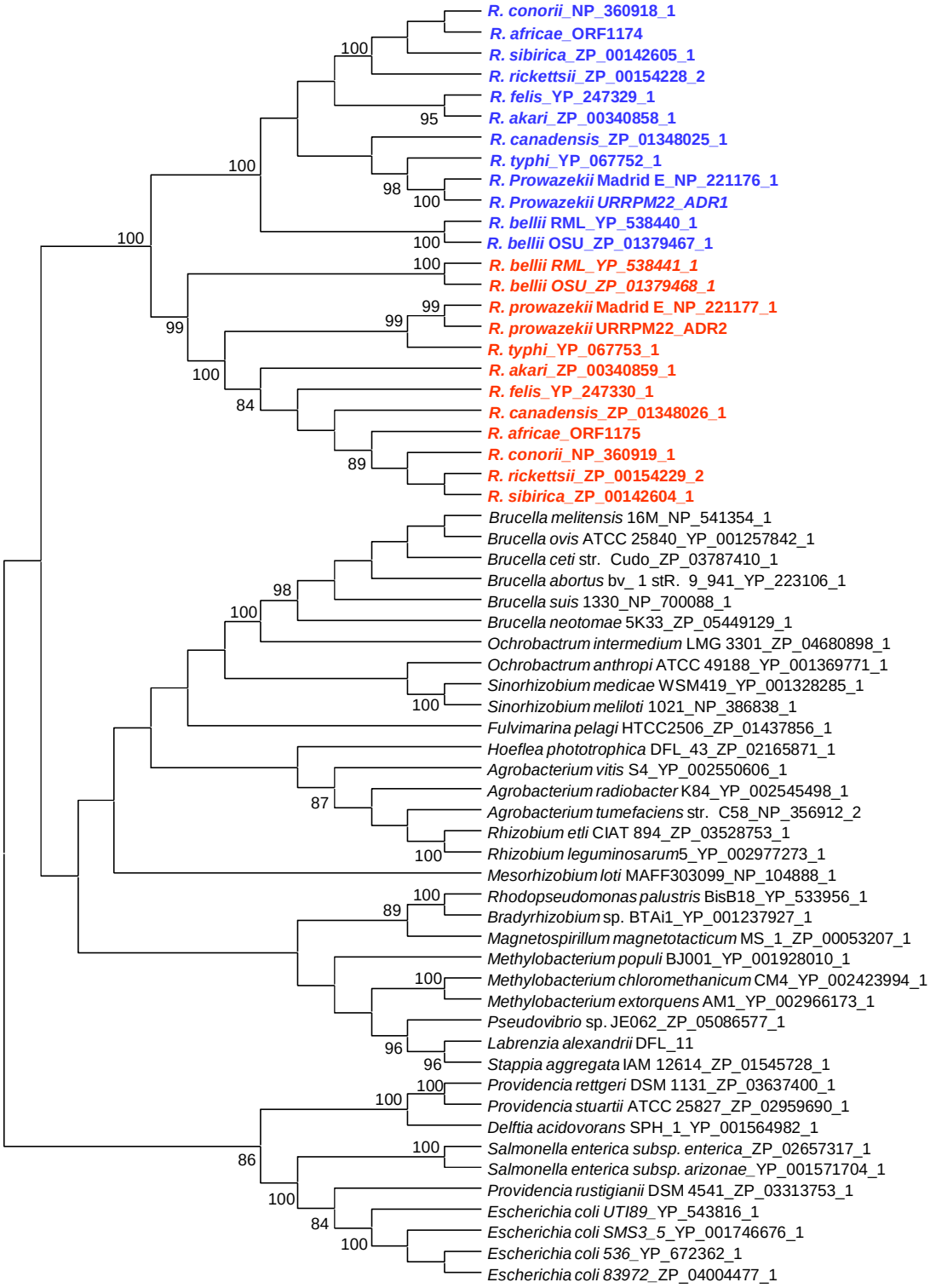


Figure 2

Recognition of adhesins Adr1 and Adr2 using the overlay assay

Rickettsial proteins were separated in the first dimension over a pH gradient (pH of 6–13) and then separated using SDS-PAGE in the second dimension. The 2D gel was silver stained for MS based identification of the spots (A) or transferred to a nitrocellulose membrane and subjected to an overlay assay (B).

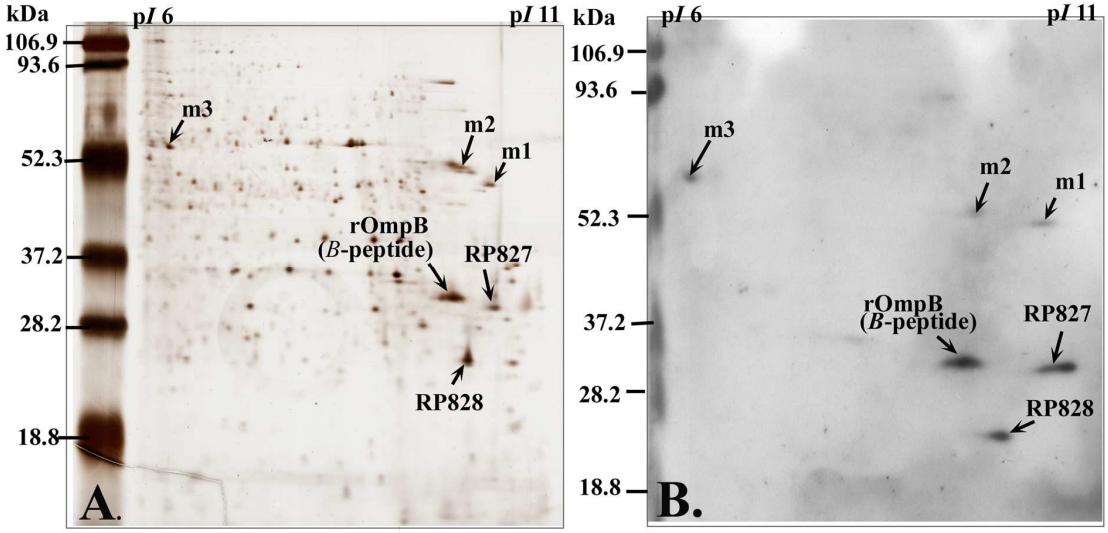


Figure 3
Purification of the rickettsial adhesin Adr2
During the purification process, different fractions of the protein extract were separated using SDS-PAGE and stained with Coomassie blue (A). The identity of purified protein was confirmed using western blot with an anti-his antibody (B). T = Total, S = Soluble, W = Wash, E = Elution.

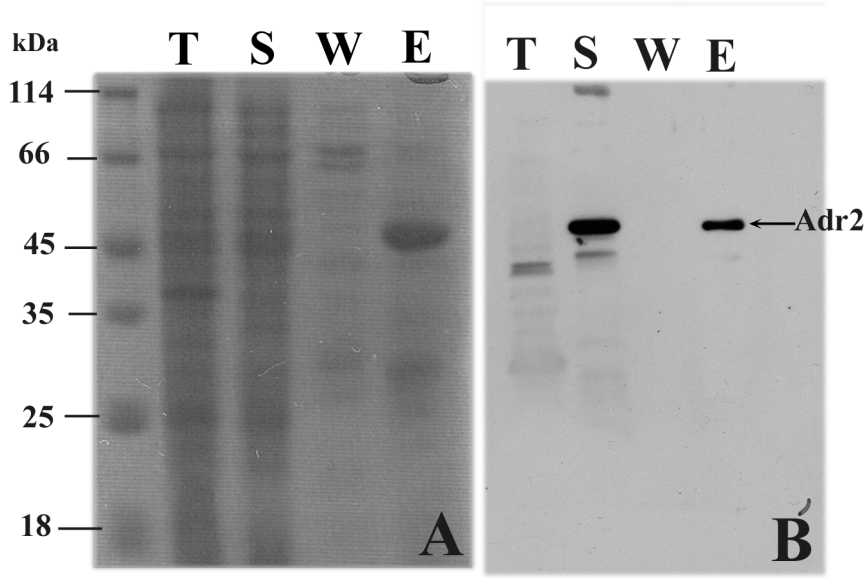


Figure 4

Specificity of monoclonal antibodies against Adr2

Detection of rickettsial Adr2 (RP828) using monoclonal antibody obtained from immunized mice. The *R. prowazekii* protein sample was separated using 2D-electrophoresis and visualized using silver staining (B) or western blot with anti- Adr2 (RP828) monoclonal antibody (A)

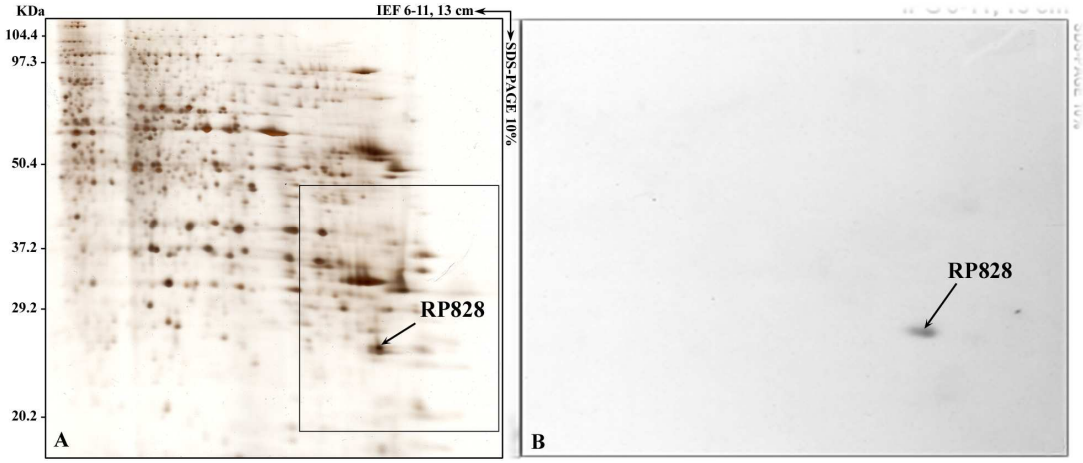


Figure 5

Inhibition of *R. prowazekii* MadridE-induced cytotoxicity with anti-Adr2 monoclonal antibody

R. prowazekii MadridE (3x103 bacteria per well) was pretreated for 20 min with increasing titers of anti -Adr2 (RP828), anti-rOmpB, anti-groEL and anti-RP059 mAbs before infection on L929 cells at the sampling points post-infection: 1h, 8h, 24h, 120h (5 days) and 168h (7days). The percentage of remaining viable L929 cells was estimated by staining with neutral red at each time of sampling. To estimate the relative cytotoxicity levels, the cytotoxicity level of *R. prowazekii* MadridE pretreated with buffer alone was considered to be 100%. The negative control consists on uninfected stained L929 cells. The % of inhibition of rickettsial cytotoxicity was calculated for each mAb.

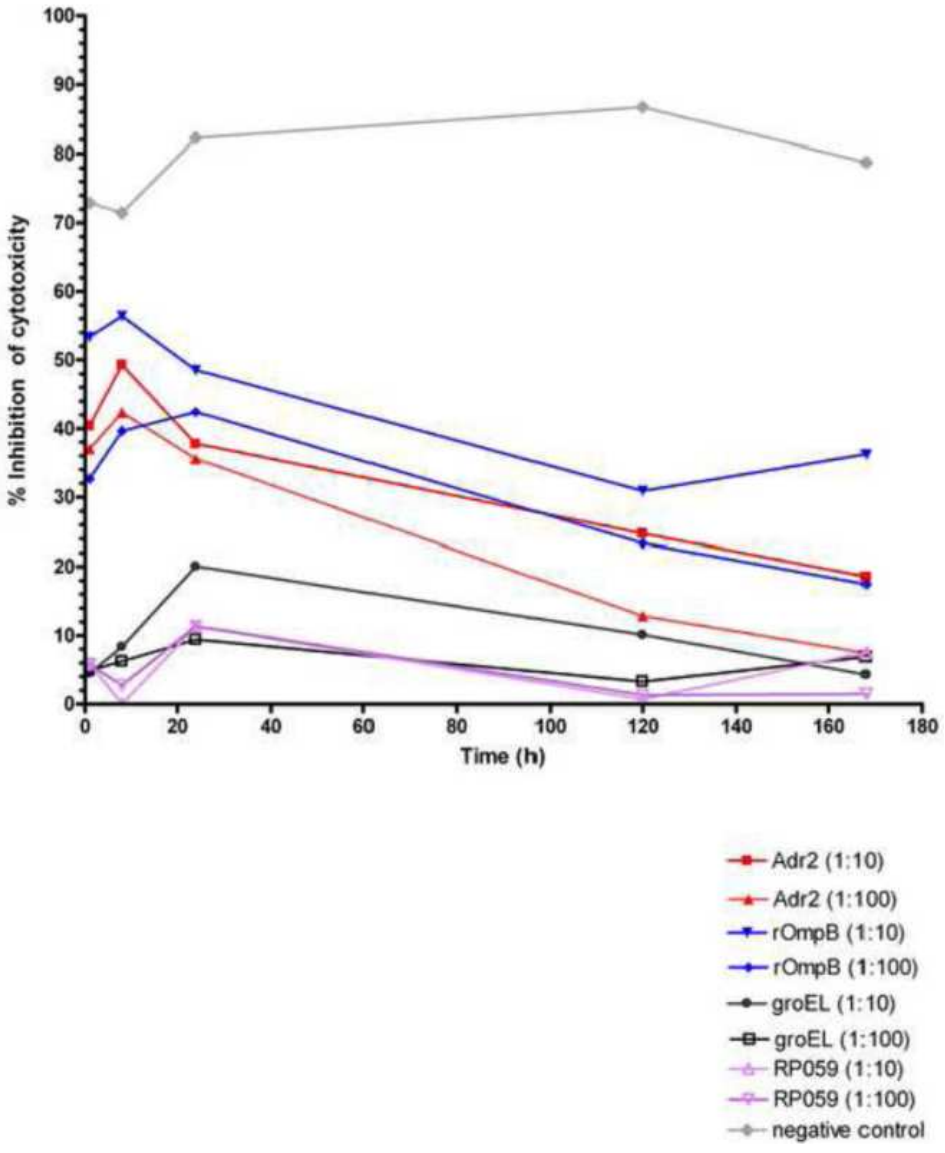


Figure 6
Model of *R. prowazekii* interaction with mammalian cells
The rickettsial entry to the host cell is likely governed by interactions between rOmpB and its receptor Ku70, most likely by the adhesins Adr1 and Adr2, by unknown mechanism. Based on the model of SFG *Rickettsiae*, we can suppose that Sca2 is also involved in bacterial entry; however, Sca2 of TG shares only 25% of homology with SFG Sca2 protein. Mammalian cholesterol is involved in bacterial interaction. Following rickettsia entry into host cells through induced phagocytosis, bacteria rapidely escape from the vacuole (possible role of PLD, TlyC) to gain the cytosolic compartiment and possibly the nucleus of mammalian cell where *R. prowazekii* replicates. The mechanism of cell-to-cell spreading for immobile TG *Rickettsiae* remains unknown. The rickettsial secretion system T4SS translocates effectors that should contribute to the intracellular survival of *R. prowazekii*.

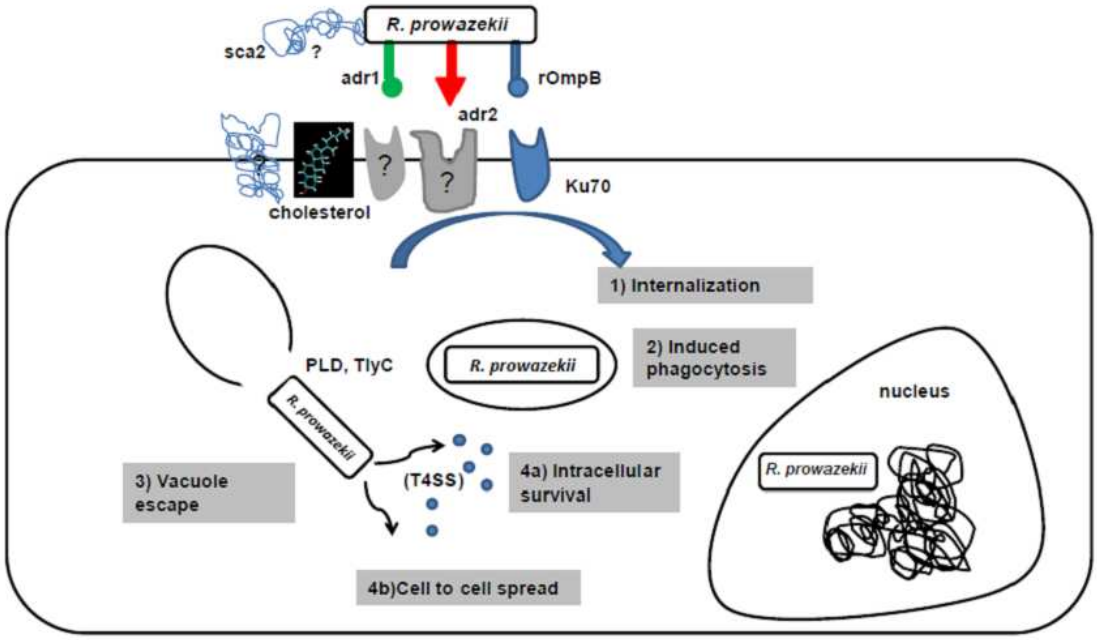


Table 1

Gene	Protein	Strain	Primers used
RP827	Dsbc-Adr1	MadridE/RP22	F:5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC Ttcgatcatgatatgaattgttctgtag -3'
			R:5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC CTA catatcaaatcttaatcctgccattaag- 3'
RP828	DsbC-Adr2	MadridE/RP22	F:5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC Ttcgagtgcattgataatgaatgg- 3'
			R:5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC CTAtataccaaatcttacacctactgtc- 3'
RP827	TRX-Adr1	MadridE/RP22	F:5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGT-GATCATGATATGAATTGTTCTGTAGATTCA-3'
			R:5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCttatta- CATATCAAATCTTAATCCTGCC- 3'
RP828	TRX-Adr2	MadridE/RP22	F:5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGT-GAGTGCATTGATAATGAATGG- 3'
			R:5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCttatta-TATACCAAATCTTACACCTACTGTC-3'
60 KD CHAPERONIN (groEL) RP626	TRX-groEL	MadridE	F:5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGT-ACAACGAAACTTATTAAACACG-3'
			R:5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCttatta-GAAGTCCATACCACCCATGCCAC-3'
Stage 0 sporulation protein J (spo0J) RP059	TRX-spo0J	MadridE	F:5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGT-GTGAAAAATAAAGGGCTAGGGC-3
			R:5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCttatta- ATTTAATTTTGATAATATTAAAT-3'

Supplementary material

Suppl.M1: Similarity of Adr sequences in *Rickettsia* spp.

The sequences similarity of the putative adhesins Adr1 and Adr2 for all sequenced rickettsial species.

Sequences	Africae-raf_ORF1174	Akari-ZP_00340858_1	BelliiOSU-ZP_01379467_1	BelliRML-YP_538440_1	Canadensis-ZP_01348025_1	Conorii-NP_360918_1	Felis-YP_247329_1	Prowazeki-NP_221176_1	Rickettsii-ZP_00154228_2	RP22_Adr1_Frame1	Sibirica-ZP_00142605_1	typhi-YP_067752_1	Africae-raf_ORF1175	akari-ZP_00340859_1	BelliiOSU-ZP_01379468_1	BelliiRML-YP_538441_1	Canadensis-ZP_01348026_1	Conorii-NP_360919_1	Felis-YP_247330_1	Prowazeki-NP_221177_1	rickettsii-ZP_00154229_2	Rp22_Adr2_Frame3	Sibirica-ZP_00142604_1	Typhi-YP_067753_1
Africae-raf_ORF1174	ID	65	63	63	74	97	65	69	95	62	98	66	42	42	42	41	42	42	43	40	42	36	43	40
Akari-ZP_00340858_1	65	ID	54	54	62	64	86	58	65	53	66	57	36	37	38	38	37	36	38	35	36	32	36	36
BelliiOSU-ZP_01379467_1	63	54	ID	100	60	62	55	56	62	51	63	56	42	42	43	43	40	41	43	40	42	35	42	40
BelliRML-YP_538440_1	63	54	100	ID	60	62	55	56	62	51	63	56	42	42	43	43	40	41	43	40	42	35	42	40
Canadensis-ZP_01348025_1	74	62	60	60	ID	74	63	68	73	63	75	69	42	42	42	42	41	42	43	40	42	35	42	40
Conorii-NP_360918_1	97	64	62	62	74	ID	65	68	95	62	98	65	41	41	42	41	42	41	42	40	41	36	42	40
Felis-YP_247329_1	65	86	55	55	63	65	ID	59	65	54	66	58	38	38	37	36	39	37	39	37	37	33	37	36
Prowazeki-NP_221176_1	69	58	56	56	68	68	59	ID	68	90	69	85	42	42	43	43	42	42	43	41	42	37	43	41
Rickettsii-ZP_00154228_2	95	65	62	62	73	95	65	68	ID	62	96	65	41	42	42	42	43	41	43	41	41	36	42	40
RP22_Adr1_Frame1	62	53	51	51	63	62	54	90	62	ID	63	80	37	36	37	37	36	37	37	36	37	36	37	35
Sibirica-ZP_00142605_1	98	66	63	63	75	98	66	69	96	63	ID	67	41	42	42	41	42	41	43	40	42	36	42	40
typhi-YP_067752_1	66	57	56	56	69	65	58	85	65	80	67	ID	41	40	41	41	41	41	42	41	41	36	41	40
Africae-raf_ORF1175	42	36	42	42	42	41	38	42	41	37	41	41	ID	87	61	61	81	97	92	77	97	70	97	76
akari-ZP_00340859_1	42	37	42	42	42	41	38	42	42	36	42	40	87	ID	63	63	83	87	92	78	89	70	89	76
BelliiOSU-ZP_01379468_1	42	38	43	43	42	42	37	43	42	37	42	41	61	63	ID	99	61	62	65	60	63	54	63	61
BelliiRML-YP_538441_1	41	38	43	43	42	41	36	43	42	37	41	41	61	63	99	ID	62	63	65	61	63	55	63	61
Canadensis-ZP_01348026_1	42	37	40	40	41	42	39	42	43	36	42	41	81	83	61	62	ID	82	83	74	83	67	83	70
Conorii-NP_360919_1	42	36	41	41	42	41	37	42	41	37	41	41	97	87	62	63	82	ID	93	76	97	69	97	75
Felis-YP_247330_1	43	38	43	43	43	42	39	43	43	37	43	42	92	92	65	65	83	93	ID	78	92	70	92	77
Prowazeki-NP_221177_1	40	35	40	40	40	40	37	41	41	36	40	41	77	78	60	61	74	76	78	ID	78	91	79	86
rickettsii-ZP_00154229_2	42	36	42	42	42	41	37	42	41	37	42	41	97	89	63	63	83	97	92	78	ID	71	99	77
Rp22_Adr2_Frame3	36	32	35	35	35	36	33	37	36	36	36	36	70	70	54	55	67	69	70	91	71	ID	71	79
Sibirica-ZP_00142604_1	43	36	42	42	42	42	37	43	42	37	42	41	97	89	63	63	83	97	92	79	99	71	ID	77
Typhi-YP_067753_1	40	36	40	40	40	40	36	41	40	35	40	40	76	76	61	61	70	75	77	86	77	79	77	ID

ADR 1 Sequences

ADR 2 Sequences

ADR 1 Sequences

ADR 2 Sequences

Suppl.M2: The sheet of Adr2 recombinant protein production

Adr2

Elution in 50% buffer B.

Pool fractions wash E4-E7 Vol: 6 mL ; 0,09 mg/mL soit 0,56 mg.

Pool fractions **élution** F9-F4 Vol : 9mL ; 1,12 mg/mL soit 10,1mg.

Conservation on 50% glycérol : Pool 18 ml in :

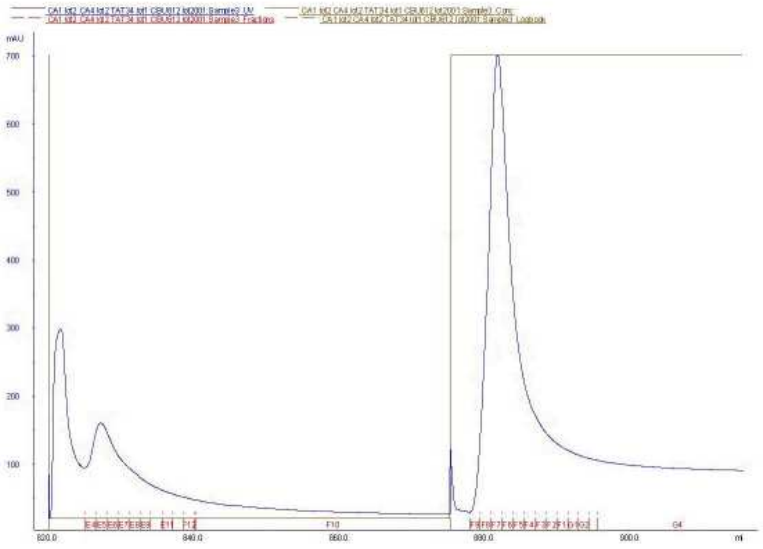
Tris 25 mM pH 8, NaCl 150 mM, Imidazole 125 mM, Glycerol 50 %

2,5 of the pool is loaded on a PD10 equilibrated in Tris 25 mM, 150 mM NacL, pH7,2.
elution in 3,5 ml

Concentration of the pool (Nanodrop): 0,39 mg/ml (1,4 mg in total).

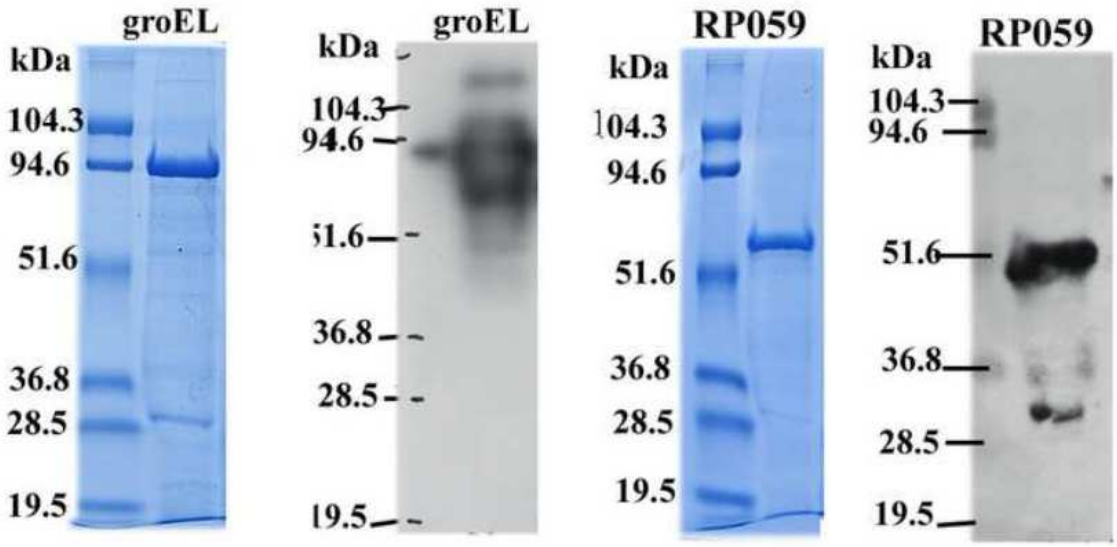
NICKEL AFFINITY: AKTA XPRESS

Date	Column	Buffer A	Buffer B
06/06/08	His Trap Ni 5 mL crude	Tris 50 mM pH 8.0, NaCl 300 mM, Imidazole 10 mM,	Tris 50 mM pH 8, NaCl 300 mM, Imidazole 500 mM



Suppl.M3: SDS-PAGE and corresponding Western blot performed with mAbs anti-groEL and anti-RP059.

The recombinant protein (10µg) groEL and RP059 respectively were resolved on 10% acrylamide SDS-PAGE. The corresponding Western blot was performed with either anti-GroEL or anti-RP059 monoclonal antibody at the dilution 1:10.



Article 2 – Preamble

SFG *rickettsiae* are obligate intracellular pathogens able to manipulate the actin cytoskeleton, thus enabling cell-to-cell spreading during infection. The genomic comparison of motile SFG with the non-motile TG bacteria allowed to evidenced that bacteria from the SFG, able to form actin comets and to move in the cytoplasm, encode for a protein sharing a domain organization similar to the actin assembly-inducing protein ActA, responsible for actin polymerisation in *Listeria* species (Ogata H *et al.*, 2001). It was later demonstrated that the *R. conorii* RickA can effectively activate Arp2/3 and induce actin polymerization *in vitro* (Gouin *et al.*, 2004, Jeng *et al.*, 2004). The precise molecular mechanisms leading to RickA-mediated rickettsia motility were not elucidated. First, and based on the lack of peptide signal, its localization as a membrane protein was for long questionnable. In addition, and while bacterial factors involved in motility are usually polarized, in the case of RickA the polarization was not clearly determined (Carlsson and Brown, 2006, Stevens *et al.*, 2006).

The aim of this work was thus to carefully analyze the localization of RickA, using *R. conorii* as model. Two approaches were used starting by immunofluorescence assays on infected cells. Localization was then refined by immunogold coupled to transmission electron microscopy analysis. The results obtained were depicted in the paper presented above.

Article 2

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Transmission electron microscopy as a tool for exploring bacterial proteins: model of RickA in *Rickettsia conorii*

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SUMMARY

Rickettsia conorii, the etiologic agent of Mediterranean spotted fever, belongs to the spotted fever group of *Rickettsia*. It is an obligate intracellular bacterium that grows within the cytoplasm of its eukaryotic host cells. It is motile in the cytoplasm of infected cells and RickA is reported as critical protein in this aspect. However, the subcellular localization of RickA remains uncertain. We describe a simple method allowing RickA protein to be localized by immunofluorescence assay (IFA) and transmission electron microscopy (TEM). By using IFA we showed the global expression of surface protein RickA in *R. conorii* organisms.
The TEM results showed that RickA is widely expressed over the entire bacterial surface of *R. conorii*.

KEY WORDS: Rickettsia conorii, RickA, Immunogold, Monoclonal antibody, Transmission electron microscopy

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INTRODUCTION

Rickettsiae are bacteria highly specialized for obligate intracellular existence in both mammalian cells and arthropod vectors (Raoult and Roux, 1997; Winkler, 1990). Historically, they have been classified into three groups based on immunological cross-reactivity and vector species: "spotted fever group" (SFG) with agents *R. conorii*, *R. rickettsii* and *R. raoultii*, the "typhus group" (TG) with *R. prowazekii* and *R. typhi* and the "scrub thyphus" (STG) (Raoult and Roux, 1997). However, this classification is probably simplistic, because some *Rickettsia* spp. do not conserved these criteria of classification (Merhej *et al.*, 2009; Merhej and Raoult, 2010). For example, SFG rickettsiae are definied as living in ticks, but exceptions include *R. akari* (transmitted by mites) and *R. felis* (transmitted by cat and dog fleas) (Merhej *et al.*, 2009; Merhej and Raoult, 2010). Recently, a "transitional group" including these 2 rickettsial species (*R. felis* and *R. akarii*) has been proposed (Gillespie *et al.*, 2007; Gillespie *et al.*, 2010; Merhej *et al.*, 2009). The SFG group bacteria, in contrast to TG, have the capacity to move from cell to cell and within the cells.

Exploitation of the host-cell actin cytoskeleton is crucial for several microbial pathogens to enter and disseminate within cells, thus avoiding the host immune response (Carlsson and Brown, 2006; Stevens *et al.*, 2006).

It was proposed that actin in rickettsial tails is nucleated by host Arp2/3 complex and the bacterial proteins *rickA* (Balraj *et al.*, 2008a; Gouin *et al.*, 2004) and recently discovered Sca2 (Haglund *et al.*, 2010). The rickettsial gene r*ickA* of SFG *Rickettsiae* was identified through a comparative analysis of *R. conorii* and *R. prowazekii* genome (Ogata *et al.*, 2001).

It encodes for a 517- amino acid protein *rickA* (Gouin *et al*., 2004; Gouin *et al*., 2005) which shares some similarities in its carboxy-terminal region with human WASP family proteins able to activate Arp2/3 *in vitro* (Gouin *et al*., 2004; Jeng *et al*., 2004). Because genetic manipulations are still difficult, the role of *rickA* in the motility of *Rickettsiae* has not been formerly demonstrated (Balraj *et al.*, 2008b).

Its function was in part supported by the absence of motility of *R. peacockii*, a strain for which *rickA* is disrupted by an insertion sequence IRSpeI (Simser *et al*., 2005). *RickA* activates the Arp2/3 complex *in vitro* and stimulated motility of *rickA*-coated beads in *Xenopus* extracts (Gouin *etal*., 2004; Jeng *et al*., 2004). Therefore, several points remain unclear. *RickA* was found to be expressed on the bacterial surface (Gouin *et al*., 1999; Gouin *et al*., 2004), but the amino-acid sequence of *rickA* does not display any signal sequence or C-terminal motif that could act as a membrane anchor (Gouin *et al*., 2004). The experiences with *rickA* transfected cells designed to drive expression of the protein in the inner face of the plasma membrane, showed that *rickA* is a surface protein expressed on *R. conorii* involved in Arp2/3 activation and inducing actin polymerization (Gouin *et al*., 2004). It has been shown that *rickA* protein was expressed on the surface of *R.conorii* using immunofluorescence (IFA) (Gouin *et al*., 2004) and in *R. raoultii* by using monoclonal antibody through western blot (Balraj *et al.*, 2008c).

However, it is unknown how *rickA* is addressed to the bacterial surface and whether the type IV secretion system predicted by the genome sequence is involved in targeting to the surface (Gouin *et al*., 2004) (Figure 1). Indeed, the ultrastructural studies of fine structure of *Rickettsiae* by using electron microscopy were conducted in late 1980^{thies} (Hase, 1985; Silverman *et al.*, 1974; Silverman *et al.*, 1978; Silverman, 1991; Silverman and Wisseman, Jr., 1978) and aimed to compare the

physical conformation of the outer envelope of *Ricketsiae* by electron microscopy, revealed some differences within *Rickettsiae* from TG and SFG when compare to *O. tsutsugamushi* (Silverman *et al*., 1978;Silverman and Wisseman, Jr., 1978). The TG and SFG *Rickettsiae* shared together with *E. coli* very similar configuration of the outer envelopes (Figure 2) (Silverman and Wisseman, Jr., 1978). However, together with *O. tsutsugamushi*, the SFG Rickettsiae possess additionally to "microcarpuscular layer", the slime layer, external to the cell wall which is probably the locus of major group-specific antigens (Silverman *et al.*, 1978). Based on the model of *R. conorii* surface expressed protein (Gouin *et al.*, 2004), the aim of the present study was to demonstrate the surface localization of *rickA* protein in the *R. conorii* by using combined approaches: immunofluoresce assay using anti-*rickA* monoclonal antibody (Balraj *et al.*, 2008c) and TEM analysis through immunogold labeling.

MATERIALS AND METHODS

Eukaryotic cell lines and bacterial strains

R. conorii strain seven were propagated within murine fibroblast monolayers, L929 cell line (ATCC CCL 1) or African green monkey kidney cells (Vero cell, ATCC C1587) in Eagles minimum essential medium (MEM, Gibco, Invirtogen, Paisley, UK) supplemented with 4% foetal calf serum (FCS, Gibco) and 1% L-glutamine (Gibco) in 150 cm2 tissue culture flasks at 32ºC as described (Balraj *et al.*, 2008b). The *rickettsiae* were harvested when the Vero cells were engorged by bacteria (3 to 7 days), which corresponded to exponential phase of growth. Supernatant of infected rickettsial cell culture, containing rickettsiae and detached host cells, were collected and centrifuged at 200 x g for 10 min to eliminate cells and free rickettsiae were pelleted by 8000 x g for 10

min. This bacterial sample was used to prepare immunofluorescence assay (IFA) slides. Bacterial growth was monitored by Gimenez staining (Gimenez, 1964). Additionally, the quantification of bacterial DNA has been performed as internal control of replication. The standard curve used in routine diagnostic was applied for DNA quantification at the same sampling times as for monitoring by Gimenez staining. The specific primers to detect genomic DNA from *R. conorii* were used, coding for putative acetyltransferase F: 5'-TTG-GTAGGC- AAG-TAG-CTA-AGC-AAA-3' and R: 5'-GGAAGT- ATA-TGG-GAA-TGC-TTT-GAA-3', sondeFAM-GCG-GTT-ATT-CCT-GAA-AAT-AAG-CCGGCA TAMRA (Bechah Y *et al.*, 2011;Bechah *et al.*, 2007).

Immunofluorescence assay

Anti-RickA monoclonal antibody was previously described (Balraj *et al.*, 2008c). Bacterial suspension was spotted on 18well slides using pin head nib and slides were air dried and fixed with 100% methanol for five minutes at room temperature (RT). Slides were incubated for 30 minutes at RT in humidified condition with mouse monoclonal anti-*rickA* antibody (1:100) diluted in PBS-Tween (0.1%) with bovine serum albumin (BSA 3%, Euromedex, France).

After two times PBS-Tween (0.5%, 5 min each) washes, bound antibody were probed with antimouse IgG conjugated biotin (1:1000; Beckman Coulter Company, France) diluted in PBS-Tween (0.1%) with BSA (3%) for 30 minutes at RT. Further washing was performed in PBS-Tween (0.5%, 5 min each) for two times. Then slides were incubated with streptavidin conjugated to fluorescein isothiocyanate (1:500; Bioscience BD pharmingen, France) for 30 minutes at RT. After two washes with PBS-Tween (0.5%, 5 min each) slides were air-dried and cover slips were mounted on slides with DAPI (4, 6- diamidino-2- phenylindole, Prolong

Gold Antifade Reagent. Molecular Probes) from a ready to use solution and examined under an olympus BX-51 epifluorescence microscopy at X 100 magnification for image analysis. A naive mouse serum was used for negative control. Table 1 summarized the controls that were used in this study.

Transmission electron microscopy

Transmission electron microscopy (TEM) analysis was conducted on L929 cells infected with *R. conorii*. A 125 cm2 flask infected with *R.conorii* for 96 h was carefully collected and pelleted by centrifugation before fixation in 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and cacodylate buffer (0.1 M) overnight at 4°C. After washing with cacodylate buffer (0.1M), the samples were further fixed for 1 h at room temperature with 2% osmium tetroxide (0.1M), dehydrated in an ascending series of ethanol (30% to 100%) and embedded in Epon 812 resin (Electron Microscopy Sciences). Ultrathin sections (70 nm) were transferred on 300 mesh nickel Formvar/carbon grid (TAAB Laboratories, England).

The grids were pre-treated twice with 50 mM NH4Cl in PBS (5min each). After washing with PBS for four times (5 min each), the grid were pre-incubated with solving solution I (PBS, BSA (1%), normal goat serum 1% (NGS, DAKO, Denmark), Tween20 (0.2%) for two times (5 min each) in 2% osmium tetroxide (0.1M). The grids were incubated 1h30 with monoclonal mouse anti-*rickA* antibodies (1:50) diluted in solving solution I. After washing 4 times (10 min each) with solving solution I, grids were incubated 90 mins with anti-mouse IgG biotinyled antibody (1:100, Beckman Coulter Company, France) diluted in solving solution I. Following gentle washing with BSA (0.1%) in PBS for two times (5 min each), the grids were pre-incubated two times (5 min each)

with solving solution II (PBS, Fish skin gelatine (0.01%), Aurion Immuno Gold Reagents & Accessories, Netherlands). The grids were incubated for 1h30 min with streptavidine (1:40) gold 10 nm conjugate reagent (Aurion Immuno Gold Reagents & Accessories, Netherlands), diluted in solving solution II. The specimens are washed with incubation solution II for two times (5 min each).

Finally, the grids were washed in distilled water for 2 times (10 min each) and stained with uranyl acetate (3%, Prolabo, France) in water. Then, grids were allowed to dry at room temperature before examined on a Philips Morgagni 268D electron microscope (FEI Company, Limcil-Brevannes, France). A negative control was carried out by using serum of naïve mice.

Statistical analysis

One hundred individual fields were taken and gold particles were counted for inner membrane (IM), outer membrane (OM) and space around rickettsia (ECS). We have performed one-tailed paired t-test (Graphpad Prism software). The graphs were also compiled in this software.

RESULTS

Detection and localisation of *rickA* in *R. conorii*

As illustrated in figure 3A, fluorescence labelling of *rickA* was amplified using biotin-streptavidin conjugate and showed that the protein was expressed on the surface of *R. conorii*. In all negative controls (Table 1) there was no fluorescence intensity over the bacterial surface (Figure 3B). The distribution of RickA in *R. conorii* is shown in figure 4.

The number of gold particles present within inner membrane (IM) and outer membrane (OM) were less when compared with the number of gold particles present outside of rickettsiae (ECS) but not statistically

significant. The ECS corresponds to the zone which is found outside of rickettsial organism sampled from supernatant of *R. conorii* culture. However, we found that the most significant difference (p=0.0024) was observed between the number of gold particles localized on IM and OM. The significant t-test (p=0.0316) was also observed for number of gold particles localized on OM in comparison with those of ECS. However, no significant difference was observed if we compare the number of gold particles localized on IM of the cell in comparison with those of ECS (p=0.2747).

DISCUSSION

The present study showed the surface expression of *rickA* in *R. conorii* using anti-*rickA* specific monoclonal antibody in IFA (Figure 3A). Our results skewed with the work of Gouin *et al.* who demonstrated that *rickA* is localized at the surface of the bacteria using IFA evidenced where actin polymerization occurs (Gouin *et al*., 2004). We can hypothesize that *rickA* found in ECS may be indirectly involved as a nucleation-promoting factor (NPS) which mediates actin nucleation (Figure 1). Actin is one of the most abundant proteins in eukaryotic cells and exists in two forms, ATP-bound monomeric (G) actin and ADP-bound filamentous (F) actin (Stevens *et al*., 2006). Polymerization of actin requires ATP hydrolysis and it is tightly regulated by monomer- and filament- binding proteins that also maintain the free monomer pool and mediate capping, cross linking, bundling or severing of actin filaments (Stevens *et al*., 2006).

An initial nucleation step creates free barbed ends by uncapping or severing of filaments or *de novo* nucleation of monomers (Stevens *et al.*, 2006). This step is stimulated by cellular factors, as complex Arp2/3, which in turn, are activated by proteins known as NPFs such as Wiskott-

Aldrich syndrome proteins (WASP family proteins) (Figure 1). In the *L. monocytogenes* model, the conformational changes of Arp2/3 complex induced by NPFs might allow these subunits to mimic barbed ends to serve as template for polymerization (Stevens *et al*., 2006). Surprisingly, Serio *et al*. did not identify a cellular actin nucleator (Arp2/3 complexe) in *R. parkeri*, suggesting that it is not required for actin-based rickettsial motility (Serio *et al.*, 2010).

Therefore, in the case of *Rickettsiae*, the molecular mechanism of actin assembly and organization, as well as the exact role of nucleation activators like *rickA* and sca2, is still obscure (Balraj *et al.*, 2008a;Gouin *et al.*, 2004; Haglund *et al.*, 2010;Kleba *et al.*, 2010; Serio *et al.*, 2010). Both well conserved genes among SFG *rickettsiae*: *R. conorii rickA* (Gouin *et al.*, 2004) and *R. rickettsi* sca2, a member of a family of large autotransporter proteins (Kleba *et al.*, 2010), were reported to be required for motility and virulence. Indeed, when Sca2 was truncated by transposon insertion, the Sca2 mutant bacteria do not generate actin comet tails (Kleba *et al*., 2010). Probably, the sca2 N-terminus which is structural homolog of formin homology 2 domain, is involved in nucleation of unbranched actin filaments, processively associated with growing barbed ends, requires profiling for efficient elongation, and inhibits the activity of capping protein (Haglund *et al.*, 2010). *RickA* includes proline-rich regions sharing the homology with WASP proteins and is considered as NPF. The surface localization of the *rickA* protein might allow its secretion and acting as NPF involved in actin polymerization. However, the contribution of *rickA* protein in this process has not been completely elucidated.

Many questions remain unanswered: the mechanism of *rickA* secretion how is *rickA* targeted to the surface of host cell, as well as identification of other NPFs and the role of T4S (Figure 1). With respect to recent data,

the mechanism of actin-based motility is still under study and the rickettsial as well as host cell factors involved in this process remain to be determined. The recent work of Serio *et al*. (Serio *et al.,* 2010) showed that numerous host cell proteins are involved in *R. parkeri* infection and actin-based motility (profiling, fimbrin/T-plastin, capping protein and ADF/cofilin) (Serio *et al.,* 2010).

Interestingly, Fimbrin/T-plastin and profiling are required for *R. parkeri* motility, but they are not indispensable for *L. monocytogenes* and *S. flexenerii* motility (Serio *et al.,* 2010). The bacterial motility depends on bacterial species and can differ among SFG different strains and species. In this report we address only the question of *rickA* protein localization in *R. conorii* bacterium (Figure 1). IFA is commonly used technique to monitor the global expression of bacterial proteins. However, this technique is frequently performed in combination with other modern approaches which yielded better image resolution. Indeed, TEM enables the study of small details in the cell down to near atomic levels.

The possibility for high magnifications has made the TEM a valuable tool in both medical and biological research (Robinson, 1986). TEM has been successfully applied to determine the subcellular localization of bacterial protein Hfq (Diestra *et al.,* 2009) and the extracellular site evidence of virulent plasmid pYV harbored by *Yersinia pseudotuberculosis* (Simonet *et al*., 1990), as well as expression of IcsA and ActA on the surface of *Shigella flexneri* (Nhieu and Sansonetti, 1999), *Listeria monocytogenes* (Cossart and Kocks, 1994) and surface expression of *rickA* in *R. raoultii* (Balraj *et al.,* 2008c). However, by using TEM, we demonstrated that RickA is widespread in *R. conorii* (Figures 4 and 5). It has been shown that other bacterial components like IcsA, ActA, or BimA are known to be responsible for intracellular motility and exhibit a polarized distribution (Goldberg and Theriot, 1995; Kocks *et al*., 1993; Stevens *et*

al., 2006). Such polarization was not observed for *rickA* which was found to be expressed over the entire bacterial surface in *R. conorii* in the study of Gouin *et al*. (Gouin *et al*., 2004) as in our study (Figures 3 and 4). Thus, our results skewed with the results of this group (Gouin *et al*., 2004).

CONCLUSION

In conclusion, we have shown the global expression of *rickA* in *R. conorii* cell by using IFA approach (Figure 3). The results of TEM showed that gold particles were distributed over the entire surface of *R. conorii*. This result emphasizes the importance of disclosing the detailed mechanism of *rickA* secretion and it's targeting to the host cell surface, and to determine the host receptors and factors involved in the dynamics of actin-tail formation and its motility inside the cell. For future prospects it will be suitable to fractionate the different bacterial compartments and to demonstrate the presence or absence of *rickA* in each compartment. Localization of proteins in cells has largely relied upon the use of specific antibodies. The results presented here show that anti-*rickA* monoclonal antibodies provided the same labeling pattern over almost the entire bacterial surface.

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Table 1. The controls included in IFA experiments
+/- indicates whether the antibody added or not.

Negative controls	*Monoclonal antibody Rick A*	*Normal mouse serum*	*Anti-mouse biotin*
1	-	+	+
2	+	-	-
3	-	-	+
4	-	-	-

+/- indicates whether the antibody added or not.

Figure 1

Summary of *rickA* protein roles in rickettsial physiopathology

rickA is involved in actin polymerization (transformation of monomeric actin G to filamentous actin F). An initial nucleation step creates free barbed ends by uncapping or severing of filaments or *de novo* nucleation of monomers. The complex of actin- relating protein (Arp) 2/3 involved in actin nucleation seems to be activated by nucleation-promoting factors (NPFs) as WASP proteins and *rickA*. However, the mechanism of actin polymerization in the model of *Rickettsiae* has not been completely elucidated. Several questions remain without response (grey boxes): (i) *rickA* protein secretion, (ii) how the Arp2/3 complex of actine is activated by *rickA*, (iii) There are other bacterial cofactors involved in actin polymerization, (iv) Is the T4S is involved in targeting *rickA* to the cell surface? (v) Is T4S is involved in host genes regulation? (vi) How *Rickettsiae* spread in the cell and from cell to cell? The question which is the object of this study concerns the *rickA* localization in *R. conorii* cell. To respond to this question, IFA and TEM were performed.

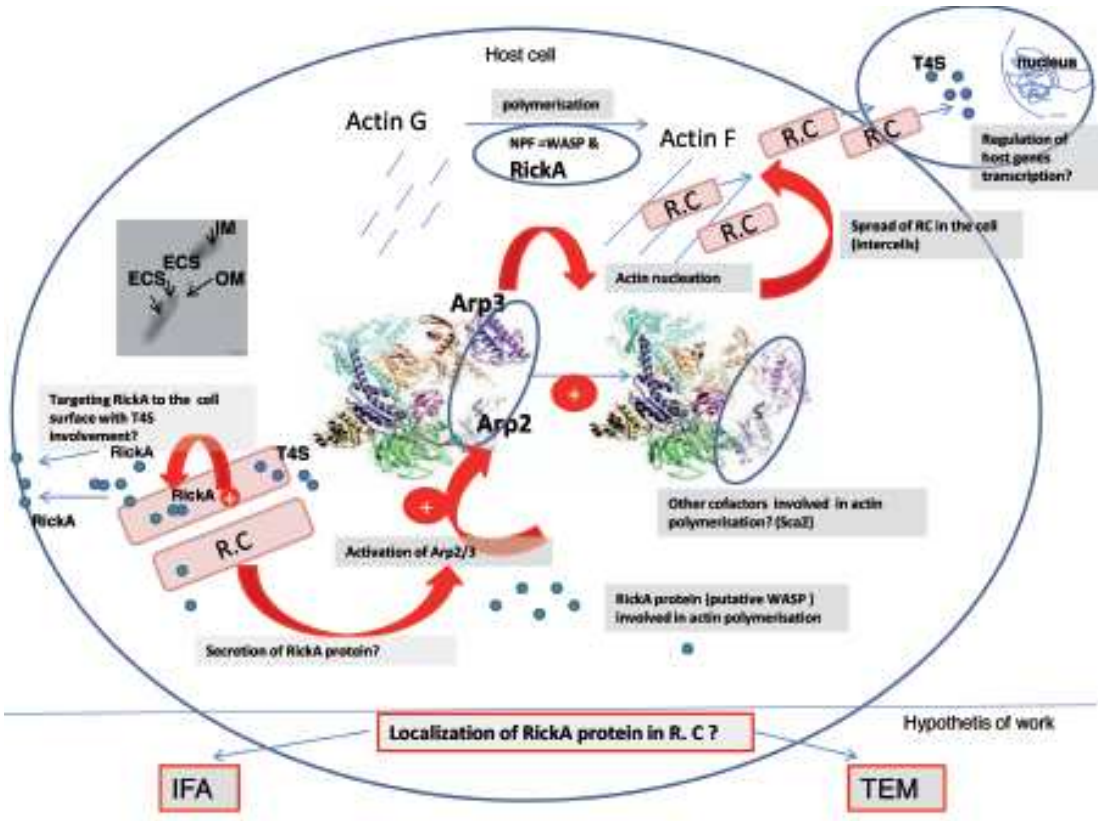
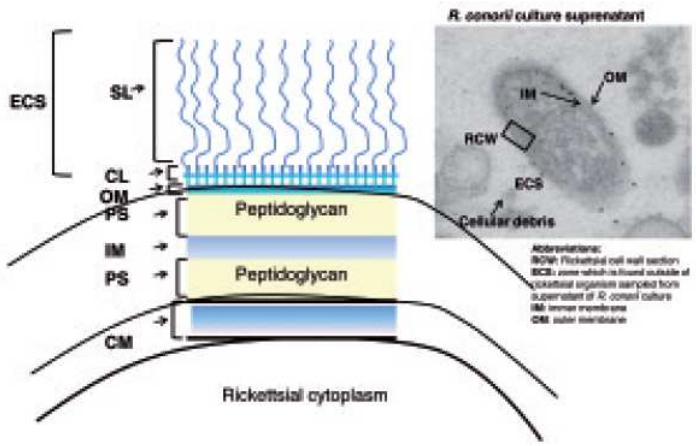


Figure 2

A) Schema of the cell membrane and B) Model of rickettsial surface membrane assembly. a) Schematic model of TG and SFG *Rickettsiae* of the cell membrane, outer envelope (cell wall) and adjacent extracellular layers. *Rickettsiae* are characterized by a specific membrane structure. The outer envelopes of SFG Rickettsiae are as follows: 1)An outer leaflet (OM) with additional "microcorpuscular layer" (ML) and ended with external slime layer (SL), 2) The peptidoglycan layer (PS) is localized between the OM of the cytoplasmic membrane (CM) and the inner leaflet (IM) of the cell wall; (Adapted from Silverman et al., (Silverman and Wisseman, Jr., 1978) b) Surface membrane assembly of *Rickettsiae*. In the rickettsial assembly, the rickettsial body formed first, and the rickettsial envelope subsequently formed over the body (Hase, 1983). The previously proposed mechanism of rickettsiae assembly is as follow: 1. The body of nascent Rickettsia took a definitive form, a fuzzy material mainly composed of lipoproteins is formed over the body, and graduatly separate the emerging rickettsia from the surrounding cytoplasm. 2. The assembly of the rickettsial limiting membrane on the ricketsial surface along the fuzzy zone occurs in close association with ribosomes. 3. The surface ribosomes are associated with rickettsial plasma membrane, although the plasma membrane of the assembling rickettsia is difficult to recognize. 4. The short projections of membrane extended from the surface ribosomes into the fuzzy zone, and as rickettsial double membrane assembled, these projections of membrane, form the septa of membranewhich stay connected with the surface of ribosomes and the outer membrane (OM) across the periplasmic space (PS). (Adapted from (Hase, 1985) and the image of ribosome has been freely available on internet:http://biology.kenyon.edu/courses/biol114/Chap05/RNA_ribosomes.gif)

A


R. conorii culture supernatant
ECS
SL
CL
OM
PS
Peptidoglycan
IM
PS
Peptidoglycan
CM
Rickettsial cytoplasm
IM
OM
RCW
ECS
Cellular debris

B

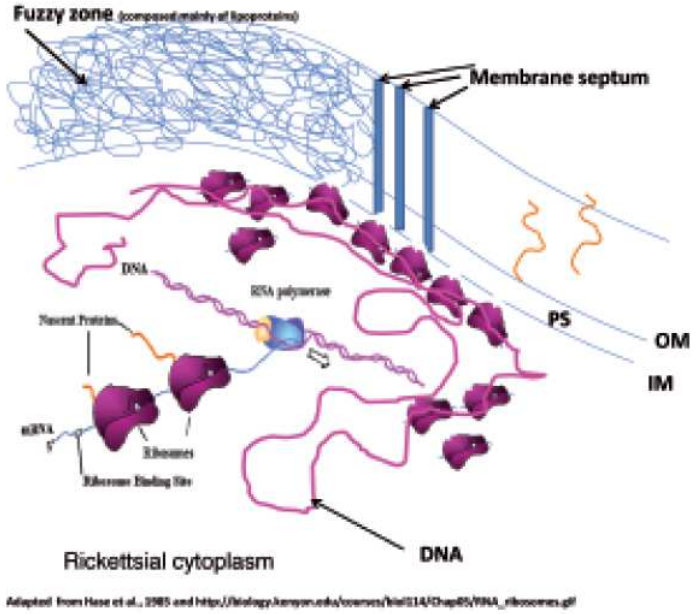

Fuzzy zone
Membrane septum
DNA
RNA polymerase
PS
OM
IM
mRNA
Ribosomes
DNA
Rickettsial cytoplasm

Figure 3

Detection of surface expression of *rickA* protein by indirect immunofluorescence

3A. Detection of RickA expression by IFA. Host cell-free *R. conorii* was fixed in methanol, incubated with *rickA* anti-mouse monoclonal antibody (1:100) followed by an anti-mouse biotin (1:1000), stained with streptavidin FITC (1:500) and visualized by epifluorescence microscopy (magnifications 100X), showed that *rickA* was expressed at the surface of *R. conorii*. **3B**. the right panel corresponds to negative control.

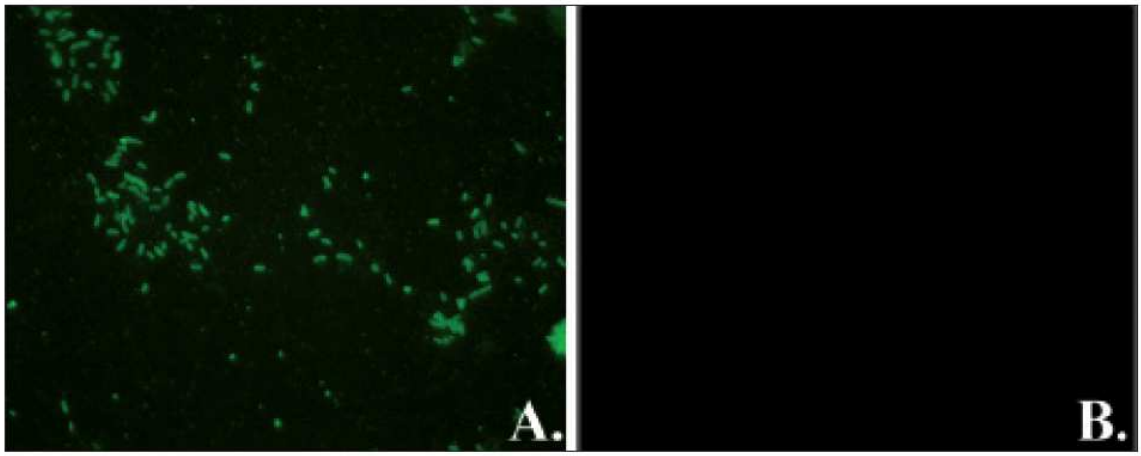


Figure 4
Localization of *rickA* (TEM)
4A. TEM analysis performed on *R. conorii* cultured on L929 cells using *rickA* anti-mouse monoclonal antibody followed by biotin and with streptavidin gold (10 nm); the arrows indicates the distribution of *rickA* in *R.conorii* cells inner membrane (IM), outer membrane (OM) and extracellular space around rickettsies (ECS). 4B. Negative control performed using serum of naive mice.

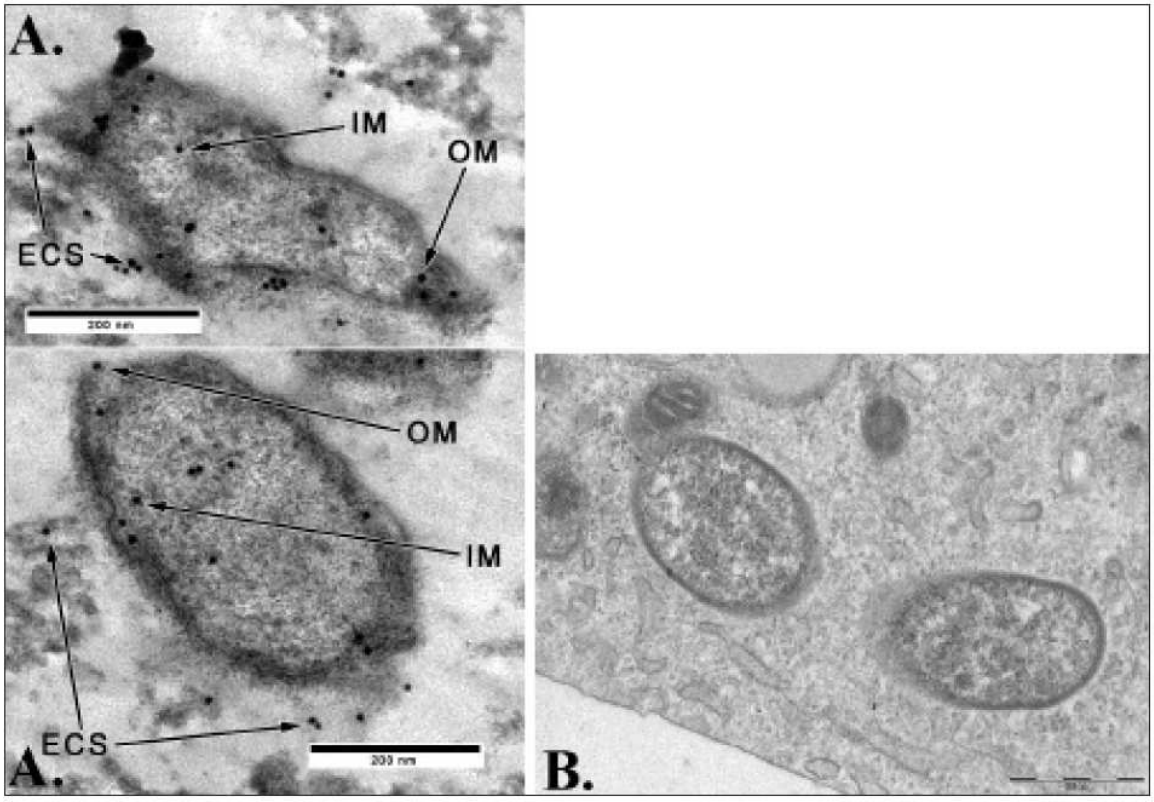
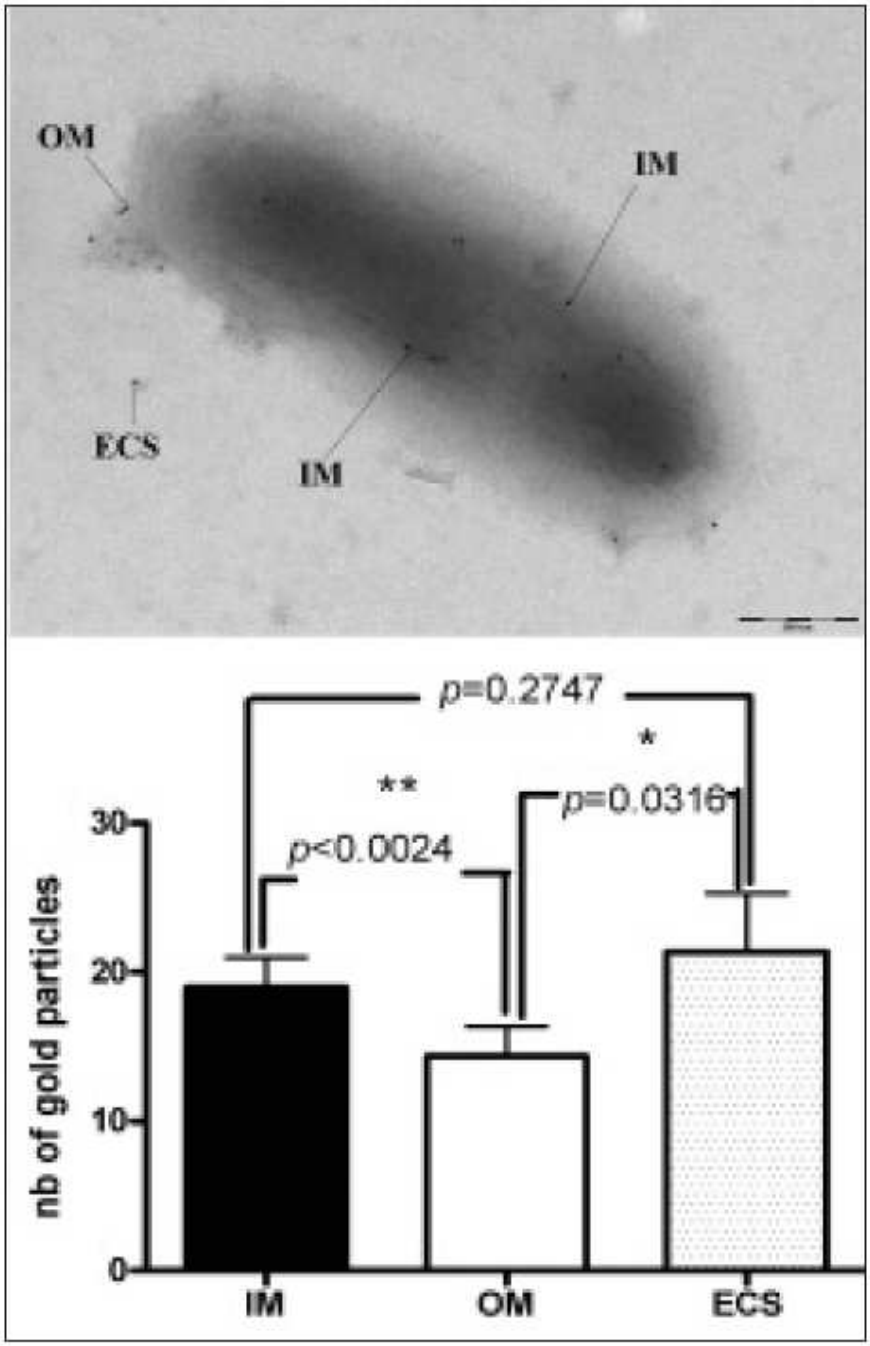


Figure 5

Histograms showing the distribution of gold particles in *R. conorii*

Gold particles were counted for one hundred individual fields. The gold particles were localized in inner membrane (IM), outer membrane (OM) and extracellular space around rickettsies (ECS). A graph was plotted by using graphpad prism software.



Article 3 – Preamble

While serology is the most used diagnostic method for rickettsial infections, the lack of specificity and sensitivity remain a major drawback (La scola and Raoult, 1997). Cross-reactions probably result from antigenically similar epitopes, but the possibility of co-infections can not be excluded. Many studies have reported great interest in using recombinant proteins rather than purified bacteria in immunodiagnosis. The aim of the present work was to propose an efficient diagnostic test, based on recombinant proteins, for the detection of *R. prowazekii* and *R. rickettsii*. To realize our purpose, 45 and 48 target genes of *R. prowazekii* and *R. rickettsii* were selected for recombinant expression using Gateway technology. The choice of targets was not arbitrary, but resulted from the large expertise of our laboratory in the field of rickettsiae. Twenty of the recombinant proteins obtained were screened by ELISA with sera of rickettsioses patients. Results obtained demonstrated a satisfactory performance allowing to select discriminating markers of *R. typhi* and *R. conorii* infection, respectively which may be useful for detection of rickettsiae in clinical samples.

Article 3 (*in revision*)

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Proteins candidates for the serodiagnosis of rickettsioses

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Keywords:	Rickettsia prowazekii, Rickettsia rickettsii, Rickettsia typhi, recombinant proteins, serodiagnosis, test-operating parameters, ELISA

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ABSTRACT

Rickettsia is Gram-negative obligate intracellular bacteria that cause arthropod-borne diseases of humans, including typhus (*R. typhi* and *R. prowazekii*) and spotted fevers (*R. conorii*, *R. rickettsii*). Diagnosis of rickettsioses is usually based on diverse serological testing of patient serum. The diagnostic antigen used for indirect immunoflorescence assay (IFA) considered as the reference method is done with whole purified bacteria. Deficiencies of this antigen include (i) potential of crossreactivity within different rickettsial species, as well as with other pathogens, (ii) the difficulty to obtain sufficient amount of antigen due to the requirement for highly specialized laboratory platform in intracellular bacteria culture; (iii) finally, discriminate diagnosis of rickettsioses is still a great challenge, considering the fact that clinical picture is most often not specific. There is therefore a need for serodiagnostic tests improvements, especially for a test able to make discrimination between Rickettsia from typhus group (TG) from Rickettsia of spotted fever group (SFG). In this aim, we have cloned and expressed several proteins of *R. prowazekii* and *R. rickettsii* using GATEWAY approach. Then, 20 recombinant protein targets were screened with sera of patients with rickettsioses by ELISA. We have identified several potential markers which allowed discriminating infection due to R. typhi with those caused by *R. conorii*. These antigens may be useful for the detection of Rickettsiae in clinical samples.

INTRODUCTION

Members of the genera *Rickettsia* are fastidious bacterial organisms that are obligate intracellular parasites that reside in the cytosol of the host cells and in an arthropod host (La & Raoult, 1997; Socolovschi, Mediannikov, Raoult, & Parola, 2009). *Rickettsiae* have undergone evolutionary genome reduction as results of the loss of functions that are provided by the host, i.e., genes encoding metabolic enzymes.

There are four Rickettsia species that frequently cause incapacitating, life threatening illness: *Rickettsia prowazekii*, *R. rickettsii*, *R. conorii*, and *R. typhi*.

Actually, the clinical manifestations of most rickettsioses are characterized by a continuous spectrum gaped by appearance of some worldwide reemerging cases. However, some examples showed inconsistent clinical manifestations hardly correlated with geographical context, which makes clinical diagnosis uncertain. To date, laboratory diagnosis of rickettsioses is based on various PCR assays, DNA sequencing which allows convenient and rapid identification of rickettsiae, even in non referenced laboratories (La Scola& Raoult, 1997). However, the diagnosis of rickettsial illness is confirmed by serological testing (La Scola& Raoult, 1997). Several conventional methods were used in serology: historic Weil-Felix test (Weil & Felix, 1916; Eremeeva, Balayeva, & Raoult, 1994), the complement-fixation test (Shepard, Redus, Tzianabos, & Warfield, 1976), the microagglutination test (Fiset, Ormsbee, Silberman, Peacock, & Spielman, 1969) and the indirect hemagglutination test detects antibodies to an antigenic erythrocyte-sensitizing substance (Anacker, Philip, Thomas, & Casper, 1979). Lately, these methods became obsolete and were replaced in the early 1980th 20 by others easier to handle and guaranteed better sensitivity and specificity: (i) ELISA, first introduced for detection of antibodies against

R. typhi and *R. prowazekii* (Halle, Dasch, & Weiss, 1977) considered as highly sensitive and reproducible, allowing the differentiation of IgG and IgM, then extended to diagnosis of RMSF (Clements *et al*., 1983) and scrub typhus (Dasch, Halle, & Bourgeois, 1979; Crum, Hanchalay, & Eamsila, 1980), (ii) IFA in format of micromethod which is to date, considered as reference test (Philip, Casper, Ormsbee, Peacock, & Burgdorfer, 1976). The advantage of micro-IFA is the simultaneous detection of several antibodies to a number of rickettsial antigens in a single well with the same drop of patient serum. It allows isotyping of Ig: IgG, IgM and IgA which with detection of IgM provides a strong evidence of recent active infection, although the diagnosis may be compromised. Western blot and antigen adsorption has been also used in routine and is considered as powerful serodiagnostic tool for seroepidemiology, especially applied for doubtful cases and allows confirmation of serologic diagnosis obtained by conventional methods (Sompolinsky *et al*., 1986; Raoult & Dasch, 1989b; Raoult & Dasch, 1989a; Raoult & Dasch, 1995). The drawback of ELISA, IFA and adsorbed western blot in routine, is that they require the laboratory platforms specialized in culturing of Rickettsiae and in antigen purification. However, the serologic evidence of infection occurs no earlier than the second week of illness for any of rickettsial diseases (La Scola& Raoult, 1997). In practical, several diagnostic methods are used for Rickettsiae detection. In the specialized laboratories, molecular biology, serodiagnostic with IFA and adsorbed western blot and shell vial culture are used systematically. Because it is difficult to diagnose rickettsial infection early after infection occurs, administration of antibiotic treatment before a definitive diagnosis is made (Pelletier & La, 2010). Preventive measures are complicated because of the lack of effective and safe rickettsial vaccines (Walker, 2007). To detect

efficiently bacteria in clinical samples, we need to dispose of highly sensitive, specific and available detection tests.

The aim of the present work was to propose an efficient diagnostic test based on recombinant proteins for detection of *R. prowazekii* and *R. rickettsii*. To realize our purpose, first we have selected for in vivo expression 45 and 48 genes targets of *R. prowazekii* and *R. rickettsii*, respectively. From this selection, we have successfully attempt to express about 50% of targets using Gateway technology (Vincentelli R *et al*., 2011). Finally, we have screened 20 of all these recombinant proteins by ELISA and selected discriminate markers of *R. typhi* and *R. conorii* infection, respectively which may be useful for detection of Rickettsiae in clinical samples.

MATERIAL & METHODS

2.1) Choice of protein targets for cloning and expression

The choice of protein targets was defined according to previous studies showing an important role of rickettsial proteins which can be detected by human antibodies (Renesto *et al*., 2005; Renesto et al., 2006), as well as, proteins involved in physiopathological processes: RickA (Balraj et al., 2008; Balraj, Nappez, Raoult, & Renesto, 2008), rOMPB, rOMPA, adr2 (Renesto *et al*., 2006) which therefore offer opportunities for their application in medical diagnosis/vaccine and subsequent studies (Table1). This list of genes to be cloned was subsequently enlarged for *R. prowazekii* and *R. rickettsii* because of the low success rate. Indeed, in the first series of targets (13 target for *R. prowazekii* and 12 targets for *R. rickettsii*) to be cloned, respectively 6 and 3 clones have been obtained for these pathogens (SM1) (Vincentelli R *et al*., 2011). Since the cloning and protein expression of intracellular bacteria such as Rickettsiae cause problems in case of membrane proteins, insoluble and soluble form etc,

so we decided to select the majority of soluble target (SM1). The nucleic acid sequences of ORFs were extracted from genomic library (NCBI). The predicted signal peptide (http://bp.nuap.nagoya u.ac.jp / sosui / sosuisignal / SOSUIsignalDB /) sequence was removed.

2.2) Construction and identification of recombinant expression plasmids

DNA of *R. prowazeki* strain *Madrid E* and *R. rickettsii* strain *Sheila Smith* was extracted using commercially available kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions. Twenty targets were subsequently PCR amplified (Expand High Fidelity PCR System, Roche Diagnostics, Meylan, France) using specific primers containing at their 5' and 3' ends the respective attB1 and attB2 recombination sites. Each purified PCR product was transferred according to manufacturer's instructions (Gateway Cloning Technology/Invitrogen Life Technologies) in a first recombination step (BP) into the pDONR201 vector to generate an entry clone used in a second recombination step (LR) with the destination Gateway vector pETG-20A to generate expression clones contain an N-His6 tag plus a fusion protein thioredoxin (TRX) (Canaan et al., 2004; Vincentelli R *et al*., 2011) that enhances expression of the fusion partner (Vincentelli et al., 2003; Vincentelli, Canaan, Offant, Cambillau, & Bignon, 2005; Vincentelli R *et al*., 2011). The resulting entry and expression clones were transformed into E. coli DH5α cells, and constructions were confirmed by DNA sequencing and PCR screening, respectively.

2. 4) Expression and purification of recombinant proteins

All steps of expression and purifications were performed as previously described (Sekeyova *et al*., 2010; Vincentelli R *et al*., 2011). Briefly,

expression vectors carrying the 20 targets were transformed into *E. coli* strain Rosetta (DE3) pLysS (Novagen). The growth conditions, induction and harvest was done as previously described (Vincentelli R *et al*., 2011). The bacterial pellet was resuspended in lysis buffer (50mM Tris-HCl pH 8.0, 300mM NaCl, 0.1% Triton X-100, 1mM ethylenediaminetetraacetic acid [EDTA], 0.25 mg/ml lysozyme and 1mM phenylmethylsulphonyl fluoride [PMSF]) and frozen - 80°C for at least 1 hour. After thawing the bacterial pellets and the addition of DNAse I (2µg/ml) and MgSO4 (20 mM) the lysed cells were centrifuged to separate the soluble fraction from the bacterial debris. The pellet was used for subsequent steps of purification. The proteins were purified by affinity chromatography based on the affinity of the Histidine tag (HHHHHH) with Nickel ions. The pellet fraction of the lysate was solubilised in buffer A (50mM Tris-HCl, 300mM NaCl, 250mM Imidiazole pH 8.0) containing 8M GnHCl and centrifuged to separate the supernatant containing the recombinant proteins and pellet with the cellular debris. The solubilzed proteins were loaded on a Nickel affinity chromatography Histrap (GE Healthcare) and eluted in denaturant condition in the buffer B (buffer A + 6M urea, imidazole 250mM, pH 8.0). The fractions containing proteins were pooled and stored in 50% glycerol at -20°C. Total expression was visualized by SDS-PAGE according to standard protocols (Cleveland, Fischer, Kirschner, & Laemmli, 1977; Towbin, Staehelin, & Gordon, 1992). The identity of recombinant protein was confirmed by mass spectrometry.

2.5) ELISA

Modified ELISA assay was performed as previously described (Sekeyova *et al*., 2010). Briefly, 96 well plates (immunolon4, vwr) were coated overnight at +4°C with purified recombinant protein (10 µg/ml, 100µl per

well) diluted in carbonate-bicarbonate buffer (15mM Na2CO3, 35mM NaHCO3, pH 9.6). The following steps were performed according to standard protocols (Sekeyova *et al*., 2010). The human sera were diluted 1/1000 in PBST-milk. Alkaline phosphatase-conjugated goat anti-human IgG (whole molecule) (Sigma) (1/5000), alkaline phosphatase yellow para-nitro-phenyl phosphate (pNPP) (Sigma) were used as described (Sekeyova *et al*., 2010). The reaction was read with a microplate reader (Multiskan EX, Labsystems, Thermo Fisher Scientific, Waltham, MA) at a wavelength of 405 nm and data analysed by GraphPad Prism (San Diego, CA). A positive control consisted in positive serum with active R. typhi and *R. conorii* infection; a negative control consisted in negative serum. Each serum sample was tested at least in duplicate. The cut-off was determined as described (Sekeyova *et al*., 2010). Any samples exhibiting absorbance above the cut off value was considered as positive (Figure 1A&B).

2.6) Human sera

In this study, 10 patients sera (group *R. typhi*) with an infection due to *R. typhi* and 28 sera the patients diagnosed for active *R. conorii* (group R. conorii) infection diagnosed at the Unité des Rickettsies
(Marseille, France) were included in this study after giving informed consent (Table2). The diagnosis was based on serology and PCR assays targeting (Socolovschi *et al*., 2009). A control group (group HBD) consists in 10 healthy blood donors**.**

3) RESULTS AND DISCUSSION

Here, we describe the tools for detection of Rickettsiae in clinical samples using recombinant rickettsial proteins. In this aim, we produced 20 and 23 recombinant proteins of *R. prowazekii* and *R. rickettsii* with 20 which we used for ELISA. Finally, we screen them for the best serodiagnosticmarkers for *R. typhi* and *R. conorii* discriminate serodiagnosis.

Selection of genes targets for protein expression

Initially, 12 and 13 protein targets have been selected for cloning and expression of *R. prowazekii* and *R. rickettsii*, respectively (Table 1, SM1). However, the success rate of cloning and expression was very low (46% and 25%, respectively). *Rickettsiae* are obligatly intracellular bacteria, their genetic manipulation is strongly limited in these conditions (Renesto, Ogata, Audic, Claverie, & Raoult, 2005). The first attention was to choose the genes described as immunogenic for patient's or immunized animal's sera (Table 1, SM1). Consequently, the list of targets was enlarged taking care to remove highly hydrophobe-, membrane proteins of high MW known to be difficult to manipulate. This choice was determined by technical limitations and it is controversial when considering that the majority of immunogenic proteins are surface proteins, described as sca family proteins within rickettsial species, i.e., rOmpB ubiquitous in all Rickettsiae and rOmpA presents only in SFG group.

Indeed, the genes sca5 (rOmpB) and rOmpA are also used in diagnosis by PCR (Parola, Paddock, & Raoult, 2005). They are the most reacting proteins in adsorbed western blot. This study opened the opportunity to screen for diagnostic usage other not yet known in clinics the protein targets (SM1). In the context of growing interest of synthetic gene

synthesis, optimization of sequence for codon usage which has been identified as the single most important factor in prokaryotic gene expression (Lithwick & Margalit, 2003). Therefore, the improved *E. coli* strain for codon usage (Rosetta BL21 pLysS) was used. However, an analysis of codon usage remains to be performed. Most frequently, the problems of protein expression occur after cloning showing mutation or another unknown phenomenon (Vincentelli *et al*., 2005). A low yield of expression may probably be due to their cellular toxicity or another of numbered parameters required for successful protein expression.

ELISA a diagnostic tool for detection of rickettsioses

Two species of the typhus group, *R.typhi* and *R.prowazekii,* are pathogenic for human beings. *R. typhi* causes murine typhus (MT), a flea-transmitted disease that occurs in warm climates (Bechah, Capo, Mege, & Raoult, 2008). *R. prowazekii* is responsible for epidemic typhus (ET), a disease of cold months when poor sanitary conditions are conductive to lice proliferation (Bechah *et al*., 2008). ET was thought to be a sporadic disease (Bechah *et al*., 2008), but now is considered as a re-emerging due to its increasing prevalence during political conflicts associated with large human migration i.e. camps of refugees associated with breakdown of social conditions (Gillespie, Ammerman, Beier-Sexton, Sobral, & Azad, 2009) and variation in ecology of rat-flea cycle of R. typhi infection in North and Central America, involves commensal rats, opossum, cat flea (Gillespie *et al*., 2009). Outbreaks of MT were reported in Africa, Australia, Thailand, China, Kuweit, Spain and Portugal, but it remains often unrecognized in Africa (Mouffok, Parola, & Raoult, 2008) and in South-West Asia (Niang *et al*., 1998; Watt & Parola, 2003). The cohort of patients infected by R. typhi in the present study is only n=10. Considering infection due to *R. typhi* as sporadic in Europe (Bechah *et*

al., 2008), our cohort represents 1 year collection of patients diagnosed in Rickettsial Diagnosis Reference Unit, Marseille, France. Almost all of our patients are imported MT from a travel from endemic zones (Parola, Davoust, & Raoult, 2005; Bitam *et al*., 2009). Even if prevalence of MT is worldwide, remains under diagnosed because of unspecific clinical symptoms. Not all (<50% of cases) of patients from our study presents rash (Table 2) considered as hallmark of rickettsial diseases often transient or difficult to observe. Athralgia, myalgia, or respiratory and gastrointestinal symptoms, as well as, neurologic signs may also occur. Thus, the clinical picture of MT can be confounded with other diseases (Azad, 1990). Moreover, the clinical features, such as fever, rash, regional lymphoadenopathy are commonly present in tick-borne rickettsioses, i.e. MSF caused in the Mediterranean area and Europe by *R.conorii*. The main clinical difference between MT and MSF is presence of coetaneous eschar following the tick bite in *R. conorii* infected patients. However, in the absence of eschar, the clinics of both, MT and MSF can be easily confounded. Serologic tests are the most frequently used and widely available methods for diagnosis of rickettsioses (La & Raoult, 1997; Shepard *et al*., 1976). However, the cross reactions between the different rickettsial species make discrimination difficult. Adsorbed western-blot is very helpful in diagnosis (SM2). In the present study, the results of western blot were decisive in establishment of diagnosis and were performed for the majority of patients diagnosed in our laboratory. When the non adsorbed WB doesn't allow concluding, then adsorbed WB is performed (SM2). In some cases, WB performed on absorbed serum (SM2 (F, H)) hardly contributes to diagnosis. Finally diagnosis is based on clinics and several diagnostic tests. To facilitate the discrimination between *R. typhi* and *R. conorii* patients, we screened in this aim 20 recombinant proteins (10 of *R. prowazekii* (group typhus) and

10 of *R. rickettsii* (group MSF)). Four recombinant proteins were found to cross-react with both, sera infected with *R. typhi* and *R. conorii* (Table 3). This result is not surprising because they belong to well conserved bacterial proteins: groEL, Adr2, murC and EF-Tu (Table 3). Adr2 is ubiquitously presents within Rickettsiae and acts as one of putative ligand recognized by host cell surface proteins. Rickettsial entry into the host cell is mediated by the rOmpB protein, which attaches to the host cell receptor Ku70, a component of the DNA-dependent protein kinase (Uchiyama, Kawano, & Kusuhara, 2006; Uchiyama *et al*., 2006). Several tested in this format recombinant protein were discriminate for diagnosis of infection due to *R. typhi*, despite their origin: targets from *R. prowazekii* (RP016, groEL, RP173), as well as, targets from *R. rickettsii* (PLD, Sca10, EF-Tu, A1G_00215) supporting already documented the cross-reactivity among Rickettsia species. Interestingly, among these targets we found Sca10 protein belonging to a large family of outer membrane proteins known as the surface cell antigen (Sca) family proteins and PLD involved in rickettsial adherence and invasion of Vero cells. However, these results may be underestimated considered a small cohort of *R. typhi* infected patients. Thus, it will be suitable to validate these diagnostic targets on larger study population. None individual protein could be enough discriminate for diagnosis of SFG, except three targets already used for diagnosis of both, MT and MSF: groEL, adr2 and EF-Tu .Considered the results, diagnostic test by ELISA can be interesting to use in routine, because of rapidity of its realization, low cost and possible development of high put screening which required only small amount of patient's sera (less than 1µl). However, ELISA will be recommended to be use in parallel with IFA reference method and adsorbed western-blot which is more sensitive and allows an earlier diagnosis than IFA. The main drawback of adsorbed western blot is time-

consuming, required large amount of both: patient's serum samples and bacteria. Moreover, the specific choice of bacteria to be tested is based on patient's anamnesis and the epidemiological data. Optimization of recombinant proteins based ELISA may be an interesting alternative for diagnosis of rickettsial diseases.

Conclusion

This study was designed for production of recombinant proteins useful for Rickettsiae detection in clinical samples. We have successfully cloned and expressed 43 rickettsial proteins. Finally, we selected several promising antigenic markers of *R. typhi* and *R. conorii* infection by using ELISA which can be alternative method for rickettsial diseases diagnosis. Despite interest of this test in clinical routine, the larger panel of sera should be tested before and not yet tested recombinant proteins remain to be screened.

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Abbreviations:

RT, *R. typhi*, RP, *R. prowazekii*, RC7, *R. conorii* strain Malish7, RMAS, *R. massiliae*, RAES, *R. aeshlimanii*, RF, *R. felis*;

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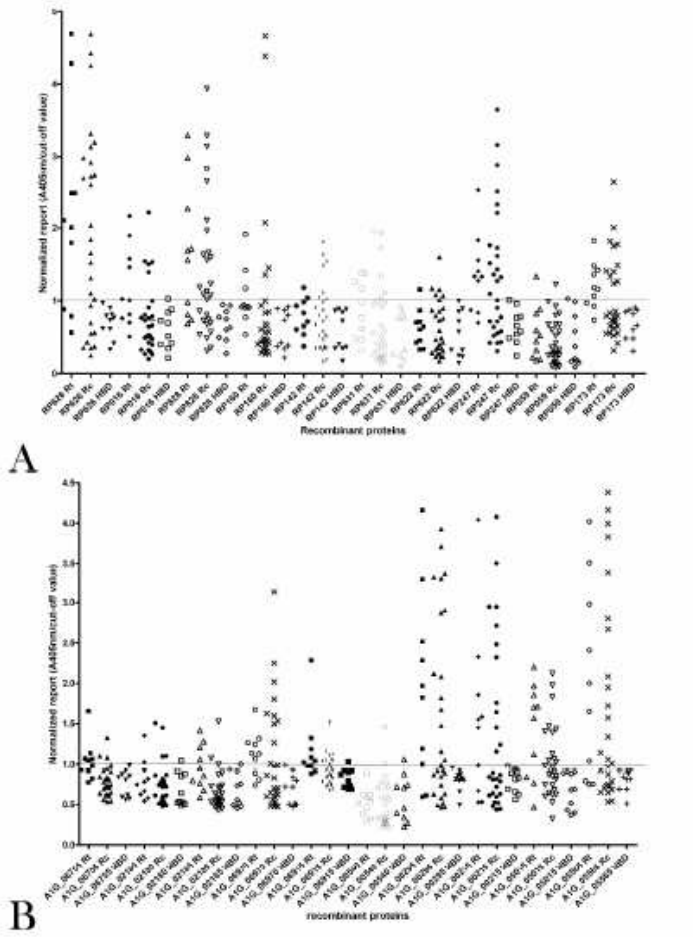
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Figure 1

The reactivity of sera from patients infected by *R. typhi*, *R. conorii*, as well as sera from blood donors (control group) against the various recombinant proteins were tested by ELISA. The different recombinant proteins used as antigens are listed along the x axis (A. The recombinant proteins of *R. prowazekii*, strain MadridE; B. recombinant proteins of *R. rickettsii* strain Sheila Smith) The axis y shows the normalized results (unity of absorbance A405/cut-off measured for each antigen). The points of scatter which exhibit the value of report >1 are considered as positive. The cut264 off value was defined as mean ±1.5 SD of A405 value obtained with control group.



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Table 1
Orthologs of *R.conorii* in *R.prowazekii* and *R.rickettsi*-selection of protein targets for cloning and expression based on immunoproteomic studies

	R. conorii	gene	*R. prowazekii*	*R. rickettsii*
1	RC0019	sca1	RP016 RP017 RP018	A1G_00130
2	RC0178	RplA	RP137	A1G_01020 (rplA)
3	RC0184	pepA	RP142	A1G_01050
4	RC0233	dnaK	RP185	A1G_01335 (DnaK)
5	RC0300	unknown	-	A1G_01695
6	RC0397	unknown	-	-
7	RC0968	GroEL	RP626	A1G_05315
8	RC1008	Tuf	RP661	A1G_05565
9	RC1085	rOmpB	RP704	A1G_06030
10	RC1234	atpC	RP800	A1G_06755 (atpC)
11	RC1266	maf	RP815	A1G_06950
12	RC1281	adr1	RP827	A1G_07045
13	RC1282	adr2	RP828	A1G_07050

Table 2
Base-line characteristics of the 48 subjects included in this study

Characteristics	Cases	Cases	Control group
	***R. typhi* infected subjects**	***R. conorii* infected subjects**	**Healthy blood donors (HBD)**
	n=10	n=28	n=10
Demographic factors			
Sex			
Male	6	21	No data available
Female	4	7	No data available
Age (years) (Mean ±SD)	41.7±9	52.5±16.9	No data available
Age (years) male (Mean ±SD)	45.7±9.3	50.4±16	No data available
Age (years) female (Mean ±SD)	35.75 ±4.3	58.7±20	No data available
Diagnosis	clinical picture, serology, WB, molecular biology		

Table 3

Test-operating parameters of 20 recombinant proteins included in the present study

		R.typhi						*R.conorii*						*R.typhi & R.conorii*						Marker of rickettsiose
protein name	**locus_tag**	**Se**	**Sp**	**VPP**	**VPN**	**L**	**Λ**	**Se**	**Sp**	**VPP**	**VPN**	**L**	**Λ**	**Se**	**Sp**	**VPP**	**VPN**	**L**	**Λ**	
***R.prowazekii* ME**																				
groEL; 60 kD chaperonin	RP626	70	100	100	77	-	0.3	68	100	100	53	-	0.321	68.4	100	100	45.45	-	0.316	Marker *R.typhi&R. conorii*
Cell surface antigen Sca1 (SPLIT GENE)	RP016	60	90	86	69	6	0.44	18	90	83.3	28	1.785	0.913	29	90	92	25	2.9	0.79	Marker *R.typhi*
Putative outer surface protein (Adr2)	RP828	60	100	100	71	-	0.4	61	100	100	48	-	0.39	60.5	100	100	40	-	0.39	Marker *R.typhi&R. conorii*
DOMAINE PFAM 447-768 omp1	RP160	30	100	100	59	-	0.7	18	100	100	30	-	0.82	21	100	100	25	-	0.79	
Aminopeptidase A [EC:3.4.11.1](pepA)	RP142	20	100	100	55.55	-	0.8	25	100	100	32.25	-	0.75	23.7	100	100	25.64	-	0.76	
hypothetical protein RP631	RP631	21	100	100	62.5	-	0.6	21	100	100	31.25	-	0.785	26	100	100	26	-	0.74	
3-demethylubiquinone-9 3-methyltransferase	RP622	10	100	100	53	-	0.9	21	100	100	31.25	-	0.785	18	100	100	24	-	0.82	
UDP-N-acetylmuramate--L-alanine ligase	RP247 (murC)	70	90	87.5	75	7	0.33	57	90	94	43	5.71	0.48	60.5	90	96	37.5	6.05	0.44	Marker *R.typhi & R. conorii*
stage 0 sporulation protein J (spo0J)	RP059	10	90	50	50	1	1	3.6	90	50	25	0.36	1.07	5.3	90	67	20	0.53	1.05	
signal recognition particle protein	RP173	70	100	100	77	-	0.3	39	100	100	37	-	0.67	47	100	100	33	-	0.53	Marker *R.typhi*
***R. rickettsii* Sheila Smith**																				
F0F1 ATP synthase subunit epsilon	A1G_06755 (atpC)	50	100	100	67	-	0.5	11	100	100	28.5	-	0.89	21	100	100	25	-	0.79	
hypothetical protein A1G_02180 (VapC1)	AG1_02180 (VapC1)	20	90	67	53	-	0.89	14	90	80	27	1.43	0.95	16	90	86	22	1.58	0.935	
hypothetical protein A1G_02185 (VapB1)	A1G_02185 (VapB1)	40	90	80	60	4	0.67	7	90	67	26	0.71	1.03	16	90	86	22	1.58	0.935	
hypothetical protein A1G_06970 (PLD)	A1G_06970 (PLD)	70	100	100	77	-	0.3	36	100	100	36	-	0.64	45	100	100	32	-	0.55	Marker *R.typhi*
cell surface antigen-like protein Sca13	A1G_06915 (Sca13)	0	90	0	47	0	1.11		90	0	24	0	1.11	0	90	0	16	0	1.11	
soj protein	A1G_00540	0	90	0	47	0	1.11	7	90	67	26	0.71	1.03	5	90	67	20	0.53	1.05	
cell surface antigen-like protein Sca10	A1G_00295 (Sca10)	70	100	100	77	-	0.3	50	100	100	42	-	0.5	55	100	100	37	-	0.45	Marker *R.typhi*
dihydrofolate reductase	A1G_00215	60	100	100	71	-	0.4	43	100	100	38	-	0.57	47	100	100	33	-	0.52	
Hypothetical protein A1G_05015 (RickA)	A1G_05015 (RickA)	70	100	100	77	-	0.3	39	100	100	37	-	0.61	47	100	100	33	-	0.52	Marker *R.typhi*
elongation factor Tu	A1G_05565	70	100	100	77	-	0.3	53	100	100	43	-	0.46	58	100	100	38	-	0.42	Marker *R.typhi&R. conorii*

Test-operating parameters :
Sensitivity (Se) = positive result with patients/total number of patients
Specificity (Sp) = negative result with control group/total number of individual blood donor
Positive predictive value (PPV) = TP/(TP+FP); true positive (TP), false positive (FP)
Negative predictive value (NPV) = TN/(TN+FN); true negative (TN), false negative (FN)
L = Se/(1-Sp) = (TP/patients) (FP/individual blood donor)
Λ = (1-Se)/Sp = (FN/patients) (TN/individual blood donor)

For Peer Review

Supplementary Material
SM1
Listing of all rickettsial ORFs selected for cloning and expression. The table is divided onto *R. rickettsii* and *R. prowazekii* targets. In grey is presented the first series of experiments targeting immunoreactive proteins.

	Protein name	locus_tag	Strain	Expression &purification	ELISA	peptide signal or THM
1	cell surface antigen	A1G_00130	RR Sheila Smith	-		MNKLTEQHLLKKSRFLKYSLLASIAVGAAIPFE
2	50S ribosomal protein L1	A1G_01020 (rplA)	RR Sheila Smith	-		
3	leucyl aminopeptidase	A1G_01050	RR Sheila Smith	-		
4	molecular chaperone DnaK	A1G_01335 (DnaK)	RR Sheila Smith	-		
5	hypothetical protein A1G_01695	A1G_01695	RR Sheila Smith	-		
6	chaperonin GroEL	A1G_05315	RR Sheila Smith	-		
7	elongation factor Tu	A1G_05565	RR Sheila Smith	-	tested	MAKAKFERTKPHVNIGTIGHVDHGKTSLTAAITIVLAKTGGAQA
8	outer membrane protein B (cell surface antigen sca5)	A1G_06030	RR Sheila Smith	-		MAQKPNFLKKLISAGLVTASTATIVASFAGSAMGAAI
9	F0F1 ATP synthase subunit epsilon	A1G_06755 (atpC)	RR Sheila Smith	+	tested	MNATILVKIITPLSIA
10	Maf-like protein	A1G_06950	RR Sheila Smith	+		
11	hypothetical protein A1G_07045	A1G_07045	RR Sheila Smith	+		MKKLLLIAAASTALLTSGLSFA
12	hypothetical protein A1G_07050	A1G_07050	RR Sheila Smith	-		MKKLLLIAATSATILSSSVSFA
				3/12=25%		
13	outer membrane assembly protein (asmA)	A1G_02675	RR Sheila Smith	-		THM : KYSLIIFISIILLLIVIPFFIPL
14	asmA DOMAINE 1-705 aa	A1G_02675	RR Sheila Smith	+		THM : KYSLIIFISIILLLIVIPFFIPL
15	hypothetical protein A1G_06970 (PLD)	A1G_06970 PLD	RR Sheila Smith	+	tested	THM : NNKFIEISIAFILGIALGI
16	hypothetical protein A1G_02185 (VapB1)	A1G_02185 VapB1	RR Sheila Smith	+	tested	
17	hypothetical protein A1G_02180 (VapC1)	AG1_02180 VapC1	RR Sheila Smith	+	tested	THM1 : MGLIIDTAIIIALER THM2 : GQTYISPIVLTELLIGVDR THM3 : KCLAFIEYVKSLFTILPFGIEEV
18	hypothetical protein A1G_02181 (VapC2)	AG1_02180 VapC2	RR Sheila Smith	+		
19	hypothetical protein A1G_07220 (VapC3)	AG1_07220 vapC3	RR Sheila Smith	-		
20	bifunctional N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase/tRNA (m7G46) methyltransferase	A1G_07200	RR Sheila Smith	+		
21	cell surface antigen-like protein Sca13	A1G_06915 Sca13	RR Sheila Smith	+	tested	
22	cell surface antigen-like protein Sca10	A1G_00295 Sca10	RR Sheila Smith	+	tested	
23	O-sialoglycoprotein endopeptidase	A1G_00390	RR Sheila Smith	+		
24	cell surface antigen-like protein Sca8	A1G_01440	RR Sheila Smith	+		
25	cell surface antigen-like protein Sca8	A1G_01445	RR Sheila Smith	-		
26	scaffold protein	AG1_04120	RR Sheila Smith	+		
27	O-antigen export system ATP-binding protein RfbE	A1G_00015	RR Sheila Smith	+		
28	Mrp protein	A1G_00940	RR Sheila Smith	+		
29	UDP-3-O-	A1G_00045	RR Sheila Smith	+		
30	UDP-N-acetylglucosamine acyltransferase	A1G_00035	RR Sheila Smith	+		
31	dihydrofolate reductase	A1G_00215	RR Sheila Smith	+	tested	
32	folate synthesis bifunctional protein	A1G_00225	RR Sheila Smith	+		
33	Sco2 protein precursor	A1G_00265	RR Sheila Smith	+		THM : IIKIFIALAMITGIIFLCLLYSS
34	soj protein	A1G_00540	RR Sheila Smith	+	tested	
35	stage 0 sporulation protein J	A1G_00545	RR Sheila Smith	-		
36	putative inner membrane protein translocase component YidC	A1G_00475	RR Sheila Smith	-		THM1 : NIINLIAAIILSLSIIFGWQYFV THM2 : AIDFGWFYIITKPVFYAMNFFYG THM3 : NFGVSILIVTVIIKLLMFTLANK THM4 : AGCLPILVQIPVFFSIYKVLYVT

37	S-adenosyl-methyltransferase MraW	A1G_04755	RR Sheila Smith	-		
38	penicillin-binding protein	A1G_04745	RR Sheila Smith	-		
39	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	A1G_04875	RR Sheila Smith	-		
40	3-deoxy-D-manno-octulosonic-acid transferase	A1G_00700	RR Sheila Smith	-		THM : YYALSFILLPVYFIIILIRLLIG
41	antitoxin of toxin-antitoxin system	A1G_04925	RR Sheila Smith	-		MAIFMTVITNRISNA
42	peptidoglycan-associated lipoprotein precursor	A1G_06560	RR Sheila Smith	-		MKTKITLAFLALCMLAGCN
43	S-adenosylmethionine synthetase (adometK)	A1G_06605	RR Sheila Smith	-		
44	hypothetical protein A1G_05015 (RickA)	A1G_05015	RR Sheila Smith	+	tested	
45	partie B-peptide de sca5 (A1G_06030)	A1G_06030	RR Sheila Smith	-		
				23 positive/45 =51% of expressed proteins	10/23 tested	15/45 with PS or TM=33% of membrane proteins
	R. prowazekii					
1	Cell surface antigen Sca1 (SPLIT GENE)	RP016	RP Madrid E	-	tested	MNKLTAQNLLKKSRFLKYSLLTSISVGAVMAIPVE
2	Cell surface antigen Sca1 (SPLIT GENE)	RP017	RP Madrid E	-		
3	Cell surface antigen Sca1 (SPLIT GENE)190 KD ANTIGEN PRECURSOR (sca1)	RP018	RP Madrid E	-		
4	50S ribosomal protein L1 (rplA)	RP137	RP Madrid E	+		
5	Aminopeptidase A [EC:3.4.11.1](pepA)	RP142	RP Madrid E	+	tested	
6	DnaK	RP185	RP Madrid E	-		
7	groEL; 60 kD chaperonin	RP626	RP Madrid E	+	tested	MAKAKFERTKPHVNIGTIGHVDHGKTSLTAAITIILAKTGGA KA
8	Elongation factor EF-Tu (tuf)	RP661	RP Madrid E	+		MAKAKFERTKPHVNIGTIGHVDHGKTSLTAAITIILAKTGGA KA
9	ompB, sca5; Outer membrane protein rOmpB	RP704	RP Madrid E	-		MAQKPNFLKKIISAGLVTASTATIVAGFSGVAMGAAM
10	atpC; ATP synthase epsilon chain [EC:3.6.1.14]	RP800	RP Madrid E	-		
11	maf; Nucleotide-binding protein implicated in inhibition of septum formation	RP815	RP Madrid E	+		
12	Unknow/ADR1	RP827	RP Madrid E	-		MKKLLLIAATSTALLTSGISFA
13	Putative outer surface protein/ADR2	RP828	RP Madrid E	+	tested	PS :MKKLLLIATASATILSSSVSFA *THM :* LLLIATASATILSSSVSFAECID
				6/13=46%		
14	DNA repair protein RECN (recN)	RP182	RP Madrid E	-		
15	patatin B1 precursor (pat1)	RP602	RP Madrid E	-		
16	UDP-N-acetylmuramate--L-alanine ligase	RP247 murC	RP Madrid E	+	tested	
17	) 3-demethylubiquinone-9 3-methyltransferase	RP622	RP Madrid E	+	tested	
18	hypothetical protein RP631	RP631	RP Madrid E	+	tested	
19	adenylate kinase	RP638	RP Madrid E	-		MIVIFLGPPG
20	response regulator PleD	RP237	RP Madrid E	-		
21	) hypothetical protein RP673	RP673	RP Madrid E	+		
22	UDP-3-O- lpxC	RP254	RP Madrid E	-		
23	hypothetical protein RP688	RP688	RP Madrid E	-		THM :SYTQNLLSFKNIIGLMLIIFAGI
24	hypothetical protein RP689	RP689	RP Madrid E	+		SFKNIIGLMLIIFAGILFYAYIL
25	hypothetical protein RP691	RP691	RP Madrid E	-		
26	O-antigen export system ATP-binding protein RFBE (rfbE)	RP003	RP Madrid E	-		
27	capsular polysaccharide biosynthesis protein CapD	RP333	RP Madrid E	-		
28	SOJ protein (soj)	RP058	RP Madrid E	-		
29	stage 0 sporulation protein J (spo0J)	RP059	RP Madrid E	+	tested	
30	preprotein translocase subunit SecB	RP070	RP Madrid E	-		

30	preprotein translocase subunit SecB	RP070	RP Madrid E	-		
31	DOMAINE PFAM 447-768 omp1	RP160	RP Madrid E	+	tested	THM :KIISISKLTILLLTIFYYHISFA
32	minor teichoic acids biosynthesis protein ggab (ggab)	RP339	RP Madrid E	+		MKQNIYSPLVSIIIPVYN
33	signal recognition particle protein	RP173	RP Madrid E	+	tested	
34	hypothetical protein RP789	RP789	RP Madrid E	+		
35	hypothetical protein RP527	RP527	RP Madrid E	-		
36	S-adenosyl-methyltransferase MraW	RP569	RP Madrid E	+		
37	methionyl-tRNA synthetase	RP683	RP Madrid E	-		
38	preprotein translocase subunit SecA	RP0575	RP Madrid E	-		
39	DOMAINES Cterm 606-906 SecA	RP0575	RP Madrid E	+		
40	) translation-associated GTPase	RP604	RP Madrid E	-		MTLKLGIVGLPNVG
41	thioredoxin reductase (trxB1)	RP445	RP Madrid E	+		MKITTKVLIIGSGPAGLSAAIYTAR
42	peptidoglycan-associated lipoprotein precursor (pal)	RP771	RP Madrid E	+		MKTKITLAFLALFMLAGCN
43	HEAT shock protein (hsp22)	RP273	RP Madrid E	-		MLKYIPAIFAIILSSNIA
44	outer membrane assembly protein (asmA)	RP347	RP Madrid E	-		THM : KYSLIIFITIILLLIIIPFFIPL
45	DOMAINE asmA 1-711 aa	RP347	RP Madrid E	-		
46	hypothetical protein RP819 (PLD)	RP819 PLD	RP Madrid E	-		THM :FIAVSISFILGIALGIYVESTYY
47	S -adenosylmethionine synthetase metK (adometK)	RP777	RP Madrid E	-		
48	ompB, sca5; Outer membrane protein rOmpB (sca 5 1353-1643, RP704, partie B-peptide)	RP0688	RP Madrid E	-		
				20/48=42 % expressed proteins	10/22 tested by ELISA	17/48 with PS or THM=35%

43/93=46% iin total expressed proteins

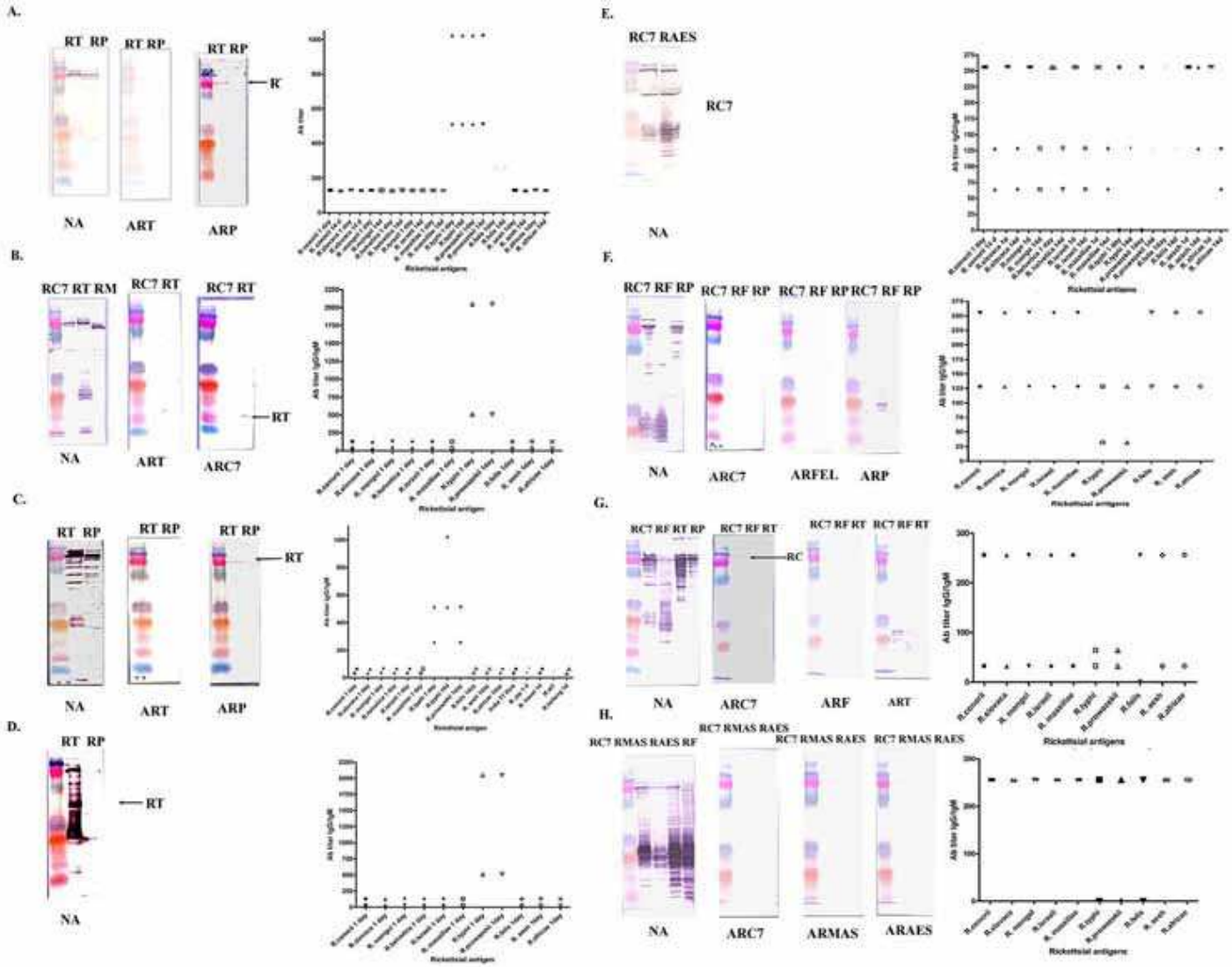
In bold and table in grey : first series of experiment, THM: transmembranary region;

SM2

On the left part of figure (A to D) are shown some examples of cases with *R. typhi* infections.

On the right part of figure (E to H) are shown some examples of cases with *R. conorii* infections. The graphs which display the results from IFA are shown on the right. On the axis X are shown the different rickettsial antigens screened with patient's serum, on the axis Y, are shown the Ab titer IgG/IgM.

The first WB corresponds to primary WB performed with not adsorbed serum of patients. The following WBs, if present, is performed with adsorbed by different rickettsial antigens (chosen according to the clinical context and results of primary WB) sera.


A.
RT RP
RT RP
RT RP
R
NA
ART
ARP
Rickettsial antigen
B.
RC7 RT RM
RC7 RT
RC7 RT
RT
NA
ART
ARC7
C.
RT RP
RT RP
RT RP
RT
NA
ART
ARP
D.
RT RP
RT
NA
E.
RC7 RAES
RC7
NA
F.
RC7 RF RP
RC7 RF RP
RC7 RF RP
RC7 RF RP
NA
ARC7
ARFEL
ARP
G.
RC7 RF RT RP
RC7 RF RT
RC7 RF RT
RC7 RF RT
RC
NA
ARC7
ARF
ART
H.
RC7 RMAS RAES RF
RC7 RMAS RAES
RC7 RMAS RAES
RC7 RMAS RAES
NA
ARC7
ARMAS
ARAES

CONCLUSION
AND PERSPECTIVES

The *Rickettsia* genus is a group of obligate intracellular α-proteobacteria that includes human pathogens responsible for the typhus disease and spotted fevers, and which are associated with arthropods vectors (Raoult and Roux, 1997). Last ten years, the advent of whole genome sequencing has fundamentally improved research in rickettsial pathogenicity. The putative role of some proteins in critical steps of rickettsiae-host cell interactions was highlighted (Walker, 2007; Balraj *et al*., 2009). However, and while these post-genomic investigations contributed to gain a better knowledge about rickettsia pathogenicity, several points remain to be clarified.

The aim of my thesis was to use mAbs as new specific tools to explore rickettsia pathogenicity. Indeed, and as reviewed in the Introduction section, antibodies can indeed be used not only for diagnostic, but also for experimental purpose.

Our first objective was to further characterize rickettsial adhesins Adr1 and Adr2 from *R. prowazekii*. Because the failure to express recombinant Adr1 protein, we focused our investigations on Adr2. Using an overlay assay coupled with mass spectrometry, we first confirmed its role as a bacterial ligand recognized by host cell proteins. Recombinant *R. prowazekii* Adr2 was then expressed through fusion with Dsbc in *E.coli*, purified and concentrated, thus allowing production of specific mAbs, as shown by western blot assays. The capacity of mAbs to inhibit rickettsiae-induced cytotoxicity, firmly demonstrated the role of Adr2 as a virulence factor (**Article 1**). These findings led us to conceive some complementary investigations. Thus, and while we evidenced that inhibition of rickettsiae-induced cytotoxicity occurred *in vitro*, infected animal models could also be used to confirm these results and reinforce the crucial role played by Adr2. In this respect, anti-Adr2 mAbs could be intraperitoneally administered to mice prior the rickettsial challenge.

Non-challenged, anti-Adr2 treated and challenged, untreated mice will be used as controls. Body weight, physical behavior and dealth also be recorded both prior and post- infection (Chan *et al*., 2011). Another main concern to be addressed in the field of rickettsial entry, is to identify the eukaryotic proteins interacting with Adr2. Such investigations could be carried out by yeast two-hybrid approach.

Exploitation of the host-cell actin cytoskeleton is crucial for several microbial pathogens to enter and to disseminate within cells, thus avoiding the host immune response. *R. conorii* has the capacity to use the actin-based motility system for promoting cell-to-cell spreading (Teysseire *et al*., 1992). The RickA protein that contains a domain with homologies with WASP-family proteins was thought to function as a nucleation-promoting factor that directly activates the Arp2/3 complex (Gouin *et al*., 2004; Simser *et al.*, 2005). From *in vitro* actin branching assay performed with recombinant RickA, the involvement of additional bacterial or eukaryotic factors in reorganizing Arp2/3 complex generated Y-branched networks into parallel arrays was also suggested (Jeng et al., 2004). Because genetic manipulations were unfeasible, the role of RickA in the motility of rickettsiae was not formerly demonstrated. Instead, some points remained unclear. Thus, and while RickA was found to be expressed on the bacterial surface, both signal sequence and hydrophobic domain that are respectively required for secretion and membrane anchorage of this protein are lacking (Gouin *et al*., 2004). Moreover, experiments achieved on *R. raoultii* evidenced that the motile phenotype could be dependent on the host cells and unrelated to the level of RickA expression (Balraj *et al*., 2008). The results obtained in our study (**Article 2**) confirm that RickA is expressed over the entire bacterial surface of *R. conorii* and do not exhibits a polarized distribution as other bacterial components known to be responsible for intracellular motility including IcsA, ActA or BimA

(Goldberg et al., 1993; Kocks et al., 1993; Stevens et al., 2005). Our data fit well with the recently published work of Kleba *et al.* (2010). Indeed, these authors took advantage of recent development of mariner-based transposon systems for rickettsia transformation and demonstrated that Sca2 mutant does not produce actin comet tails, suggestive of its role in actin-based motility.

The third part of my research focused on the serodiagnostic test improvement. In diagnostic approach, various methods were replaced by new method which is easier to handle and guaranteed better sensitivity and specificity. For this reason efficient diagnostic test based on recombinant proteins was started. Twenty recombinant proteins targets were screened with patient sera by ELISA. We believe that some of these markers could be helpful in discriminating the infection due to *R. typhi* and *R. conorii* from clinical samples (**Article 3**). While the number of studies involving engineering recombinant proteins is still low, they could offer an interesting alternative to improve the diagnosis of infection with these fastidious microorganisms.

In conclusion, and while clearly experiments remain to be done, I believe that this work has to a better knowledge of the molecular mechanisms involved in rickettsia pathogenicity.

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