DETECTION OF IMMUNOGENIC PROTEINS FROM *Rickettsia conorii* AND *Rickettsia africae* BY PROXIMITY LIGATION ASSAY BASED WESTERN BLOTTING (PLA WB)

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ABSTRACT

Rickettsia conorii, the causative agent of Mediterranean spotted fever (MSF) and *Rickettsia africae*, the etiologic agent of African tick-bite fever (ATBF), are strict intracellular bacteria transmitted by ticks belonging to the spotted fever group (SFG) *Rickettsia*. The laboratory diagnosis is based on serology, but remains not enough specific to provide the diagnosis at the species level. Here, we attempted to detecty immunogenic proteins specific from *Rickettsia conorii* and *Rickettsia africae* by proximity ligation assay based Western blotting (PLA WB). In general, we obtained better sensitivity and specificity of bands detection in PLA WB in case of proteins probed with IFA *R. conorii* or *R. africae* positive sera. In parallel, we have screened 20 rickettsial recombinant proteins by ELISA with sera infected by *R. conorii* and *R. africae*, respectively. We identified several potential markers which allowed infection due to *R. conorii* to be discriminated from those due to *R. africae*. Never the less, this technique must be optimized for use in laboratory diagnostic routine.

KEYWORDS: *Rickettsia africae, Rickettsia conorii,* Traditional Western Blot, Proximity Ligation Assay, Recombinant Proteins, Serodiagnosis, Test Operating Parameters

The spotted fever group (SFG) Rickettsia is Gram-negative obligated intracellular bacteria, contains about 25 different species including *Rickettsia africae* and *Rickettsia conorii* (Eremeeva and Dasch 2015). *Rickettsia africae* is responsible for the African tick bite fever (ATBF), transmitted by ticks of the *Amblyomma* genus, endemic in sub-Saharan Africa and the French West Indies (Parola and Paddock, 2012) (Parola *et al.*, 1998). *R. conorii,* the agent of Mediterranean spotted fever transmitted by the brown dog tick *Rhipicephalus sanguineus*, is endemic in the Mediterranean area and in a few sub-Saharan countries (Parola *et al.*, 2009) (Merhej *et al.*, 2009). Actually, the clinical manifestations of these diseases show common symptoms such as flu-like manifestations (fever, chills, myalgia, and headache) (Parola and Paddock, 2012). The laboratory diagnosis of rickettsioses is based onserological testing (La and Raoult, 1997), cell cultureand molecular techniques (La and Raoult, 1997). Diagnosis by real-time PCR and PCR suicide is rapid and sensitive (La and Raoult, 1997). The genes most commonly used for detection are the *16S rRNA*, the *citrate synthase* (*gltA),* *rOmpA* and *rOmp,* respectively (Roux *et al.*, 1997) (Renvoise *et al.*, 2012). Recently, real-time PCR gain sensitivity and specificity by using new generation primers (Renvoise *et al.*, 2012). Molecular detection was simplified by using directly the eschar swabs samples (Mouffok *et al.*, 2011). ELISA (Fuller Laboratories) methods are also currently available for non-referenced laboratories which are focused on more specific reactivities to the lipopolysaccharide (LPS) antigens (IgG screening) and rOmpB protein antigens (IgG/IgM). To date, serological testing only allows identification of Rickettsiae at the group or clade level. Western blot and antigen adsorption have been also used in routine procedures allowing confirmation of serologic diagnosis obtained by conventional methods (Renvoise and Raoult, 2009). However, the major limitation that the cross-adsorption presents is the large amount of antigen needed and that the technique is not always successful (La and Raoult, 1997). Diagnosing rickettsioses is still a great challenge. Research has been focused on new diagnostic applications that can increase sensitivity and specificity.

In this context, the objective of our work isto increase the sensitivity and specificity of protein detection. We used the proximity ligation assay based on western Blotting (PLA WB) for the first time to detect *R. conorri* and *R. africae* proteins. The proximity ligation assay (PLA) allows powerful localized signal amplification. This technique based on dual antibody recognition offers increased detection sensitivity and specificity (Fredriksson *et al.*, 2002) (Blokzijl *et al.*, 2010) (Liu *et al.*, 2011). Here, we have adapted an ultrasensitive proximity ligation assay based on western Blotting (PLA WB) method for the detection of protein bands of *R. conorii* and *R. africae* at the early stage of infection. We performed a classical 1SDS-

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PAGE Western Blot which enabled us to compare both of these techniques.

In parallel, we have screened 20 rickettsial recombinant proteins by ELISA (Kowalczewska *et al.*, 2012) with sera infected by *R. conorii* and *R. africae*, respectively. All together, these combined methods allow us to propose a few discriminate protein candidates for serodiagnostic test development.

MATERIALS AND METHODS

Rickettsiae and Cells

Rickettsia conorii (strain Seven Malish, ATCC VR-630) and *Rickettsia africae* (strain ESF-5) were propagated on a confluent monolayer of murine fibroblast L929 cells line and purified on renographin density gradient centrifugation as previously reported (Eremeeva *et al.*, 1994a). Purified bacteria were resuspended in PBS and stored at -80°C until its use. All purification steps were monitored by Gimenez staining (Gimenez, 1964). Protein concentration was determined by modified Bradford method (Kowalczewska *et al.*, 2008).

Preparation of Crude Extracts for SDS-PAGE

The whole rickettsial cells purified were solubilized in Laemmli buffer (Laemmli, 1970) and separated by electrophoresis on a 10 % sodium dodecyl sulfate-polyacrylamide gel for 1 h at 150V.All IEF (Immobiline Dry Strips gels (7 cm, pH 3–10, GE Healthcare) rehydrated with 15 µg (7 cm) of solubilized proteins) and 2-D electrophoresis steps were performed as previously described (Kowalczewska *et al.*, 2008). The proteins were resolved by electrophoresis through a 10% SDS-polyacrylamide gel (EttanTM DALT, GE Healthcare) at 5 W/gel for 30 min, followed by 17 W/gel for 4–5 h. Following electrophoresis, the gels were either silver-stained or transferred onto nitrocellulose membranes. Digital images were generated using transmission scanning (Image Scanner, GE Healthcare).

Western Blotting

Rickettsial extracts (15 µg) separated in one dimension by SDS-PAGE or 2D gel electrophoresis (7cm, pH 3-10) were transferred onto nitrocellulose membranes (Trans-blot transfer medium, pure nitrocellulose Membrane 0.45 µm, BioRad) at 100 V for 2 h. Membranes were then blocked in PBS supplemented with 0.2% Tween 20 and 5%

non-fat dry milk (PBS-Tween-milk) for 1.5 h at room temperature before incubation with sera of infected patients (dilution 1:100 in the blocking buffer) or with rabbit sera (dilution 1:1000 in the blocking buffer). After 1 h of incubation, the membranes were washed three times for 10 min in 0.2% PBS-Tween 20 and probed with horseradish peroxidase-conjugated goat anti-human IgG (1:1000; GE Healthcare). The blots were washed three times as indicated above, and chemiluminescence kit (ECL™ Western blotting Analysis System, GE Healthcare) was used to detect protein bands. The resulting signal was detected on Hyperfilm™ ECL and subsequently developed using an automated film processor (Hyperprocessor™, GE Healthcare).

Serum Cross-Adsorption

Sera from ten patients determined to have had *R. conorii* and from ten patients determined to have had *R. africae* were randomly selected for cross-adsorption and Western blotting studies. Two aliquots of the 10 selected sera (100 ml each) were each diluted 1:10 separately in an *R. africae* antigen suspension previously adjusted to contain 2 mg of protein/ml and in a *R. conorii* antigen suspension previously adjusted to contain 2 mg of protein/ml and shaken for 24 h at room temperature. After centrifugation at 10,000 x g for 10 min, the supernatant was removed and treated twice again as described above (Jensenius *et al.*, 2004). The cross-adsorption assay using *R. africae* and *R. conorii* antigens and followed by WB on the resulting supernatant wasperformed as previously described (Hechemy *et al.*, 1983). We considered assays where adsorption with *R. africae* antigen removed both homologous and heterologous antibodies and adsorption with *R. conorii* antigen removed homologous antibodies only as serological evidence of *R. africae* infection. If these criteria were not met, the result was classified as undetermined SFG rickettsial infection. The cross-adsorption assay was performed on convalescent-phase sera from all cases classified as undetermined SFG rickettsial infection by IFA and WB and with IgG titers of > 1:64 by IFA (La and Raoult, 1997).

Proximity Ligation Assay Based on Western Blotting (PLA WB)

The whole cells rickettsial proteins (15µg) were separated using 1D SDS-PAGE (10 %) or 2D gel electrophoresis (7cm, pH 3-10), and transferred onto

nitrocellulose membranes (Bio-Rad) using a semidry transfer unit (Hoeffer Scientific) as described previously (Kowalczewska *et al.*, 2008). Membranes were blocked in PBS supplemented with 0.2% Tween 20 (PBST) and 5% non-fat dry milk (PBST-milk) for 1.5 h at room temperature before incubation with sera of infected patients (dilution 1:100 in PBST-milk) or with rabbit sera (dilution 1:1000 in the blocking buffer). After the wash, the membrane was incubated for 1 h at room temperature under gentle rotation with Duolink ® II PLA Plus probe (OLINK Bioscience) as described by the manufacturer. The detection probes (0.2-1 µg/ml) were diluted in PLA probe diluent buffer (0.5 mg/ml BSA, 5 µg/ml salmon sperm DNA, 5mM EDTA, 0.05 % Tween20 in TBS buffer). The membrane was then briefly rinsed and washed for 2 x 10 min and incubated at gentle orbital rotation for 40 min at 37°C with T4 DNA ligase (Fermentas) (0.01-0.03 U/µl) and backbone and splint oligonucleotides (1-90 nM) supplied by the manufacturer (OLINK Bioscience) and were diluted in oligo ligation buffer. Next, the membrane was washed for 5 min in PBST and after that incubated with phi29 DNA polymerase (GE Healthcare) (0.01- 0.125 U/µl) in rolling circle amplification buffer for 1 h at 37°C (OLINK Bioscience). After two rinses, the membrane was incubated with dye Cy5 (5-45 nM) (Integrated DNA Technologies) in detection buffer (2xSSC, 0.5 mg/ml BSA, 2 µg/ml salmon sperm DNA, 0-5 % formamide, 0.05 % Tween20) for 30 min at 37°C. Finally, the membrane was rinsed twice and washed for 3 x 10 min in PBST and rinsed quickly in PBS to remove Tween20. Fluorescence signals were captured by using a Typhoon™ FLA 9000 imager (PMT, 450 V) (GE Healthcare).

ELISA

Modified ELISA performed with 20 recombinant rickettsial proteins, was performed as previously described (Kowalczewska *et al.*, 2012). All steps were performed according to standard protocols (Kowalczewska *et al.*, 2012). 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 (Kowalczewska *et al.*, 2012 ;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. africae 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 (Kowalczewska *et al.*, 2012) (Sekeyova *et al.*, 2010). Any samples exhibiting absorbance above the cut off value was considered as positive (Figure 1 A & B).
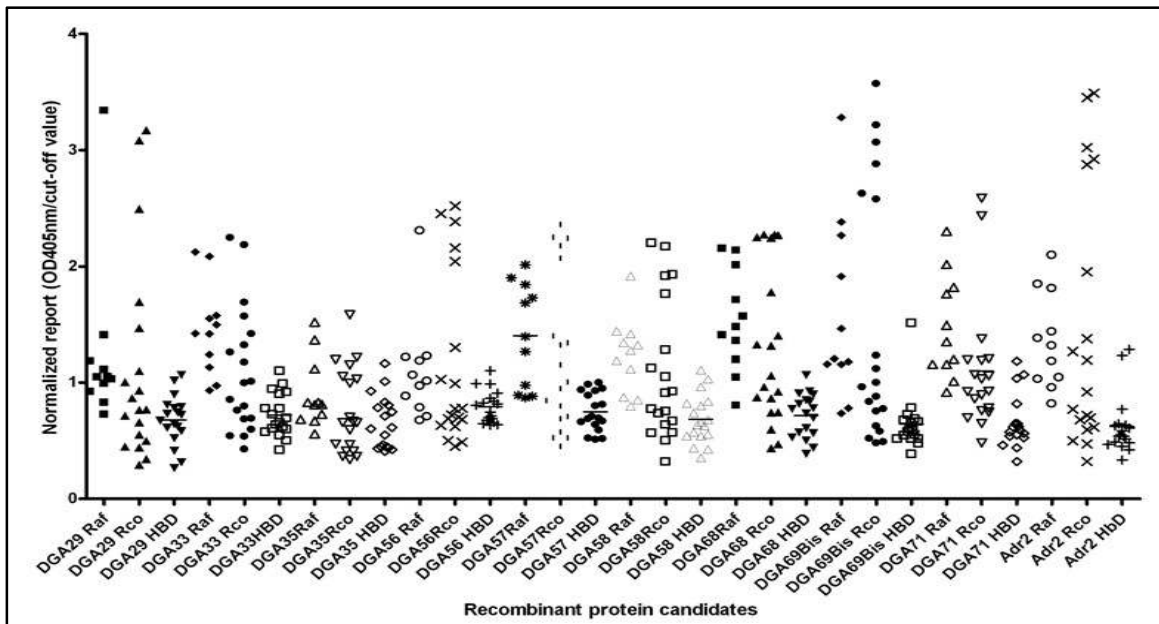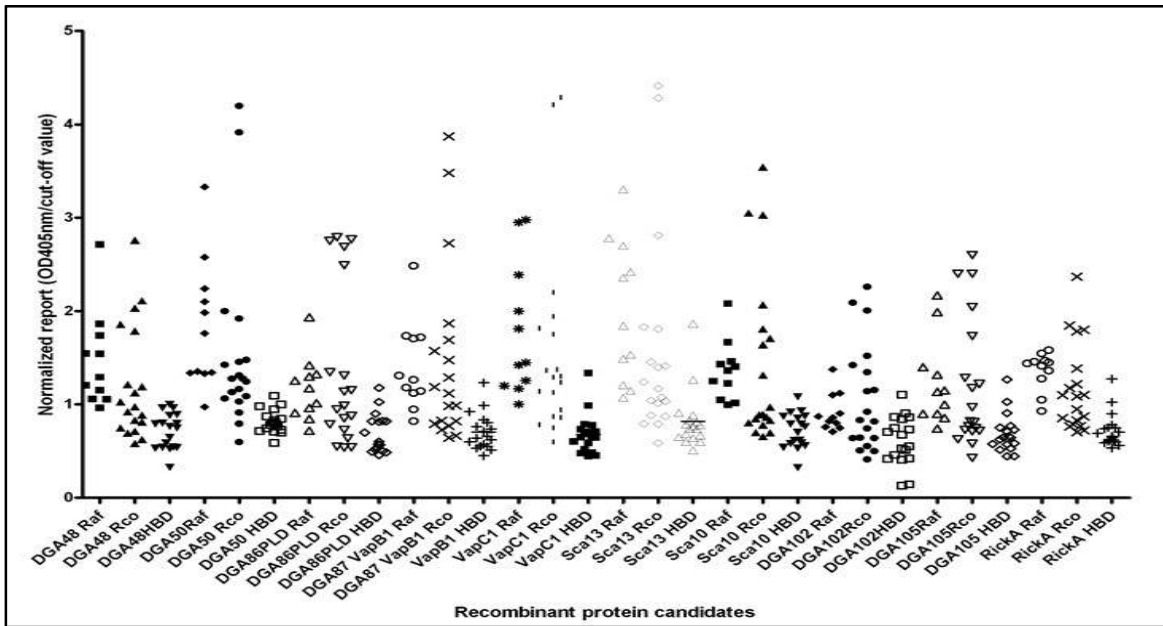

Figure 1A: The Recombinant Proteins of *R, prowazekii*, Strain MadridE

Figure 1B: The Recombinant Proteins of *R, prowazekii*, Strain Sheila Smith

Human Sera and Sera of Rabbits

For Western Blot, 22 patient's sera with an infection due to *R. africae* and 20 sera the patients diagnosed for active *R. conorii* infection diagnosed at the Unité des Rickettsies (Marseille, France) were included in this study (Kowalczewska *et al.*, 2018). The sera of rabbits infected with *R. conorii* and *R. africae* were obtained by 2 immunizations with the bacterial suspension of *R. conorii* and *R. africae* purified by the renographin gradient method.

For ELISA, we used the same sera as for Western Blot: 11 sera of patients with *R. africae*, 19 sera with *R. conorii* infection and we enlarge to18 of healthy blood donors (HBD). The diagnosis was based on serology and PCR assays targeting (Socolovschi *et al.*, 2009).

RESULTS AND DISCUSSION

Serum Cross-Adsorption

The Western blot allows earlier diagnosis, but its interest lies in the accuracy of the species rickettsia or by only detecting antibodies against the species in question, or after cross adsorption eliminating non-specific antibodies (an example of this technique is illustrated in Fig. 2). We can observe a strong reactivity with the band of 50 kDa but weakly with bands of 34 kDa, 30 kDa and 17 kDa *R. conorii* antigen (Figure 2). These protein bands (20-50 kDa)

are specificto the lipopolysaccharide in *Rickettsia* (Eremeeva *et al.*, 1994b). Cross adsorption is achieved by mixing serum studied bacteria are suspected to be cause. Resulting in a loss of homologous antibodies and heterologous adsorption occurs when the bacteria responsible of the disease, while only homologous antibodies disappear when the adsorption is performed with the bacterium responsible for the cross reactivity (Figure 2) (La and Raoult, 1997). A specific serological diagnosis of ATBF vis-a`-vis other SFG rickettsioses is sometimes of clinical and epidemiological importance. The major limitations with the cross-adsorption assay are the large amounts of antigen needed, high costs, and the fact that conclusive reactions are usually only seen in samples with pronounced immune response (e.g., with IgG titers of >1:64) (La*et al.*, 2000). Indeed, 10 sera used here showed negative serology, which makes this approach limited for early stage infections. The technique is not always successful because only 4 out of 20 cases (20 %) of patient's analyzed sera have identified the *Rickettsia* species responsible for the disease. Serological differentiation of 20 % is similar to what has been observed in *Rickettsia prowazekii* and *Rickettsia typhi* (La *et al.*, 2000).
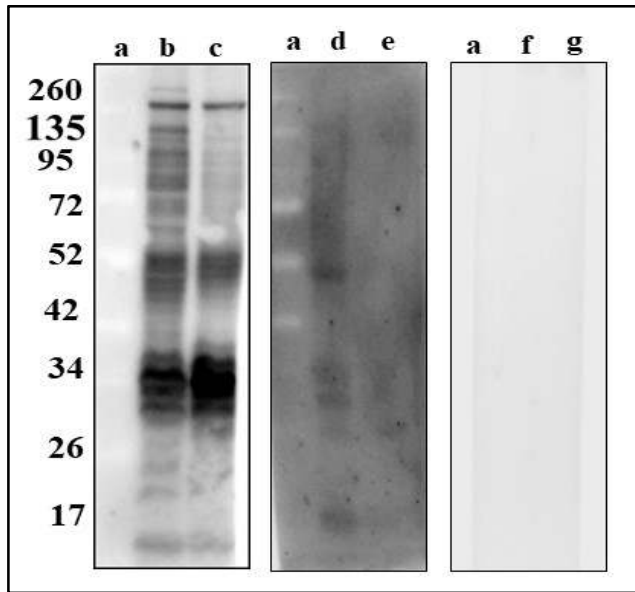
Figure 2: Example of western immunoblotting of serum samples from a patient with ATBF before and after cross-adsorption assay with *R. conorii* or *R. africae*

Considering that only 20% of samples can be confirmed by this in routine usage technique, which is so far the most useful in rickettsial species discrimination, we decided to run a challenge for rickettsial markers discovery by two approaches: proximity ligation WB and traditional 1D SDS-PAGE, respectively.

Detection of Protein Bands by 1D PLA WB Method

To increase signal amplification and detection sensitivity, we adapted PLA technique to detect rickettsial proteins bands. To determine if -*in situ*- PLA can provide enhanced sensitivity in WB, we compared 1D PLA WB and traditional 1D-WB. We limited to compare the number of detected bands and just visually estimate the intensity of the signal. In general, we obtained better sensitivity and specificity of bands detection in PLA WB in case of proteins probed with IFA *R. conorii* or *R. africae* positive sera (Figure 3).We noted the enhanced signal in PLA WB when comparing with traditional WB. Thus, PLA is known to enhance considerably the sensitivity (20-50 folds) (Liu *et al.*, 2011) (Clausson *et al.*, 2011). The improved sensitivity can also permit analysis of small amounts of sample and reducing consumption of precious samples. These results allow us to make 2D PLA WB in order to detect and identify immunogenic proteins of Rickettsia.
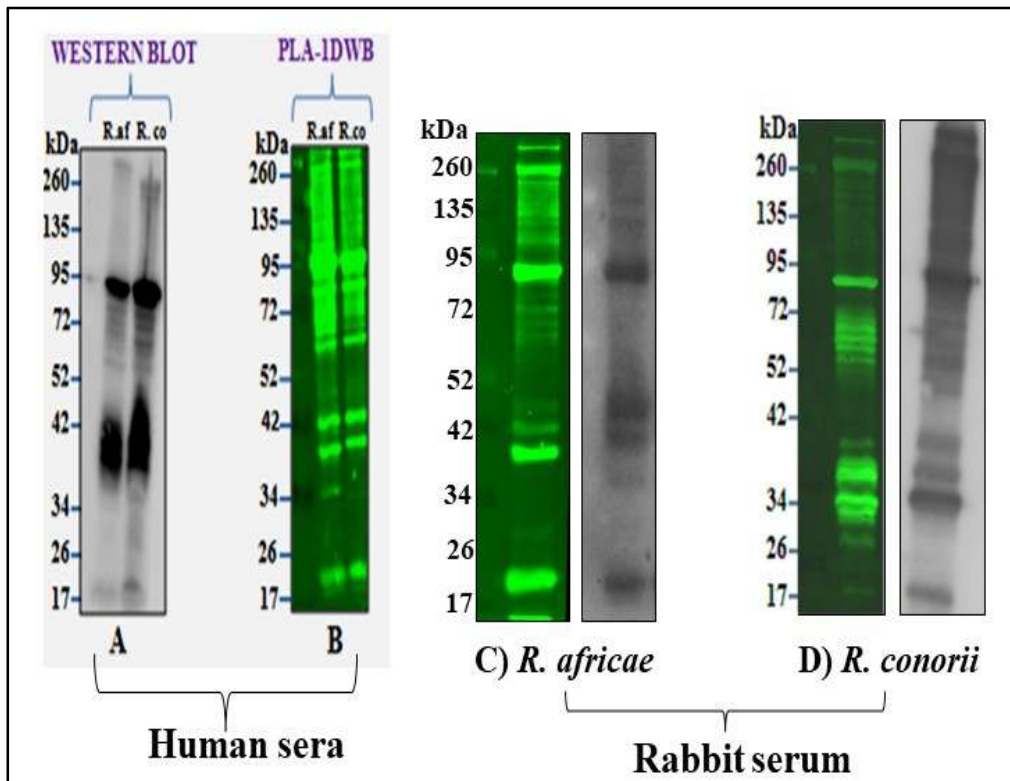
Figure 3: Example of detection of rickettsial proteins bands using traditional WB and PLA-WB

Immunoreactivity Patterns Revealed by 2-D PLA WB

Here, we describe the preliminary results obtained with of rabbit sera for the detection of immunogenic proteins of *Rickettsia conorii* and *Rickettsia africae* with the 2D PLA WB technique (Figure 4). We adapted PLA technique to detect rickettsial proteins. To determine if *in situ* PLA can provide enhanced sensitivity in WB, we compare 2D PLA WB to traditional 2D WB. The immunoreactivity profiles obtained by PLA 2D WB were very similar to those obtained with traditional WB. Mainly, the zone in pH range 3-4.5 was highly reactive with major rOmpB spot (Kowalczewska *et al.*, 2018). Considering that the aim of our study was global sera reactivity patterns screening, we did not perform the detailed quantifications

of signals obtained by both techniques. We limited to compare the number of detected spots and just visually estimate the intensity of signal. In general, we obtained the better sensitivity and specificity of spot detection in PLA WB in case of proteins probed with IFA *R. conorii* or *R. africae* positive rabbit sera (Figure 4). Therefore, we noted the enhanced signal in PLA WB when comparing to traditional WB. Thus, PLA is known to enhance considerably the sensitivity (20-50 folds) (Liu *et al.*, 2011) (Clausson *et al.*, 2011). All together, the immunoreactivity profiles obtained for both *R. africae* and *R. conorii* were very similar and homogeneous and this in case for both techniques: 2D PLA-WB and traditional 2D-WB, respectively (Kowalczewska *et al.*, 2018).
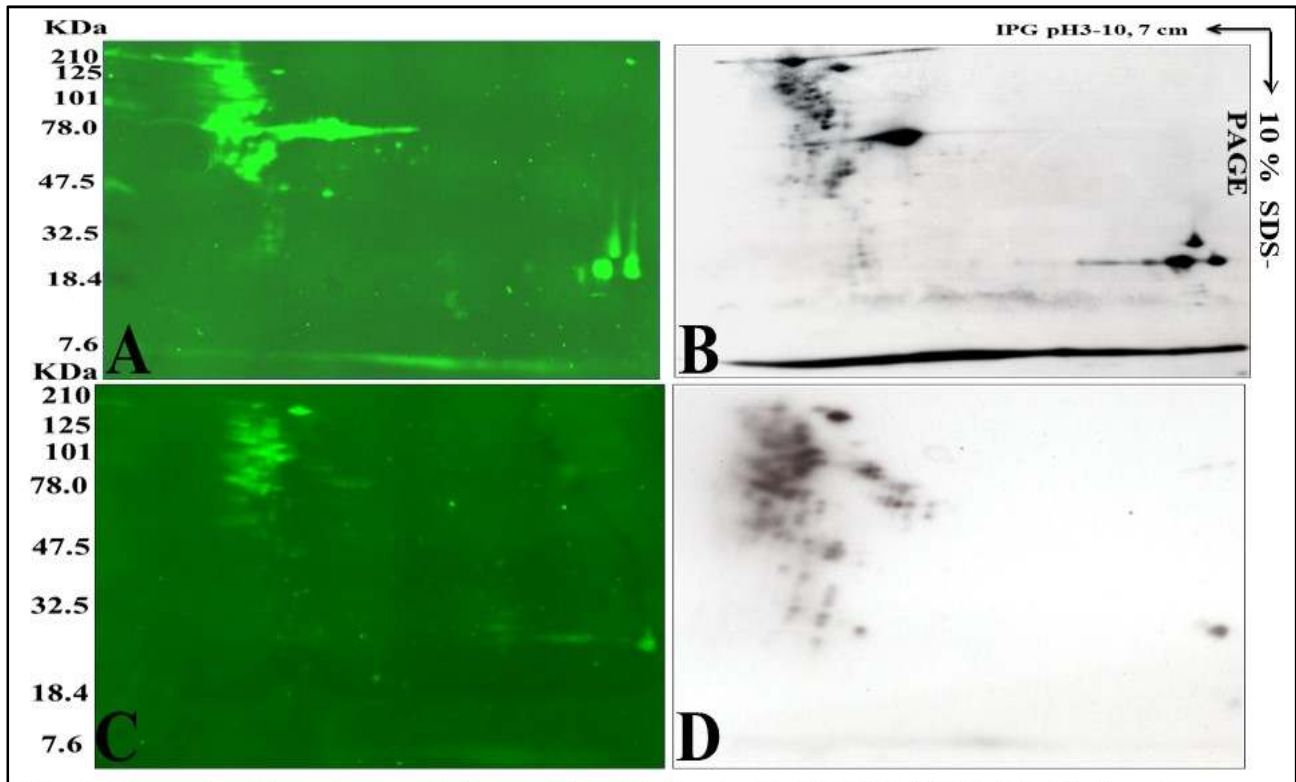

Figure 4: Example of immunoreactivity profiles obtained with PLA 2D WB (pI 3–10, 7 cm)

Discriminate Recombinant Proteins

To facilitate the discrimination between *R. africae* and *R. conorii* patients, we screened in this aim 20 recombinant proteins from a previous study (Kowalczewska *et al.*, 2012). Several recombinant proteins were found to cross-react with both, sera infected with *R. africae* and *R. conorii* (Table 1) with the best hits for detection of both

rickettiae: sca13, VapB1, atpC, spoJ. However, the reactivity of sera from *R. africae* infected patients was more intense than for *R. conorii*. This conform the results obtained with immunoblots. Not suprising that *R. rickettsii* proteins reacted better with both *R. africae* and *R. conorii*, than *R. prowazekii* recombinant proteins. Thus, several proteins were shown specific and sensitive for detection of *R. africae* (best hits: Sca10, EF-Tu, VapC1, pepA, and

peptide β). However, it is important to consider a small cohort of *R. africae* infected patients (n=11). As in previous study, we did not find the highly discriminate markers of *R. conorii* infection (Kowalczewska *et al.*, 2012). Moreover, the best hits for diagnosis both *R. conorii* and *R. typhi* differs from the best hists found in the present study for diagnosis of *R. conorii* and *R. africae* infection. In the fact in both studies we observe effect of cohort over the serological parameters. Thus, it will be suitable to validate these diagnostic targets on larger study population. 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.

Table 1: Test-operating parameters of 20 recombinant proteins included in the present study

DGA			R.africae						R.conorii						R.africae&R.conorii						This study	previousstudy(1)
			Se	Sp	VPP	VPN	L	Λ	Se	Sp	VPP	VPN	L	Λ	Se	Sp	VPP	VPN	L	Λ		
R.prowazekii ME																						
DGA35	groEL; 60 kDchaperonin	RP626	25	89	60	64	2.25	0.844	37	89	78	57	3.316	0.711	33	89	83	44	3	0.75		Marker *R.typhi&R. conorii*
DGA29	Cell surface antigen Sca1 (SPLIT GENE)	RP016	72	89	80	84	6.54	0.306	37	89	78	57	3.316	0.711	50	89	89	52	4.5	0.563		Marker *R.typhi*
Adr2	Putative outer surface protein (Adr2)	RP828	81	89	81	89	7.36	0.2	47	88	81	61	4.26	0.59	60	89	90	57	5.4	0.45		Marker *R.typhi&R. conorii*
DGA69 Bis	DOMAINE PFAM 447-768 omp1	RP160	82	94	90	89	14.72	0.1925	42	94	89	60	7.57	0.61	56	94	94	56	10.2	0.45	marker Raf	
DGA33	Aminopeptidase A (EC:3.4.11.1) (pepA)	RP142	82	94	90	89	14.72	0.1925	52	94	90	65	9.474	0.502	63	94	95	60	11.4	0.388	marker Raf	
DGA58	hypotheticalprotein RP631	RP631	72	89	80	84	6.54	0.306	42	89	80	59	3.79	0.651	53	88	88	53	4.8	0.525		
DGA57	3-demethylubiquinone-9 3-methyltransferase	RP622	64	100	100	82	-	0.363	53	100	100	66	-	0.473	57	100	100	58	-	0.433		
DGA56	UDP-N-acetylmuramate--L-alanine ligase	RP247 (murC)	54.5	94	86	77	9.818	0.481	37	94	87.5	58.6	6.632	0.669	43	94	93	50	7.8	0.6		Marker *R.typhi&R. conorii*

ID	Description	Gene																			Marker 1	Marker 2
DGA68	stage 0 sporulation protein J (spo0J)	RP059	91	94	90	94	16.36	0.09	58	94	92	68	10.42	0.445	70	94	95	65	12.6	0.317	marker Raf&Rco	
DGA71	signal recognition particleprotein	RP173	90	83	77	94	5.45	0.109	47	83	75	60	2.84	0.63	63	83	86	57	3.8	0.44		Marker *R.typhi*
***R. rickettsii* Sheila Smith**																						
DGA50	F0F1 ATP synthase subunit epsilon	A1G_06755 (atpC)	91	94	90	94	16.36	0.09	84	94	94	85	15.15	0.167	87	94	96	81	15.6	0.14	marker Raf&Rco	
DGA88 VapC1	hypotheticalprotein A1G_02180 (VapC1)	AG1_02180 (VapC1)	100	94	92	100	18	0	68	94	93	74	12.3	0.33	80	94	96	74	14.4	0.211	marker Raf	
DGA87 VapB1	hypotheticalprotein A1G_02185 (VapB1)	A1G_02185 (VapB1)	82	94	90	89	14.72	0.1925	53	94	90	65	9.47	0.50	63	94	95	60	11.4	0.39	marker Raf&Rco	
DGA56 PLD	hypotheticalprotein A1G_06970 (PLD)	A1G_06970 (PLD)	64	89	77	80	5.72	0.40	47	89	81	61	4.26	0.59	53	89	89	53	4.8	0.525		Marker *R.typhi*
DGA92 Sca13	cell surface antigen-like protein Sca13	A1G_06915 (Sca13)	100	94	92	100	18	0	74	95	93	72	13.26	0.28	93	94	96	77	15	0.176	marker Raf&Rco	
DGA105	sojprotein	A1G_00540	54	89	75	76	4.9	0.511	42	89	80	59	3.78	0.65	47	89	87.5	50	4.2	0.6		
DGA93 Sca10	cell surface antigen-like protein Sca10	A1G_00295 (Sca10)	100	94	92	100	18	0	42	94	89	60	7.57	0.61	63	94	95	60	11.4	0.39	marker Raf	Marker *R.typhi*
DGA102	dihydrofolate reductase	A1G_00215	27	94	75	68	4.9	0.77	42	94	89	60	7.6	0.61	37	94	92	47	6.6	0.67		
DGA120 RickA	Hypothetical protein A1G_05015 (RickA)	A1G_05015 (RickA)	90	89	33	94	8.18	0.102	52	88	83	64	4.74	0.532	67	89	90	61.5	6	0.375		Marker *R.typhi*
DGA48	elongation factor Tu	A1G_05565	90	94	90	94	16.36	0.09	47	94	90	63	8.52	0.56	63	94	95	60	11.4	0.39	marker Raf	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/individualblooddonor)

Λ = (1-Se)/Sp = (FN/patients) (TN/individualblooddonor)

CONCLUSION

In conclusion, we have detected of immunogenic proteins from *Rickettsia conorii* and *Rickettsia africae* by proximity ligation assay based Western blotting (PLA WB). However, it still required optimization for *Rickettsiae*. The preliminary results obtained with 20 recombinant proteins of *R. africae* and *R. conorii* which may be useful tools in the detection of Rickettsiae in clinical samples. ELISA performed with recombinant proteins is a valuable tool to enable the evaluation of the best targets. Several recombinant proteins were found to cross-react with both, sera infected with *R. africae* and *R. conorii.* Optimization of recombinant proteins based ELISA may be an interesting alternative for diagnosis of rickettsial diseases.

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