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Fractionation of the *Coxiella burnetii* Parasitophorous Vacuole

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Summary

Coxiella burnetii is a bacterial obligate intracellular pathogen that replicates within a spacious parasitophorous vacuole (PV) with lysosomal characteristics. The pathogen actively participates in the biogenesis of its PV by synthesizing proteins that mediate vesicular interactions. Both *C. burnetii* and host factors that regulate PV formation are likely localized to the PV membrane, and their identification would be aided by an efficient method for isolating the *C. burnetii* vacuole. To this end, we developed a method to separate intact PV from host cell material that relies on fusion of the vacuole with latex bead-containing phagosomes (LBP). Sequestration of latex beads by the *C. burnetii* PV increases the vacuole's buoyant density and facilitates its fractionation on a sucrose step gradient. Transmission electron microscopy confirms the isolation of intact PV-containing latex beads from infected MH-S murine alveolar macrophage-like cells. Immunoblotting demonstrates that *C. burnetii* PV lysates are dramatically enriched for the late endosome/lysosome markers LAMP-1 and LAMP-2 when compared to total host cell lysates. Conversely, PV preparations are devoid of p62 and GM130, markers of the nucleus and Golgi apparatus, respectively, indicating effective separation of the vacuole from these host cell compartments. Two-dimensional gel electrophoresis and immunoblotting reveal distinct protein differences between *C. burnetii* PV and LBP. Identification of proteins unique to the PV membrane will yield important insight into *C. burnetii*–host interactions.

Key Words: *Coxiella*; vacuole; lysosome; proteomics; membrane; latex beads; phagosome; Q fever.

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1. Introduction

Coxiella burnetii is a bacterial obligate intracellular parasite and the etiological agent of human Q fever, an acute, debilitating influenza-like illness ***(1)***. The primary route of human infection by *C. burnetii* is through inhalation of contaminated aerosols with the alveolar macrophage being the initial target host cell ***(2)***. From this site the organism can hematogenously spread to infect other tissues, including the heart where a potentially severe chronic infection can be established ***(1)***.

Following phagocytosis, *C. burnetii* is enclosed in a phagosome that matures through the endocytic pathway to ultimately fuse with the lysosomal compartment ***(3)***. However, relative to latex bead phagosomes (LBP) ***(4)***, fusion between the *C. burnetii* vacuole and lysosomes is significantly delayed with approximately 2 h required for delivery of the lysosomal enzymes acid phosphatase ***(5)*** and cathepsin D ***(6)***. Stalled lysosome interactions may be mediated by early engagement of the *C. burnetii* phagosome with the autophagic pathway ***(6)***. Exponential replication of the organism coincides with the appearance of a large and spacious parasitophorous vacuole (PV), which is visible by light microscopy ***(7)***. The mature *C. burnetii* PV is phenotypically similar to a secondary lysosome ***(8)*** (e.g., moderately acidic with lysosomal hydrolases); however, unlike primary lysosomes, the PV is unusually fusogenic with other vacuoles within the endolysosomal cascade ***(9)***.

A paradigm in cellular microbiology is that pathogens direct biogenesis of their respective intracellular compartments to result in a vacuole that supports growth. *C. burnetii* is no exception to this model as protein synthesis is required for delayed fusion between the PV and lysosomes, and for early PV interactions with autophagosomes ***(5,6)***. Moreover, promiscuous fusogenicity between mature PV and endolysosomal vacuoles, and maintenance of the large and spacious PV architecture, require the activity of *C. burnetii* proteins ***(5)***. Collectively, these data suggest that *C. burnetii* secretes protein effectors into the cytosol that modulate host functions involved in membrane trafficking. This hypothesis is consistent with the presence of a Dot/Icm type IV secretion system in *C. burnetii* that is nearly identical to that of *Legionella pneumophila* ***(10)***. Abundant evidence indicates Dot/Icm function is critical for biogenesis of the *L. pneumophila* replication vacuole, and various genetic screens have identified a number of type IV substrates ***(10)***.

Identification of secreted effector molecules and their cellular targets is critical to gaining insight into the sophisticated interplay of intravacuolar pathogens and their host ***(11)***. To this end, genetic manipulation of pathogens to generate mutants in PV formation has been a valuable method ***(12–14)***. However, recent progress in identifying effectors of vacuole maturation has also resulted from subcellular fractionation of PV coupled with proteomic

analysis ***(15–17)***. This general strategy relies on the well-known fact that proteins (both pathogen and host) involved in PV development are often targeted to the vacuolar membrane ***(12,14,17)***.

The method of PV isolation described herein will facilitate identification of both bacterial and host proteins that modulate *C. burnetii* PV biogenesis. These proteins are likely critical for the intracellular replication and virulence of *C. burnetii*, and elucidation of their function will provide needed insight into mechanisms exploited by *C. burnetii* to remodel and survive within its lysosomal-like niche.

2. Materials

2.1. Loading of Latex Beads and C. burnetii Infection of MH-S Cells

1. RPMI medium with Glutamax I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 0.05 m*M* β-mercaptoethanol (β-ME). Store at 4°C.
2. MH-S murine alveolar macrophage-like cells (CRL-2019; American Type Culture Collection).
3. Phosphate-buffered saline (PBS): 53.9 m*M*, Na_2HPO_4, 12.8 m*M*, KH_2PO_4, and 72.6 m*M*, NaCl. Sterilize by autoclaving and store at room temperature.
4. 150-cm^2 cell culture flasks (Corning, Corning, NY).
5. 6-Well cell culture plates (Corning).
6. Red FluoSpheres fluorescent latex beads (1.0 μm in diameter) (Invitrogen).

2.2. Isolation of C. burnetii PV and LBP

1. Ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA): prepare a 100X (50 m*M*) solution by dissolving 1.9 g in 80 mL water and bring to a final volume of 100 mL.
2. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES): prepare a 10X (200 m*M*) solution by dissolving 4.76 g in water. Adjust pH to 7.2 with 2 *N* potassium hydroxide.
3. Complete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany).
4. Sucrose solutions: Prepare 10, 25, 35, 40, and 62% (w/v) sucrose, 0.5 m*M* EGTA, 20 m*M* HEPES solutions by first dissolving 10, 25, 35, 40, and 62 g, respectively, of sucrose in 70 mL or less of water. Add 1 mL of 100X EGTA solution and 10 mL of 10X HEPES solution to each sucrose solution and bring the final volume to 100 mL with water. Immediately prior to preparing sucrose gradients, dissolve one protease inhibitor tablet in 50 mL of each sucrose solution.
5. Homogenization buffer: 250 m*M* sucrose, 0.5 m*M* ethylene glycol tetraacetic acid (EGTA), 20 m*M* HEPES. To prepare 100 mL, dissolve 8.5 g of sucrose in

70 mL of water. Add 1 mL of 100X EGTA solution, 10 mL of 10X HEPES solution, and bring to 100 mL with water.
6. Cell scrapers (39-cm handle, 31-mm blade) (Sarstedt AG and Co., Numbrecht, Germany).
7. 50-mL disposable conical centrifuge tubes (Corning).
8. Knotes 2-mL Dounce homogenizer with a "B" pestle (Knotes Glass, Vineland, NJ).
9. Fifteen-mL Falcon tubes (#2059).
10. Five-mL Falcon tubes (#2058).
11. Ultra-clear centrifuge tubes (25 mm × 89 mm) (Beckman Coulter, Fullerton, CA).
12. Ultra-clear centrifuge tubes (14 mm × 95 mm) (Beckman Coulter).
13. Ten-mL Luer-lok Tip disposable syringes (Becton Dickinson, Franklin Lakes, NJ).
14. Fourteen-gauge, 4-in. metal cannulas (Popper and Sons, Inc., New Hyde Park, NY).
15. Sterile 1.5-mL O-ring screw cap microfuge tubes (Sarstedt AG and Co.).

2.3. Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

1. Separating buffer (5X): 1.875 *M* Tris-HCl, pH 8.8. Filter through Whatman #1 filter paper and store at room temperature.
2. Stacking buffer (8X): 1 M Tris-HCl, pH 6.8. Filter through Whatman #1 filter paper and store at room temperature.
3. Ten percent sodium dodecyl sulfate (SDS). Store at room temperature.
4. Thirty percent acrylamide/bis solution (29:1 with 3.3% C) and *N*,*N*,*N*,*N*'-tetramethylethylenediamine (TEMED) (Bio-Rad, Hercules, CA). Store at 4°C.
5. Ammonium persulfate: prepare a 10% solution in water. Store at –20°C in 500-µL aliquots.
6. SDS-PAGE sample buffer (2X): 1 *M* Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (w/v), 1 *M* β-ME, 0.03% bromophenol blue (w/v), 40 m*M* ethylenediaminetetraacetic acid (EDTA). Store in 500-µL aliquots at –20°C.
7. Running buffer (10X): 250 m*M* Tris, 2 *M* glycine, 1% SDS (w/v). Store at room temperature.
8. Precision Plus Duel Color prestained molecular weight markers (Bio-Rad).
9. Immobilon-P transfer membrane (0.45-µm pore size) (Millipore Corp., Bedford, MA).
10. Transfer buffer (20X): 0.5 *M* sodium phosphate, pH 7.5. Store at room temperature.
11. Washing buffer: 0.1% TWEEN 20 (w/v) in PBS (PBS-T). Store at room temperature.
12. Blocking buffer: 5% (w/v) nonfat dry milk in PBS-T. Prepare fresh before each experiment.

13. Monoclonal antibodies: mouse anti-human lysosomal glycoprotein LAMP-1 (Clone H4A3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rat anti-mouse lysosomal glycoprotein LAMP-2 (clone ABL-93, BD Biosciences, San Jose, CA), mouse anti-rat Golgi membrane protein GM130 (clone 35, BD Biosciences), mouse anti-human endoplasmic reticulum (ER) protein Bip (clone 40, BD Biosciences), and mouse anti-human nuclear pore protein nucleoporin p62 (clone 53, BD Biosciences).
14. Rabbit polyclonal antibody directed against formalin-killed *C. burnetii*.
15. Horseradish peroxidase (HRP)–conjugated anti-mouse, anti-rat, and anti-rabbit immunoglobulin G (Pierce, Rockford, IL.).
16. Tracker Tape luminescent alignment tape (GE Healthcare, Piscataway, NJ).
17. SuperSignal West Pico enhanced chemiluminescent (ECL) substrate (Pierce).
18. Hyperfilm ECL high performance chemiluminescence film (GE Healthcare).

2.4. Isoelectric Focusing

1. Equilibration buffer I (reduction buffer): 0.375 *M* Tris-HCl, pH 8.8, 6 *M* urea, 2% SDS (w/v), 20% glycerol (w/v), 130 m*M* dithiothreitol. Prepare fresh before each experiment.
2. Equilibration buffer II (alkylation buffer): 0.375 *M* Tris-HCl, pH 8.8, 6 *M* urea 2% SDS (w/v), 20% glycerol (w/v), 135 m*M* iodoacetamide. Prepare fresh before each experiment.
3. ReadyStrips (NL pH 3–10, 7 cm) isoelectric focusing strips (Bio-Rad).
4. ReadyPrep sequential extraction reagent 3 (Bio-Rad).
5. ReadyPrep 2-D cleanup kit (Bio-Rad).
6. ReadyPrep 2-D rehydration/sample buffer (Bio-Rad).
7. ReadyPrep 2-D overlay agarose (Bio-Rad).

3. Methods

Isolation of pathogen PV that are spacious and/or have a complex architecture, such as those harboring *Chlamydia trachomatis* and *Salmonella typhimurium,* is problematic due to the sensitivity of these vacuoles to mechanical disruption ***(18,19)***. To circumvent this problem, we exploited the property of the large and spacious PV of *C. burnetii* to sequester low-density latex beads through fusion with LBP. The resulting increased buoyancy of the PV allows fractionation of the vacuole using a gentle method that involves flotation of the vacuole on a discontinuous sucrose gradient. This procedure is similar to one described by Desjardins et al. ***(20)*** for the purification of LBP. Because only professionally phagocytic cell types efficiently internalize inert particles such as latex beads, the murine alveolar macrophage-like cell line MH-S, which supports vigorous growth of *C. burnetii*, was chosen for this procedure.

LBP ultimately mature through the default endocytic pathway to acquire characteristics of a prototypic secondary lysosome ***(21)***. The ability to purify

large quantities of LBP to near homogeneity has allowed their proteome to be extensively analyzed at various stages of maturation. Mature LBP contain over 600 different proteins with about 150 of these currently identified ***(21)***. While the *C. burnetii* PV has lysosomal characteristics similar to LBP, it represents a specialized compartment that displays distinctive behaviors such as promiscuous fusogenicity with endolysosomal vacuoles ***(8)***. Accordingly, the PV likely contains a unique subset of pathogen and/or host proteins that confer its unusual properties. To aid in identification of these proteins, parallel LBP purifications are conducted to provide a reference sample that contains proteins of a typical phagolysosome.

3.1. Generation of C. burnetii PV and LBP

1. Passage confluent MH-S cells in a single T-150 flask equally into 4 T-150 flasks. This split will provide semi-confluent monolayers in new flasks in 24–48 h. Grow cells in RPMI medium with 10% FBS and 0.05 m*M* βME at 37°C in 5% CO_2 (*see* **Note 1**).
2. Prepare a *C. burnetii* inoculum for infecting MH-S cells. Quick thaw a vial containing *C. burnetii* Nine Mile (phase II, clone 4, RSA493) (*see* **Note 2**) in a 37°C water bath, then place on ice. In a 50-mL conical tube, make 10 mL of inoculum consisting of a 1:50 dilution of the *C. burnetii* stock in the tissue culture medium. Discard the tissue culture media from the 2 T-150 flasks and add 4 mL of inoculum to each flask (*see* **Note 3**). Incubate 1 h at room temperature with slow rocking. Save the remaining two flasks to generate uninfected LBP control samples.
3. Add 40 mL of tissue culture media directly to flasks without removing the inoculum. Incubate approximately 36 h at 37°C in 5% CO_2. At this time point, small PV containing *C. burnetii* will be visible by phase contrast light microscopy (*see* **Note 4**).
4. Add 800 µL of Red FluoSpheres fluorescent latex beads to 16 mL of tissue culture media (*see* **Note 5**). Discard the tissue culture media from infected and uninfected T-150 flasks and add 4 mL of bead solution to each flask. Incubate the flasks for 20 min at room temperature with slow rocking, then incubate the flasks at 37°C in 5% CO_2 for 15 min.
5. Remove noninternalized beads from T-150 flasks using a 10-mL pipet. Wash cells gently 3X with 15 mL of tissue culture media warmed to 37°C. Discard the wash media and add 40 mL of tissue culture media to each flask and incubate at 37°C in 5% CO_2 for 12 h to allow trafficking of latex beads to the *C. burnetii* PV (**Fig. 1**) or to phagolysosomes.

3.2. Fractionation of LBP and C. burnetii PV Containing Latex Beads

1. Gently scrape the cells in each flask into the tissue culture media using a cell scraper (*see* **Note 6**). Transfer the cell suspension from each flask to a 50-mL conical tube. Make sure to label tubes containing uninfected and infected samples.

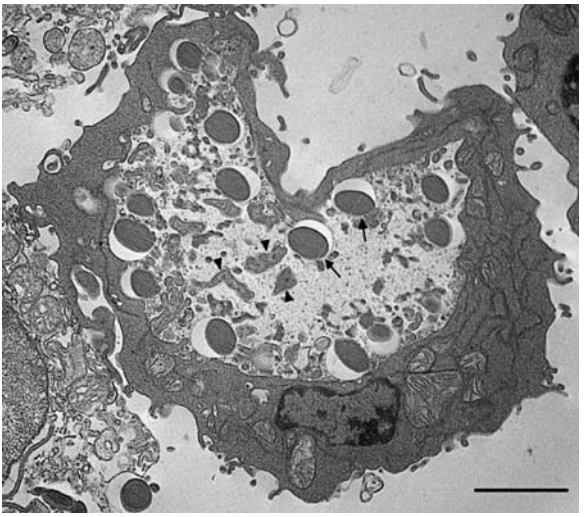

Fig. 1. Latex beads traffic to the *C. burnetii* PV in MH-S cells. MH-S murine macrophage-like cells were infected for 36 h with *C. burnetii,* followed by incubation with 1-μm latex beads for 16 h to allow trafficking of beads to the PV. This transmission electron micrograph shows a moderately sized PV harboring *C. burnetii* (arrowheads) and at least 15 latex beads (Arrows). (Bar, 2.0 μm.)

2. Centrifuge at 900*g* for 5 min at 4°C to pellet cells. Wash cells by resuspending them in 25 mL of cold (4°C) PBS and rocking the samples on ice for 15 min. At this stage in the procedure, combine the infected cell pellets together and the uninfected cell pellets together. Centrifuge at 900*g* for 5 min at 4°C to pellet cells and repeat the washing procedure one more time. Resuspend the final pellet in 2 mL of cold (4°C) homogenization buffer.
3. Transfer the cell suspension to a Kontes Dounce homogenizer that has been precooled on ice. Lyse cells by subjecting cell suspensions to approximately 150 strokes with the "B" pestle. Homogenize until at least 90% of the cells are lysed (*see* **Note 7**).
4. Transfer lysates containing *C. burnetii* PV or LBP to a 15-mL Falcon tube (*see* **Note 8**). Bring the lysate volume to 6 mL with homogenization buffer and place the tube on ice. Adjust the samples to 40% sucrose by adding 8 mL of 62% sucrose solution to the Falcon tube to result in a final volume of 14 mL.
5. Fractionate the *C. burnetii* PV containing latex beads or LBP by flotation on discontinuous sucrose gradients. For each gradient, place 3.5 mL of 62% sucrose solution into a 25 × 89 Ultra-clear centrifuge tube. Gently overlay this solution with the 40% sucrose solution (7 mL) containing *C. burnetii* PV or LBP. Sequentially overly samples with 35, 25, and 10% sucrose solutions (7 mL each). Centrifuge at 104,000*g* for 3 h at 4°C (*see* **Note 9**). *C. burnetii* PV and LBP will "float" to the interface of the 25 and 10% sucrose solutions **(Fig. 2)**.

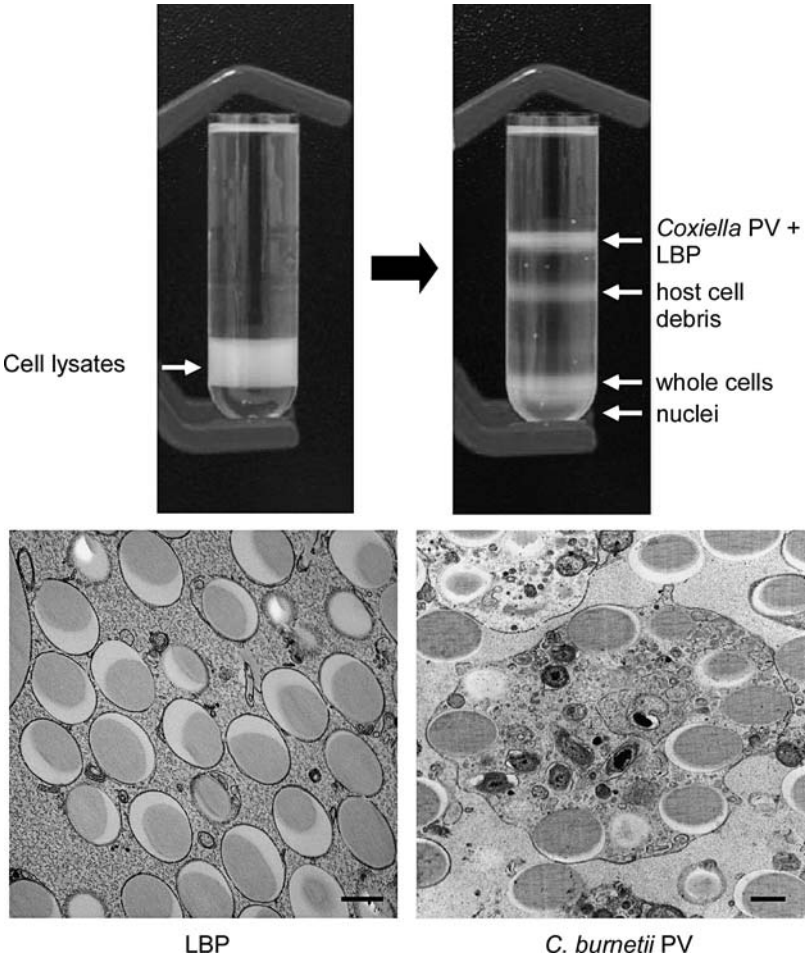

Fig. 2. Trafficking of latex beads to the *C. burnetii* PV facilitates separation of the vacuole on sucrose density gradients. **(A)** Infected MH-S cells were incubated with latex beads and lysed using a Dounce homogenizer. Cell lysates were adjusted to 40% sucrose and subjected to centrifugation of a sucrose step gradient. Following centrifugation, *C. burnetii* PV containing red latex beads band at the 10–25% sucrose interface. Control LBP prepared from uninfected cells band at the same gradient location. **(B)** Transmission electron micrographs showing fractionated control LBP and a *Coxiella* PV containing latex beads. LBPs are tightly bound by an electron-dense phagosomal membrane. The spacious *C. burnetii* PV containing latex beads is surrounded by free latex beads and LBP. Contaminating LBP likely result from the small number of uninfected cells in the cell culture or the small percentage of latex beads that do not traffic to *C. burnetii* PV in infected cells. Contaminating latex beads likely result from PV that rupture during the cell lysis procedure or beads that remain after washing that are not internalized by macrophages. (Bar, 0.5 μm.)

6. Collect the *C. burnetii* PV and LBP fractions (approximately 5 mL each) from the gradients using a 10-mL syringe and cannula. Transfer to a 15-mL Falcon tube and adjust the volume to 12 mL with cold (4°C) PBS.
7. Transfer samples to 14 mm × 95 mm Ultra-clear centrifuge tubes and pellet *C. burnetii* PV or LBP by centrifugation at 41,000*g* for 10 min at (4°C) (*see* **Note 10**).
8. Prepare the samples for subsequent protein analyses (**Subheadings 3.4.** and **3.5.**). Gently discard the PBS supernatant and resuspend the entire PV or LBP pellet in 200 µL of ReadyPrep sequential extraction reagent 3 (Bio-Rad) for isoelectric focusing, or 200 µL of SDS-PAGE sample buffer for slot immunoblotting (*see* **Note 11**). Store samples at –20°C.

3.3. Generation of Lysates of C. burnetii–Infected MH-S Cells

1. Plate 1 × 10^6 MH-S cells in one well of a 6-well tissue culture plate. Quick thaw a vial containing *C. burnetii* Nine Mile (phase II, clone 4, RSA493) in a 37°C water bath, then place on ice. In a 5-mL Falcon tube, make 200 µL of inoculum consisting of a 1:50 dilution of the *C. burnetii* stock in the tissue culture medium. Remove the tissue culture media from the well and add the inoculum (*see* **Note 12**). Incubate 1 h at room temperature with slow rocking. Add 3 mL of tissue culture media to well without removing the inoculum. Incubate for 48 h at 37°C in 5% CO_2 to allow PV to develop to the same stage as fractionated PV.
2. To prepare infected MH-S cell lysates, first remove the tissue culture media from the well using a 10-mL pipet. Wash cells with 3 mL of PBS. Remove buffer and lyse infected cells in situ by adding 200 µL of SDS-PAGE sample buffer directly to the well using a 200-µL capacity micropipetter. Pipet up and down while gently swirling the plate. Transfer the cell lysate to a 1.5-mL microfuge tube (*see* **Note 13**). Rinse the well with an additional 100 µL of sample buffer and transfer the material to the same microfuge tube for a final cell lysate volume of 300 µL. Denature proteins by boiling the sample for 5 min. Store at –20°C.

3.4. Comparison of Lysates of Infected MH-S Cells and Fractionated C. burnetii PV by Slot Immunoblotting

1. These instructions are based upon the use of Bio-Rad's Mini-PROTEAN 3 electrophoresis cell, Mini-Trans Blot cell, and Mini-PROTEAN II multiscreen apparatus.
2. Prepare a 1.0-mm-thick 12% gel by mixing 2 mL of 5X separating buffer with 4 mL of acrylamide/bis solution, 100 µL of 10% SDS, 3.85 mL of water, 50 µL of ammonium persulfate solution, and 5 µL of TEMED. Pour the separating gel, making sure to leave a space for the stacking gel. Overlay the separating gel with water and allow the gel to polymerize (*see* **Note 14**).
3. Prepare a 4% stacking gel by mixing 1.25 mL of 8X stacking buffer with 1.3 mL of acrylamide/bis solution, 7.35 mL of water, 50 µL of ammonium persulfate solution, and 10 µL of TEMED. Remove the water overlaying the separating gel.

Pour the stacking gel and insert the 2-well preparative gel comb (*see* **Note 15**). Let the gel polymerize.

4. Prepare 800 mL of running buffer by diluting 80 mL of 10X running buffer with 720 mL of water.
5. Remove the comb and rinse wells with running buffer. Assemble the gel unit and add running buffer to the upper and lower chambers. Load 100 µL of MH-S cell lysate or PV lysate in SDS-PAGE sample buffer into the large sample well (*see* **Note 16**) and 10 µL of prestained molecular mass markers into the small well.
6. Connect the gel unit to the power supply and run for approximately 80 min at 100 V. Stop the electrophoresis run when the bromophenol blue dye front is at the bottom of the gel.
7. Wet a gel-sized piece of Immobilon transfer membrane in 100% methanol, then soak the membrane in transfer buffer. In a suitably sized tray, saturate two transfer cassette sponges and two gel-sized sheets of 3MM paper. In another tray, open a blot transfer cassette with the black panel down and sequentially place one sponge and one sheet of 3MM paper onto the panel.
8. Disconnect the gel unit from the power supply. Separate the glass plates and remove the stacking gel with a razor blade. Place the gel onto the 3MM paper in the open transfer cassette. Carefully place the Immobilon transfer membrane onto the gel and saturate with transfer buffer to ensure the gel and membrane do not dry out (*see* **Note 17**). Finish assembling the transfer cassette by placing the other piece of 3MM paper over the transfer membrane, followed by a sponge, and then close the transfer cassette using the clamp. Saturate the transfer cassette with transfer buffer and insert the cassette into the transfer chamber. Make sure that the cassette is in the correct orientation for electrophoretic transfer of proteins to the membrane, i.e., with the transfer membrane between the gel and anode.
9. Place a frozen ice pack and a small stir bar into the transfer chamber. Fill the transfer chamber with transfer buffer and place it on a magnetic stirring plate. Transfer the proteins to the Immobilon membrane by electrophoresis at 35 V for 2 h with moderate stirring.
10. Upon completion of the transfer, remove the transfer cassette from the transfer chamber and disassemble. Remove the membrane from the gel with a forceps and block the unoccupied sites on the membrane by incubating in blocking buffer for 20 min at room temperature, or overnight at 4°C.
11. To allow probing in a slot blot format with multiple antibodies, clamp the blocked membrane into the multiscreen apparatus. Make sure that the protein side of the membrane is up relative to the open slots (*see* **Note 18**). Fill empty slots with blocking buffer (approximately 500 µL) (*see* **Note 19**).
12. To determine the relative amounts of selected cellular markers in cell lysate and PV samples, probe the membrane with primary mouse or rat monoclonal antibodies directed against the cellular markers LAMP-1, LAMP-2, GM130, Bip, and nucleoporin p62. Rabbit polyclonal antibody directed against *C. burnetii* will

be used to determine the relative amount of pathogen antigen in each sample. Make 600 µL of a 1:300 dilution of each monoclonal antibody, and a 1:1200 dilution of anti–*C. burnetii* antibody in blocking buffer. Remove the blocking buffer from the slot and replace with diluted primary antibody solution (*see* **Notes 20** and **21**).

13. The primary antibodies are allowed to bind to target proteins by incubating the membrane for 1 h at room temperature with gentle rocking.
14. Dump the primary antibody solutions from the slots into a discard tray. Wash each slot 3X with PBS-T.
15. To the appropriate wells, add HRP anti-mouse or anti-rat (diluted 1:5000) or anti-rabbit immunoglobulin G (diluted 1:30,000) in blocking buffer. Allow secondary antibodies to react with bound primary antibodies by incubating the membrane for 1 h at room temperature with gentle rocking.
16. The secondary antibodies are removed from the slots and the slots are washed three times with PBS-T. At this point, disassemble the slot blot apparatus and transfer the Immobilon membrane to an appropriately sized tray. Wash the membrane two more times for 20 min with 25 mL of wash buffer at room temperature with gentle rocking.
17. Toward the end of the final wash period, place one or more pieces of luminescent tape around a piece of 3MM paper larger than the Immobilon membrane. This will allow easy orientation of the final exposed film relative to the immunoblot. Prepare the ECL reagent by mixing 500 µL of peroxide solution with 500µL of enhancer solution.
18. Discard the final wash solution and thoroughly drain the washing tray. Apply the ECL reagents evenly over the Immobilon membrane using a pipet. Once a minute for 5 min, tip the tray and redistribute the ECL reagents over the blot. Remove the membrane from the tray and dab away the excess ECL reagent with a paper towel. Position the damp membrane on the 3MM paper near the luminescent tape and cover the membrane and 3MM paper with cellophane.
19. The membrane is then placed in an x-ray cassette along with film. A 1-min exposure is usually sufficient to detect bands indicative of antibody–protein interactions **(Fig. 3)**.

3.5. Comparison of Protein Composition of Fractionated LBP and C. burnetii PV by 2-D Gel Electrophoresis and Immunoblotting

1. Two-dimensional (2-D) gel electrophoresis coupled with immunobloting is performed to identify differences in protein composition between *C. burnetii* PV and LBP (*see* **Note 22**). The instructions for isoelectric focusing are based upon the use of Bio-Rad's Protean IEF Cell.
2. The fractionated PV and LBP solubilized in ReadyPrep sequential extraction reagent 3 from **Subheading 3.2.** contain salts and lipids that can result in gel artifacts during 2-D electrophoresis. Therefore, the BioRad ReadyPrep 2-D cleanup kit is used to remove these contaminants prior to isoelectric focusing.

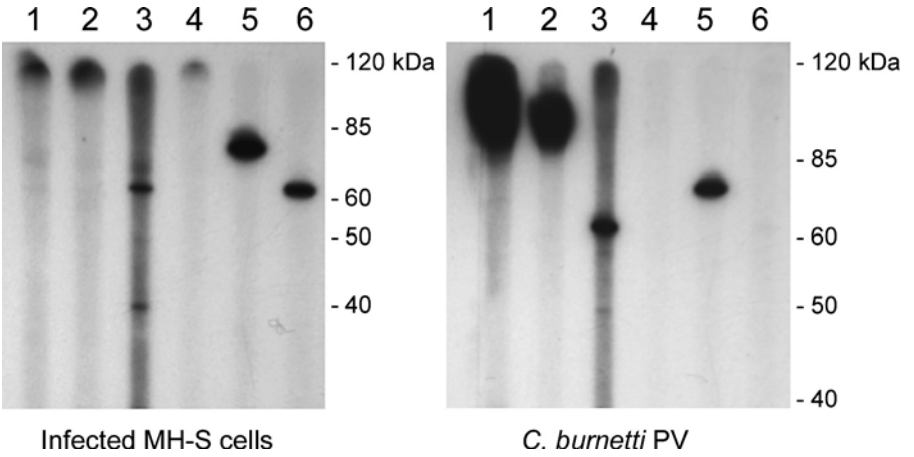

Fig. 3. *C. burnetii* PV lysates are enriched for lysosomal glycoproteins. Equal amounts of infected MH-S cell and purified *C. burnetii* PV lysates were separated by SDS-PAGE on preparative gels and transferred to an Immobilon membrane. Bound antibodies were detected by chemiluminesence. The Golgi protein GM130 (130 kDa) (Lane 4) and nuclear pore protein nucleoporin p62 (62 kDa) (Lane 6) are not detected in PV preparations, while strong signals for the lysosomal proteins LAMP-1 (120 kDa) (Lane 1) and LAMP-2 (120 kDa) (Lane 2) are observed with PV lysates relative to infected MH-S cells lysates. PV lysates are also enriched for *C. burnetii* antigen (Lane 3) relative to infected MH-S cell lysates. These data confirm that the fractionation procedure results in a substantial enrichment of the lysosome-like PV in the absence of detectable contamination by the Golgi and nuclear compartments. Signals of similar intensity for the ER protein Bip (78 kDa) (Lane 5) are associated with infected whole cell and PV lysates. ER proteins may be relevant constituents of the *C. burnetii* PV as recent work has demonstrated ER involvement in the phagocytic process of both latex beads and intracellular pathogens ***(21)***. Moreover, the PV has substantial interactions with ER-derived autophagosomes ***(6)***. Alternatively, the sheer cellular abundance of the ER makes elimination of ER material during subcellular fractionations very difficult ***(24)***. Molecular mass markers are expressed in kDa.

3. To remove contaminants from PV and LBP samples, use reagents supplied with ReadyPrep 2-D cleanup kit. Aliquote 100 µL of each sample to two 1.5-mL microcentrifuge tubes. To each tube, add 300 µL of precipitating agent 1, mix by vortexing, then incubate on ice for 15 min. Precipitating agent 2 (300 µL) is then added to each tube, the samples mixed by vortexing, and the precipitated protein pelleted by centrifugation at 15,000*g* for 5 min. Remove the supernatant immediately from tubes with a micropipetter, centrifuge briefly again, and remove the residual supernatant. Be careful not to disturb the pellet as it may be loose.

4. Wash pellets with 40 µL of wash reagent 1 by placing the reagent over the top of the pellets and centrifuging the tubes at 15,000*g* for 5 min. Remove the wash reagent with a micropipet, add 25 µL of distilled water, and vortex the samples for 10–20 s. The pellets may break apart but they will not dissolve in water. Add 1 mL of cold (4°C) wash reagent 2 and 5 µL of wash reagent 2 additive to sample tubes. Mix the samples by vortexing for 1 min, then incubate samples for 30 min at –20°C. (During this incubation period, vortex the samples for 30 s every 10 min.) Pellet the samples by centrifugation at 15,000*g* for 5 min, then discard the supernatant and briefly air-dry the pellet. Solubilize PV and LBP pellets in 75 µL of 2-D rehydration/sample buffer and combine the respective samples for a final volume of 150 µL for each sample.
5. The isoelectric focusing strips are now equilibrated with solubilized LBP and PV samples. Carefully disperse 150 µL of each sample along the entire length of the rehydration tray well. Try to keep the sample along one edge of each of the well. Place a NL pH 3–10 ReadyStrip gel side down in the well. The sample will be absorbed by the gel component on the underside of the strip. Allow the strips to soak up samples for 1 h at room temperature. Overlay the strips with 1.5 mL of mineral oil and allow the strips to continue equilibrating with samples by incubating the strips for an additional 12 h or overnight.
6. Program the PROTEAN IEF cell to run the following six-step isoelectric focusing program at 20°C: step 1, rapid ramp, 250 V for 30 min; step 2, rapid ramp, 500 V for 30 min; step 3, linear ramp, 4000 V for 2 h; step 4, linear ramp, 8000 V for 1 h; step 5, rapid ramp, 8000 V for 35000 V-h; step 6, rapid ramp, 500 V for 99 h. This final step holds the focused proteins until the strip is removed from the IEF cell.
7. To load the gel strips into the focusing tray, place wetted paper wicks over the cathode and anode electrode wires and place the (+) end of the gel strip toward the (+) electrode of focusing tray. Start the isoelectric focusing program. The run should take approximately 9 h but can vary somewhat according to salt and protein content of the samples.
8. At the end of the focusing run, the isoelectric focusing strips require equilibration in buffers compatible with separation of focused proteins by SDS-PAGE electrophoresis. Remove the isoelectric focusing strip from the focusing tray with a forceps, drain the oil from the strip, and place the strip gel side up in a clean rehydration tray well (*see* **Note 23**). Overlay the strip with 2 mL of equilibration buffer I and incubate at room temperature with rocking for 10 min. Remove the buffer and overlay the strip with 2 mL of equilibration buffer II and incubate at room temperature with rocking for 10 min. Drain the equilibration buffer from the isoelectric focusing strip and briefly dip the strip in SDS-PAGE running buffer.
9. Prepare a 12% acrylamide gel using the procedure described for slot immunoblotting in **Subheading 3.4**. To fit the isoelectric focusing strip into the large well of the preparative polyacrylamide gel, cut approximately 0.75 cm from each end of the strip. (These end portions of the strip overhang the

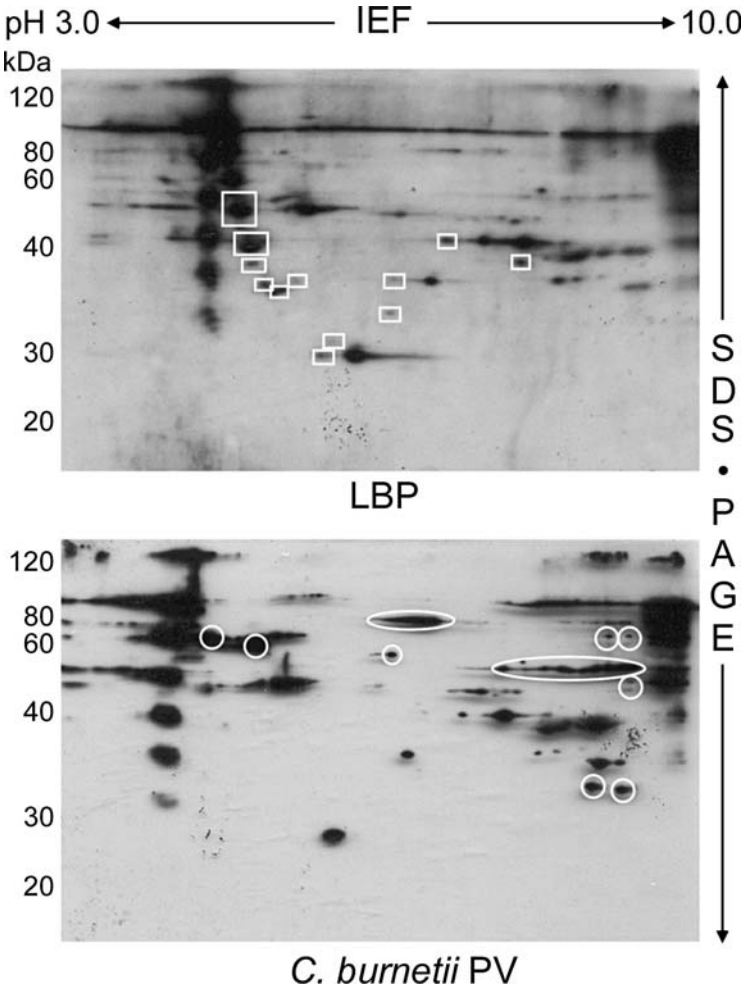

Fig. 4. Two-dimensional immunoblots reveal differences in protein composition between *C. burnetii* PV and LBP. Equal amounts of PV and LBP protein were separated by two-dimensional gel electrophoresis, blotted to Immobilon membrane, then probed with rabbit antiserum generated against total membranes from *C. burnetii*–infected rabbit kidney epithelial cells. Bound antibodies were detected by chemiluminesence. White circles and boxes denote immunoreactive proteins that are more abundant in PV and LBP, respectively. PV-specific proteins may represent host or pathogen factors that modulate the unique biological properties of this vacuole. Molecular mass markers are expressed in kDa.

electrodes in the focusing tray and do not contain focused proteins.) Overlay the isoelectric focusing strip with melted ReadyPrep 2-D overlay agarose, being careful not to fill the small well used for loading molecular weight markers. After the assembled gel is placed in the electrophoresis unit, overlay the top of the isoelectric gel strip with 50 µL of SDS-PAGE sample buffer. This will provide a visible dye front during electrophoresis. Electrophorese and transfer the proteins to an Immobilon membrane as described in **Subheading 3.4**.

10. To detect differences in the protein composition between PV and LBP, blots are probed with a 1:2500 dilution of rabbit antiserum generated against total membranes from *C. burnetii*–infected rabbit kidney epithelial cells. (Details on probing blots with primary/secondary antibodies and chemiluminescent detection of bound antibodies are found in **Subheading 3.4.**) Representative 2-D immunoblots showing differences in immunoreactive proteins between PV and LBP are depicted in **Fig. 4** (*see* **Note 24**).

4. Notes

1. We have found that other professionally phagocytic cells, such as J774.A1 murine macrophage like cells (TIB-67; American Type Culture Collection), also work well for this procedure.
2. The Nine Mile, phase II, clone 4 isolate (RSA439) can be worked with under biosafety level 2 laboratory conditions ***(22)***. All other *C. burnetii* strains or isolates are considered biosafety level 3 organisms.
3. Assuming a stock of approximately 3×10^9 *C. burnetii* RSA439 genome equivalents per mL, and approximately 2×10^7 MH-S cells per T-150 flask, this infection procedure will result in a multiplicity of infection of approximately 10. The low inoculum volume with rocking facilitates adherence and internalization of *C. burnetii*.
4. The small to medium-sized PV observed at 36–48 h postinfection work best for this procedure. The *C. burnetii* PV becomes very large and more fragile at later time points postinfection ***(7)***.
5. Fluorescent beads are not necessary for this procedure. However, the red color allows easy visualization of bead internalization and intracellular trafficking as well as the separation of PV and LBP on sucrose gradients.
6. The specific Sarstedt cell scrapers recommended for this procedure are comprised of soft rubber. Their use minimizes cell lysis during the cell detachment procedure.
7. The degree of lysis is easily assessed by placing 5–10 µL of lysate under a glass cover slip and viewing the material by phase contrast light microscopy. Greater than 90% cell lysis in the absence significant breakage of the nucleus typically requires approximately 150 strokes of the Dounce pedestal.
8. Earlier versions of this protocol included a centrifugation step to pellet nuclei and unbroken cells with the resultant postnuclear supernatant then separated on

a sucrose gradient. We found this step to be unnecessary as these components are effectively pelleted or fractionated to the bottom of sucrose density gradients **(Fig. 2)**.

9. This centrifugal force is attained using a Beckman SW28 rotor at 24,000 g.
10. This centrifugal force is attained using a Beckman SW40 rotor at 18,000 g.
11. We have found that the latex beads, when carried through the various solubilization and clean-up steps of this protocol, do not affect on the behavior of proteins in isoelectric focusing and SDS-PAGE procedures.
12. Assuming a stock of approximately 3×10^9 *C. burnetii* RSA439 genome equivalents per mL and approximately 1×10^6 MH-S cells per well, this infection procedure will result in a multiplicity of infection of approximately 10. The low inoculum volume with rocking facilitates adherence and internalization of *C. burnetii*.
13. Cell lysates resulting from the initial application of sample buffer will be viscous and difficult to remove. The second 100-µL sample buffer wash and harvest will ensure quantitative recovery of material.
14. This is sufficient for pouring two gels. When more gels are required, increase the recipe accordingly.
15. The preparative comb should consist of one small outside well for the loading of molecular mass markers and one large well extending the remaining width of the gel for the loading of sample.
16. Commercial protein assays can be used to normalize sample loadings. Alternatively, analysis of the staining intensity of SDS-separated experimental samples stained with Coomassie brilliant blue will facilitate equal loading of samples for immunoblots.
17. For complete transfer of separated proteins, it is essential that there are no bubbles between the gel and transfer membrane. Trapped bubbles can be squeezed from underneath the membrane by rolling a piece of a 10-mL plastic pipet over the top of the membrane.
18. The transferred molecular markers will take up the first slot in the slot blot apparatus. This region of the blot, and possibly the region bounded by the second slot, will not contain sample protein. Thus, these two slots should not be loaded with antibodies. The markers of the protein side of the membrane will appear the darkest.
19. A 1000-µL capacity micropipet works well for adding antibody solutions and blocking buffer to slots. To avoid bubbles in the antibody solutions, tip the slot blot apparatus up by placing it on top of a 10-mL plastic disposable pipette. Pipet the solutions to the bottom of the slots allowing the solution to fill the slot from the bottom to the top. A squirt bottle containing PBS-T works well to wash individual slots of the slot blot apparatus.
20. The optimal dilutions of primary and secondary antibodies may have to be empirically determined.

21. The same micropipet tip can be used to remove the blocking buffer and the primary antibody from an individual slot. Remember to record the slot number for each antibody. Leave blocking buffer in unused slots.
22. Silver staining of 2-D gels will also reveal unique proteins between *C. burnetii* PV and LBP.
23. Alternatively, gel strips may be frozen at this time by placing them gel side up in a 15-mL Falcon tube, sealing the tube, and storing the tube at –80°C with the gel side up until it is convenient to run the second dimension.
24. A method to identify unique or upregulated proteins involves overlaying an immunoblot film with a parallel silver-stained gel. The proteins corresponding to immunoreactive spots can then be located, excised from the gel, and identified by mass spectrometry ***(23)***.

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