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Reliable tool for detection of novel *Coxiella burnetii* antigens, using immobilized human polyclonal antibodies

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ABSTRACT

Coxiella burnetii (C. burnetii) is the etiological agent of a Q fever—the re-emerging disease with considerable economic impact. Due to many similar symptoms with commonly occurring infections, its clinical diagnosis is very difficult. Thus, a strong effort should be taken to raise the awareness and develop a robust strategy for an accurate diagnosis. The identification of specific *C. burnetii* biomarkers could be valuable for a sensitive and selective diagnosis of the disease. Herein, we described a workflow to identify immunoreactive proteins of *C. burnetii* with a high confidence. It is based on immunocapturing of bacterial antigens by biofunctionalized magnetic microspheres, followed by tandem mass spectrometry (MS/MS) identification. We detected dozens of previously reported antigens and proposed 15 novel biomarkers, which specificity was confirmed by *in silico* epitope analysis. Among them, the cardiolipin synthetase participating in the synthesis of cardiolipin was recognized. This biomarker could play a critical role in the early management of acute Q fever and prevention of Q fever endocarditis.

1. Introduction

Reliable detection and identification of bacterial species are necessary for timely response to natural or terrorist-caused outbreaks of infectious diseases and play crucial roles in efficient treatment. In order to accomplish this diagnostic task successfully, indisputable identification of bacterial biomarkers must be performed. Serological proteome analysis (SERPA) is a valuable tool in this respect due to the ability to determine characteristic antibody-antigen interactions [1,2].

Coxiella. burnetii, a Gram-negative intracellular pathogen—is the etiological agent of a zoonotic disease Q fever. The human infection is generally acquired by inhaling aerosolized microorganism, produced by infected livestock. The acute infection is typically asymptomatic or manifests as a febrile flu-like illness or pneumonia followed by a spontaneous recovery. In some cases, especially for patients with meningoencephalitis or myocarditis, there can be serious complications that require intensive care. The chronic infection is demonstrated as hepatitis or endocarditis, and serious distress or death may occur [3].

Various, mainly serological or molecular biology methods are currently used for a rapid and sensitive diagnosis of the disease, but in numerous cases, they yield to ambiguous results [4,5]. Thus, development of an analytical approach for an accurate identification of the bacterium is of utmost importance. During the last decades, several attempts have been made to investigate characteristic immunoreactive proteins of *C. burnetii* [6–15]. The majority of them relied on the two-dimensional gel electrophoresis (2-DE) followed by western blot analysis. This approach, however, has to deal with many limitations, especially those related to the high amount of serum and antigens needed for the analysis, as well as to typical drawbacks of 2-DE separation. The most interesting membrane proteins, low copy number or highly basic/acidic proteins are notably difficult to separate, using 2-DE. To overcome these disadvantages, alternative analytical methods have to be used.

In this work, we aimed to develop a platform that enables to identify novel immunodominant proteins of *C. burnetii* in their native form using a faster, less material-consuming, and more reliable approach in comparison to 2-DE based Western blotting analysis. Our workflow is based on in-solution immunoaffinity enrichment of bacterial antigens, followed by HPLC profiling and mass spectrometry identification.

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2. Materials and methods

2.1. Isolation of IgGs from human sera infected with C. burnetii using protein A coupled to magnetic beads

Three hundred microliters of Protein A magnetic beads suspension (Pure proteome, Millipore) were washed with 500 μl of phosphate buffered saline (PBS) and incubated for 60 min at room temperature (RT) under mild rotation with patient sera (250 μl, 5 x diluted with PBS). These sera were previously tested by enzyme-linked immunosorbent assay. High titers against *C. burnetii* antigens were observed in each of them (data not shown). As a control, the serum from a healthy individual was immobilized in parallel. Written and informed consents were obtained from the patients according to the institutional guidelines. The beads were then removed with a magnetic stand (Invitrogen) and washed 3 times using 500 μl of PBS. The IgGs were eluted from beads in 5 consecutive steps by 50 μl of 0,2 M glycine (pH 2,5), followed by immediate neutralization of pH with 1 M Tris (pH 9,5). In order to determine the purity of the elution fractions, absorbance at 280 and 260 nm was recorded using a spectrophotometer (BioPhotometer plus, Eppendorf). Protein concentration and purity was evaluated by the following equations: 1) protein purity: $A^{260nm}/A^{280nm} < 0{,}6$; 2) concentration of pure protein, $A^{280nm}/E^{1\%}_{280} \times 10$ mg/ml (extinction coefficient for IgG $E^{1\%}_{280} = 13{,}6$). The isolation procedure was verified also by 12% SDS-PAGE stained with colloidal Coomassie dye.

2.2. Preparation of the magnetic beads with immobilized IgGs

Sixty micrograms (1 mg/ml solution) of the isolated IgGs was subjected to covalent binding to 3 mg (100 μl according to the manufacturer instructions) of Dynabeads M-270 (2,8 μm; ThermoFisher Scientific) by two steps coupling using *N*-(3-dimethylaminopropyl)-*N'*-ethyl carbodiimide hydrochloride (EDC; Fluka) and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS; Sigma-Aldrich). Briefly, the beads were 5 times washed with a cold 25 mM MES buffer pH 5 (Calbiochem) and mixed for 30 min with 100 μl sulfo-NHS (50 mg/ml) containing 200 μl EDC (50 mg/ml) to activate the carboxylic group. The beads were removed with the magnetic stand and washed 2 times with 100 μl of 25 mM MES, pH 5,0. The binding of IgGs (in 500 μl of 0,1 M phosphate buffer pH 7,3) to the beads was achieved by overnight incubation at 4 °C under mild stirring. Before the use, the biofunctionalized beads were washed 3 times with 25 mM MES buffer pH 5,0, followed by 4 washes with 1 ml of PBS pH 7,4.

2.3. Immunocapturing of C. burnetii antigens

C. burnetii RSA493 cells were propagated for 9 days in embryonated, antibiotic-free and pathogen-free eggs as described previously [16]. The handling of live microorganism was carried out in a Biosafety Level 3 laboratory. Bacterium was inactivated with 0,5% phenol and purified by rate-zonal sedimentation in gradient of Renografin (Ultravist 370; 0,769 g/mL) at 112 500 × *g* and 4 °C for 90 min [17].

Twenty milligrams of purified cells were washed with 500 μl of miliQ H_2O and centrifuged for 30 min at 12 000 × *g* and 4 °C. Bacterium was resuspended in 700 μl of PBS pH 7,4 at presence of EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and treated with freeze-thaw cycles (10 x) using liquid nitrogen followed by 3 cycles of sonication (Sonifier Cell Disruptor SLPe, Branson) for a total of 10 min (20 s pulse, 3 min pause to cool down the sample). The lysate was clarified by centrifugation (12 000 × *g*, 30 min, 4 °C). Protein concentration in the supernatant was determined by BCA assay (ThermoFisher Scientific).

One milligram of the lysate was mixed with 200 μg of biofunctionalized (immobilized IgGs) beads and incubated overnight at 4 °C under mild stirring. To remove non-specific bindings, the beads were washed 10 x and 5 x with 500 μl of 0,1 M phosphate buffer pH 7,0 containing 0,2 M and 1 M NaCl, respectively. The excess of salt was then reduced by consecutive washing steps (5 x) using 0,1 M phosphate buffer pH 7,0.

The immunocaptured antigens were eluted for 10 min by 2 consecutive steps (50 μl) using 0,05% formic acid (Sigma-Aldrich) under agitation. The elution fractions were concentrated in Concentrator plus (Eppendorf) to half the volume and validated by separation in 15% SDS-PAGE.

2.4. Identification of immunoreactive proteins and bioinformatic data processing

The proteins in the elution fractions were reduced in 25 mM NH_4HCO_3 containing 10 mM dithiothreitol (DTT, Sigma-Aldrich) for 45 min at 56 °C, and alkylated in the dark at room temperature for 45 min in the same buffer containing 55 mM iodoacetamide (IAA, Sigma-Aldrich). After neutralization of IAA, 10 μl of trypsin solution (containing 40 ng of lyophilized sequencing grade modified trypsin dissolved in 50 mM NH_4HCO_3; Promega) was added and the proteins were digested overnight at 37 °C under gentle shaking. Enzymatic cleavage was terminated by 1 μl of 10% formic acid (Sigma-Aldrich). Peptides were concentrated to a volume of 20 μl and stored at −80 °C until MS analysis.

The peptide mixtures were analyzed by LC–MS/MS, using nanoAcquity UPLC system (Waters) and Q-TOF Premier mass spectrometer (Waters). Briefly, the peptide mixtures were separated by reverse phase chromatography using BEH 130 C18 column (75 μm diameter, 200 mm length, 1,7 μm particle size, Waters) during 30 min with 7–40% acetonitrile gradient. The spectra were acquired in data independent MSE mode when alternate scans at low and high collision energies were employed for accurate precursor MS and fragment MS/MS masses in a single run. The data were processed using the ProteinLynx Global Server v. 3.0 (PLGS, Waters) that provided noise-filtering at the following threshold parameters: low energy 60 counts, high energy 150 counts, intensity 1200 counts. The time alignment was used to initially correlate the precursor and fragment ions. All data were lockspray calibrated using [Glu1]-Fibrinopeptide B reference signal (Sigma-Aldrich). The results were searched against the full proteome of *C. burnetii* downloaded from UNIPROT (July 2014). The following search parameters were used: 1 trypsin missed cleavage, fixed—carbamidomethylated cysteine, variable—oxidized methionine, automatic precursor/fragment errors estimated from calibrant peaks. Peptide score cut-off was set at 5,35 meaning more than 95% confidence of identification.

In order to predict cell compartment localization, PSORT algorithm (http://psort.org) [18] was applied. To assign biological function to the identified proteins we used BlastKOALA (KEGG Orthology and Links Annotation; http://www.kegg.jp/blastkoala/), and BioCyc pathway database (http://biocyt.org)

2.5. Validation of immunocapturing by indirect immunofluorescence assay (IFA)

The functionalized magnetic beads were exposed to intact *C. burnetii* cells for 1 h at RT and washed with PBS. The beads were fixed on a microscopic slide with 3,7% formaldehyde (Sigma-Aldrich) in PBS for 15 min at RT. Following that, treatments with 0,1% Triton X-100 in PBS (7 min at RT) and 5% BSA in PBS (1 h, 37 °C) were performed. After additional washing steps, the beads were incubated for 1 h at 37 °C with a rabbit antiserum against *C. burnetii* (100 x diluted in PBS with 2,5% BSA). Anti-rabbit IgG secondary