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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 *RickA* 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

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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 *et al*., 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

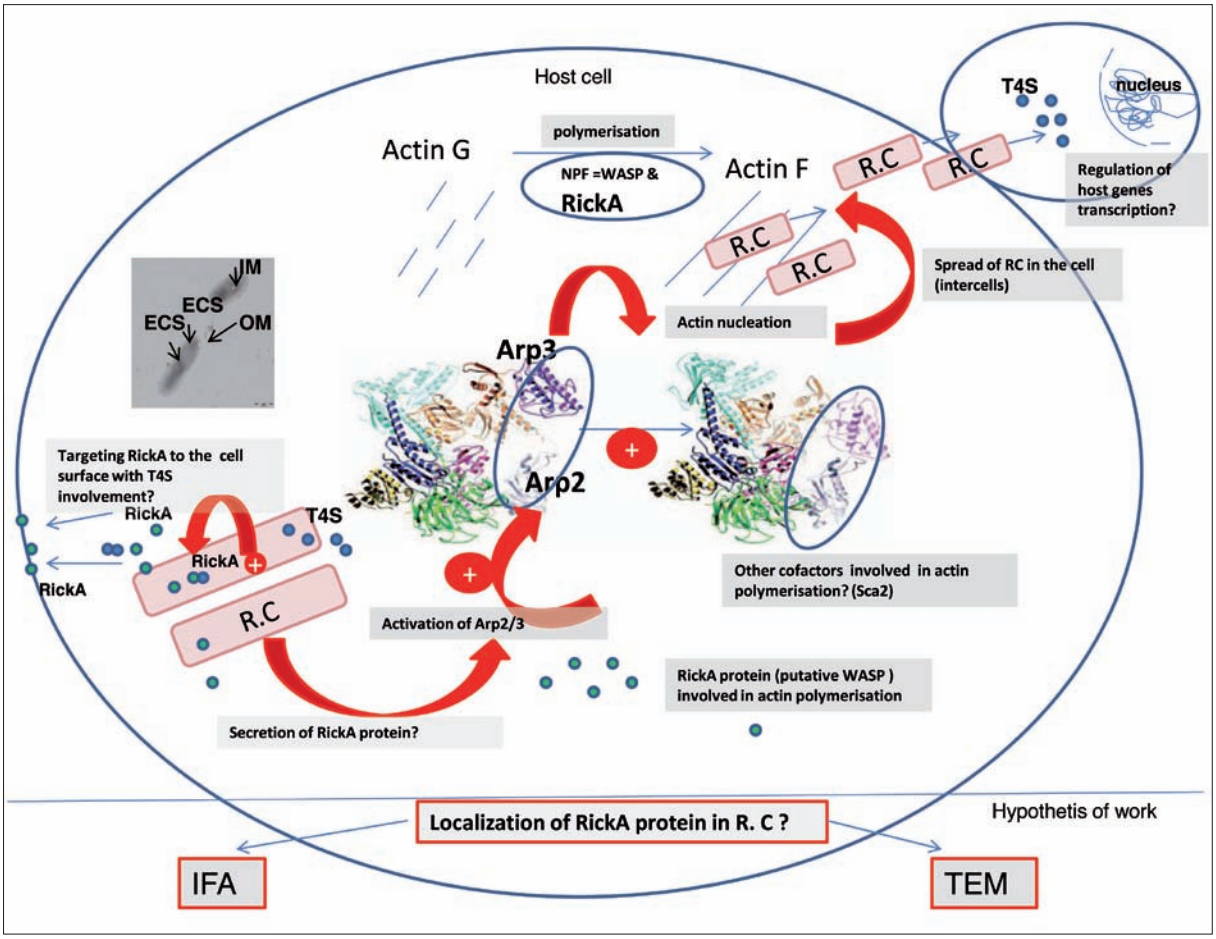

FIGURE 1 - *Summary of RickA protein roles in rickettsial physiopathology. RickA is involved in actin polymerization (transformation of monomeric actin G to filamentous actin F). An initial nucleation step creates free barbed ends by uncapping or severing of filaments or de novo nucleation of monomers. 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 unanswered (grey boxes): 1. RickA protein secretion; 2. How the Arp2/3 complex of actine is activated by RickA; 3. There are other bacterial cofactors involved in actin polymerization; 4. Is the T4S is involved in targeting RickA to the cell surface?; 5. Is T4S is involved in host genes regulation?; 6. How do 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.*

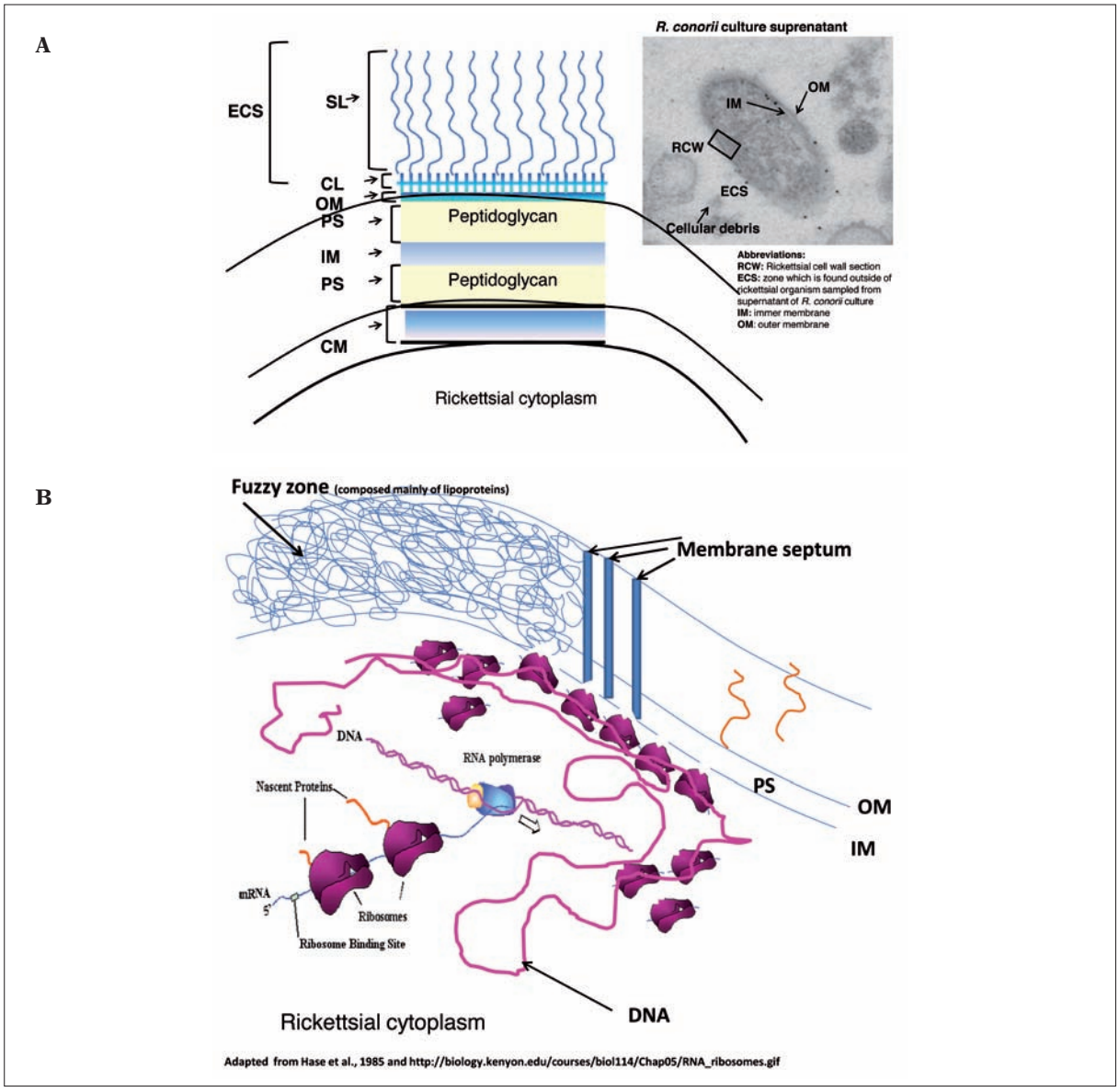

FIGURE 2 - *A) Schema of the cell membrane and B) Model of rickettsial surface membrane assembly. a) Schematic model of TG and SFG Rickettsiae of the cell membrane, outer envelope (cell wall) and adjacent extracellular layers. Rickettsiae are characterized by a specific membrane structure. The outer 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 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 follows: 1. The body of nascent Rickettsia took a definitive form, a fuzzy material mainly composed of lipoproteins is formed over the body, 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 is freely available on internet: http://biology.kenyon.edu/courses/biol114/Chap05/RNA_ribosomes.gif).*

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 strain

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 Eagle s minimum essential medium (MEM, Gibco, Invirtogen, Paisley, UK) supplemented with 4% foetal calf serum (FCS, Gibco) and 1% L-glutamine (Gibco) in 150 cm^2 tissue culture flasks at 32°C 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-GTA-GGC-AAG-TAG-CTA-AGC-AAA-3' and R: 5'-GGA-AGT-ATA-TGG-GAA-TGC-TTT-GAA-3', sonde FAM-GCG-GTT-ATT-CCT-GAA-AAT-AAG-CCG-GCA-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 anti-mouse 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.

TABLE 1 - *The controls included in IFA experiments.*

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

+/- indicates whether the antibody added or not.

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 cm 2 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 NH_4Cl 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), Tween 20 (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 labeling 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*

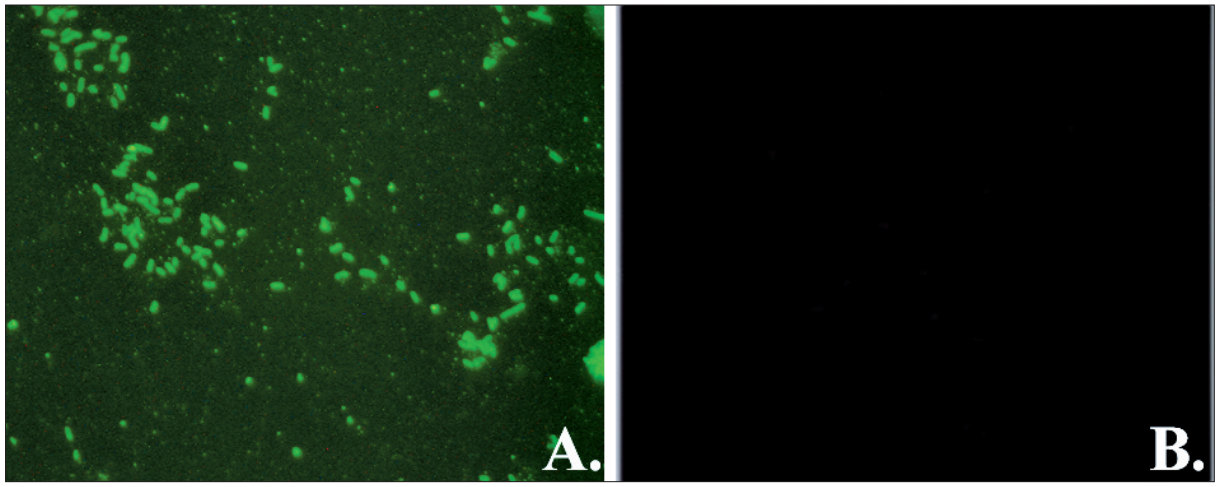

FIGURE 3 - *Detection of surface expression of Rick A protein by indirect immunofluorescence. 2A. Detection of RickA expression by IFA. Host cell-free R. conorii was fixed in methanol, incubated with RickA anti-mouse monoclonal antibody (1:100) followed by an anti-mouse biotin (1:1000), stained with streptavidin FITC (1:500) and visualized by epifluorescence microscopy (magnifications 100X), showed that RickA was expressed at the surface of R. conorii. 1B. the right panel corresponds to negative control.*

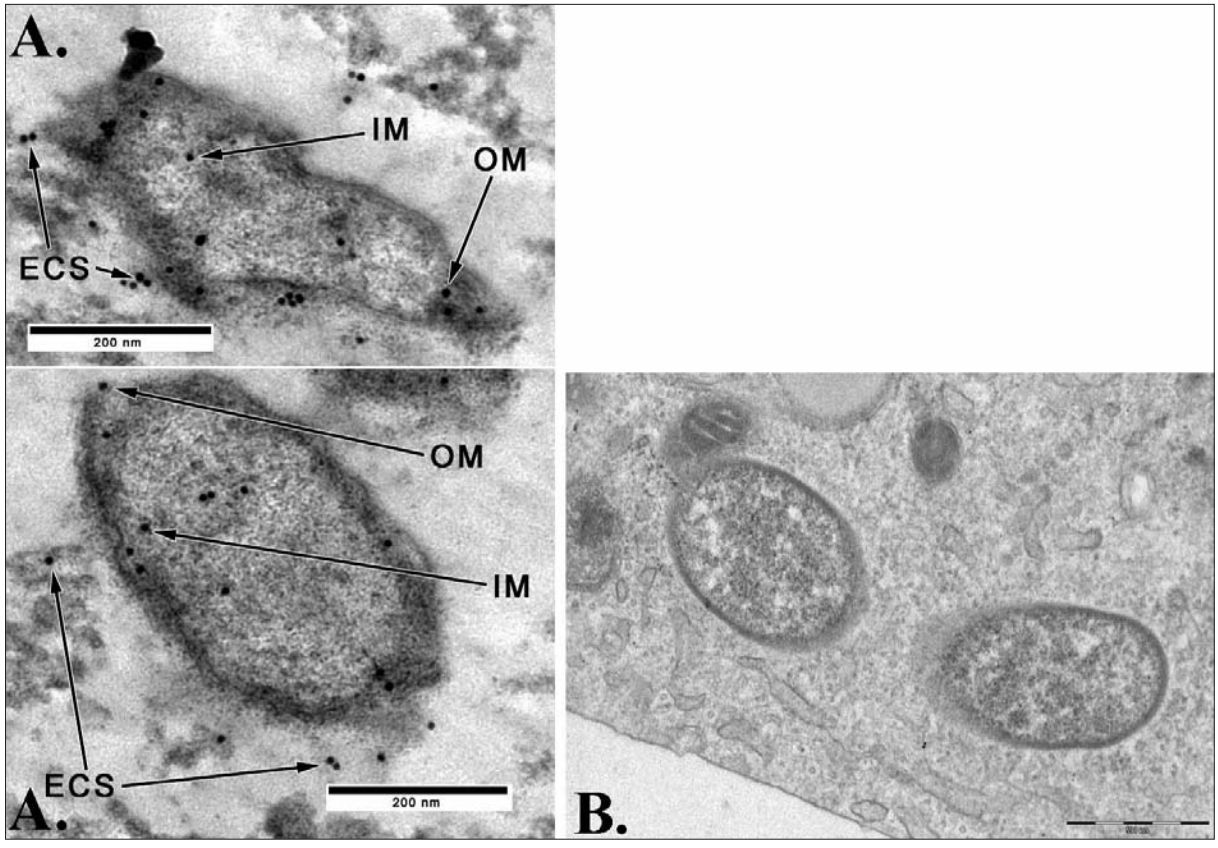

FIGURE 4 - *Localization of RickA (TEM). 3A. TEM analysis performed on R. conorii cultured on L929 cells using RickA anti-mouse monoclonal antibody followed by biotin and with streptavidin gold (10 nm); the arrows indicates the distribution of RickA in R.conorii cells inner membrane (IM), outer membrane (OM) and extracellular space around rickettsies (ECS). 3B. Negative control performed using serum of naive mice.*

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 fila-

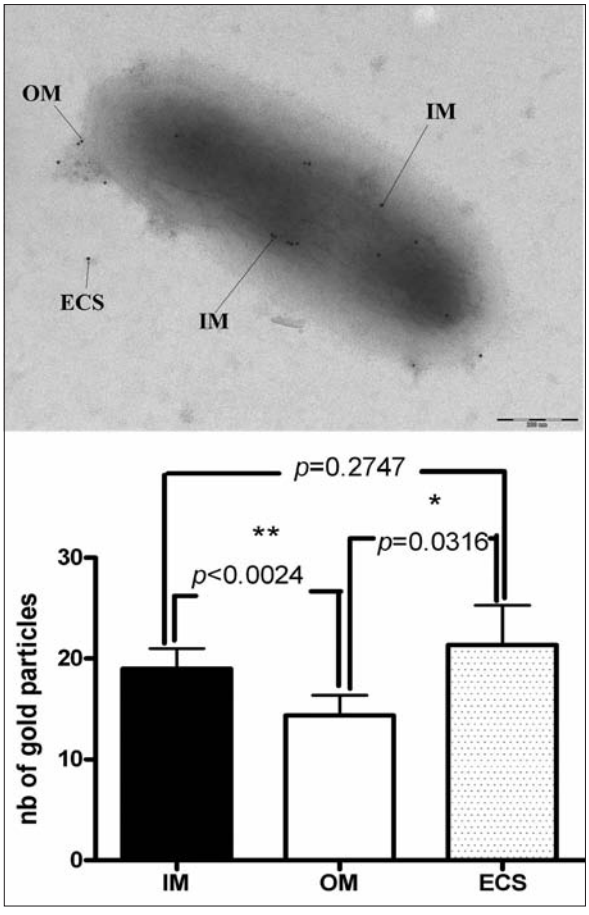

FIGURE 5 - *Histograms showing the distribution of gold particles in R. conorii. Gold particles were counted for one hundred individual fields. The gold particles were localized in inner membrane (IM), outer membrane (OM) and extracellular space around rickettsies (ECS). A graph was plotted by using graphpad prism software.*

ments, 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 its 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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