Rickettsia conorii O antigen is the target of bactericidal Weil–Felix antibodies

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Rickettsial diseases have long been diagnosed with serum antibodies cross-reactive against Proteus vulgaris (Weil–Felix reaction). Although Weil–Felix antibodies are associated with the development of immunity, their rickettsial target and contribution to disease pathogenesis are not established. Here, we developed a transposon for insertional mutagenesis of \textit{Rickettsia conorii}, isolating variants defective for replication in cultured cells and in spotted fever pathogenesis. Mutations in the \textit{polysaccharide synthesis operon (pso)} abolish lipopolysaccharide O-antigen synthesis and Weil–Felix serology and alter outer-membrane protein assembly. Unlike wild-type \textit{R. conorii}, pso mutants cannot elicit bactericidal antibodies that bind O antigen. The pso operon is conserved among rickettsial pathogens, suggesting that bactericidal antibodies targeting O antigen may generate universal immunity that could be exploited to develop vaccines against rickettsial diseases.

transposon mutagenesis | lipopolysaccharide | O antigen | Weil–Felix reaction | polysaccharide synthesis operon

Antibodies directed against bacterial surface carbohydrates, i.e., the capsular polysaccharide or the O antigen of lipopolysaccharide (LPS), activate, complement, and promote microbial killing (1). Bactericidal antibodies are acquired during colonization or invasive disease with pathogens such as \textit{Escherichia coli}, \textit{Haemophilus influenzae}, and \textit{Neisseria meningitidis} or following immunization with carbohydrate-conjugate vaccines (2–4). Bactericidal antibodies represent a correlate for immunity; however, disease protection is limited to specific pathogen serotypes and countered by the selection of variants with distinct carbohydrate antigens (5). Rickettsial pathogens rely on hemophagous arthropods for host transmission and disease pathogenesis (6). Due to their obligate requirement for intracellular replication, \textit{Rickettsia} spp. cannot be propagated on laboratory media (7). Rickettsial diseases have therefore been diagnosed with the Weil–Felix serology, the detection of IgG or IgM cross-reactive with \textit{Proteus vulgaris} (8). Increased Weil–Felix serology has been associated with positive clinical outcome and with protective immunity (9). For survivors of epidemic typhus, waning Weil–Felix serology has been observed in patients with recrudescence typhus (Brill–Zinsser disease) (10). Administration of epidemic typhus vaccines, for example extracts from lice infected with \textit{Rickettsia prowazekii}, elicits Weil–Felix antibodies and disease protection (11). Nevertheless, the rickettsial target of Weil–Felix antibodies and its contribution to rickettsial disease pathogenesis are not established.

Here, we developed a transposon mutagenesis technology enabling facile isolation of insertional mutants defective in the obligate intracellular life cycle of \textit{Rickettsia conorii}. We describe that \textit{R. conorii} variants harboring transposon insertions in the conserved \textit{polysaccharide synthesis operon} cannot produce O-antigen polysaccharides and fail to induce Weil–Felix serology with a significant virulence defect in the murine infection model. The results suggest that humans may develop protective immunity against pathogenic rickettsiae by targeting the conserved carbohydrate epitope present in the O antigen of LPS.

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The authors declare no conflict of interest.

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Data deposition: Whole-genome sequencing data for wild-type \textit{R. conorii} Malish 7 (accession no. SRR8404401) and rifampin-resistant \textit{R. conorii} (accession no. SRR8404402) are deposited in the Sequence Read Archive.

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transposon under control of the R. rickettsii rompB promoter (P_{rompB}) (Fig. 1A). The minitransposon was named kkaebi. kkaebi DNA was PCR amplified from pHTRL3 template DNA, cleaved with restriction enzyme (RE), and incubated with TsI transposase to generate transposome complexes that, when electroeluted into R. conorii and selected on Vero cell cultures in the presence of chloramphenicol, generated insertional mutants at a frequency of $5 \times 10^{-3}$/pg kkaebi. Isolated variants were cataloged with HK numbers in the R. conorii mutant library database (SI Appendix, Table S1). After 4 d of plaque expansion in Vero cells, R. conorii HK mutants were purified via gradient density centrifugation and absorption at 600 nm ($A_{600}$) was determined. Most of the kkaebi mutants replicated to the same level as wild-type R. conorii (Fig. 1B). However, $A_{600}$ and PFU for the HK2, HK24, HK27, HK28, and HK50 variants were lower than those of wild-type R. conorii (Fig. 1B). Sequence analysis of the transposon insertion sites revealed that all R. conorii variants harbored single insertions of kkaebi distributed randomly across the rickettsial genome (Fig. 1C and SI Appendix, Table S1). Alignment of the 9-nucleotide region for each recorded insertion revealed that transposition of kkaebi is not biased by specific nucleotide sequences (Fig. 1D).

Genes Involved in the Biosynthesis of the O-Antigen Polysaccharide and Weil-Felix Serology. Sequence analysis identified 2 variants, HK2 and HK15, with kkaebi insertions in Rc0457 and Rc0459. Rc0457 and Rc0459 are located in a gene cluster here designated as the rickettsial polysaccharide synthesis operon (pso) (Fig. 2A and SI Appendix, Fig. S1). The first 3 genes (Rc0456 to Rc0458) of pso are conserved among all pathogenic Rickettsia and exhibit sequence homology with 3 gene products from P. vulgaris (3074 to 3076) and Vibrio cholerae (wrbB, wbrR, and wbdD) (SI Appendix, Fig. S2). When expressed and purified from E. coli, WbrB/WbrR/WbdV catalyze synthesis of UDP-$\alpha$-QuiNAc (N-acetyl-$\alpha$-QuiNAc, a key constituent of the O antigen of V. cholerae, P. vulgaris, and Rickettsia (SI Appendix, Fig. S2)) (17–19). The Rickettsia typhi O antigen comprises the polysaccharide repeat $[\rightarrow-\alpha-D-Glc-(1\rightarrow3)-\alpha-L-QuiNAc(\rightarrow-\alpha-D-Glc-(1\rightarrow3)-\alpha-L-QuiNAc-(\rightarrow-\alpha-D-Glc-(1\rightarrow3)-\alpha-L-QuiNAc-(\rightarrow-\alpha-D-Glc-Nac(\rightarrow-1)]$ with a short side chain $[\alpha-D-Glc-Nac(\rightarrow-1)-\alpha-L-QuiNAc-(\rightarrow-\alpha-D-Glc-Nac(\rightarrow-1)]$ linked to $\alpha-L$ QuiNAc (20). Of note, the disaccharide motif, $[\alpha-L-$QuiNAc (\rightarrow-1)-$\alpha-D-$GlcAc], is also present in the O antigen of P. vulgaris OX19 (21). However, in P. vulgaris OX2, which does not generate cross-reactive Weil-Felix antibodies with R. typhi, the disaccharide unit is linked to the O-antigen repeats via a $\beta$-glycosidic bond (21).

To determine whether the R. conorii variants HK2 and HK15 exhibit O-antigen synthesis defects, we purified R. conorii LPS and analyzed molecules by acrylamide gel electrophoresis and silver staining. As expected, LPS from wild-type R. conorii migrated as a spectrum of molecules with tethered O-antigen repeats and ladder-like appearance on polyacrylamide gels (Fig. 2B). LPS isolated from the HK15 mutant exhibited a similar migration pattern (Fig. 2B). In contrast, a single LPS species was purified from the HK2 variant, which migrated faster on the polyacrylamide gel than wild-type and HK15 LPS (Fig. 2B). Immunoblot analysis with R. conorii-specific human antisera ($\alpha$-Rc0459) revealed antibodies that bound LPS from wild-type and HK15 R. conorii, but not LPS from the HK2 variant (Fig. 2C). Rabbit IgG raised against R. conorii LPS was used to immunize mice with purified LPS, and LPS-specific antibodies bound LPS from wild-type and HK15 R. conorii, but displayed only weak binding for HK2 LPS (Fig. 2D). Rabbit IgG raised against purified HK2 and HK15 LPS ($\alpha$-LPS_{HK2} and $\alpha$-LPS_{HK15}) bound to the cognate LPS antigens but did not exhibit cross-reactivity with LPS from wild type or the other mutant strain (Fig. 2D). The LPS O-antigen biosynthesis defect in R. conorii HK2 was in part restored by transformation with pHTRL8. This plasmid carries Rc0457 and upstream promoter sequences as well as Rc0458 to Rc0460 (SI Appendix, Fig. S3). Rc0457 encodes UDP-GlcNAc 4,6-dehydratase/3,5-epimerase, an enzyme that is essential for QuiNAc and O-antigen polysaccharide synthesis in V. cholerae. Next, we performed immunoblot analyses with affinity-purified LPS from P. vulgaris OX2 and OX19 and Proteus mirabilis OXK (Fig. 2E). As expected, $\alpha$-Rc0456, but not $\alpha$-O_{P. vulgaris} (human immune serum from individuals infected with Orientia tsutsugamushi) harbored Weil-Felix antibodies against OX2 and OX19 LPS (Fig. 2F). Finally, rabbit $\alpha$-LPS_{HK2}, but not rabbit $\alpha$-LPS_{HK2} or $\alpha$-LPS_{HK15}, recognized P. vulgaris OX2 LPS (Fig. 2G). As previously reported, we detected OX19 LPS-specific antibodies in naive rabbit serum, which prohibited further cross-reactivity analysis (22). Together these data indicate that R. conorii pso encodes genes for the synthesis of the O antigen of LPS, which represents the rickettsial target of Weil-Felix antibodies.

**Without O-Antigen Synthesis, R. conorii Displays Altered Outer-Membrane Protein Content and Reduced Host Cell Invasion Activity.** We wondered whether the O-antigen synthesis defects of pso mutants interfere with the assembly of rickettsial outer-membrane proteins. Outer-membrane extracts of wild-type R. conorii and the pso mutant strains HK2 and HK15 were analyzed by Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which revealed increased abundance of proteins migrating at 190, 120, and 32 kDa from the outer membrane of R. conorii HK2 (Fig. 3A). Corresponding gel slices were excised and tryptic peptides of proteins analyzed by mass spectrometry (MS) with in silico comparison of tryptic peptides derived from the R. conorii genome (SI Appendix, Table S2). The data identified rOmpA (190 kDa), rOmpB (120 kDa), and rOmpB (32 kDa) as the most abundant species. Of note, the 32-kDa gel slice harbored proteins from 2 additional outer-membrane proteins, rOmpA and Sca1. rOmpA and rOmpB are members of the autotransporter superfamily, forming surface (S) layers that contribute to...
rickettsial invasion of host cells (23–25). Autotransporters are synthesized as large precursor species with N-terminal signal peptides for secretion via the Sec pathway. The Bam complex subsequently translocates and assembles autotransporters in the outer membrane (26). During assembly, the N-terminal passenger domain is cleaved and displayed on the bacterial surface, while the C-terminal β-barrel domain functions to anchor the passenger domain in the outer membrane. Outer-membrane samples of R. conorii strains were subjected to immunoblotting with monoclonal antibodies specific for the 190-kDa passenger domain of rOmpA or polyclonal antibodies specific for the 32-kDa β-barrel domain of rOmpA. Compared to wild-type R. conorii and the HK15 mutant strain, the abundance of the rOmpA passenger and β-barrel domains was increased in R. conorii HK2 (Fig. 3B). Of note, the β-barrel domain exhibited increased mobility in R. conorii HK2, suggesting that the O-antigen synthesis defect altered not only the abundance of the autotransporter but also its proteolytic cleavage. Immunoblotting with polyclonal antibodies against rOmpB also revealed an increased abundance of this autotransporter in R. conorii HK2 compared with naive rabbit serum. (Fig. 2, B) Silver-stained polyacrylamide gel of affinity-purified LPS from R. conorii wild type (WT) or the pso variants HK2 and HK15. (A) Immunoblot of R. conorii LPS with R. conorii (α-RcLPS) or O. tsutsugamushi (α-OtLPS) convalescent human sera. (D) Rabbit antisera raised against affinity-purified LPS from R. conorii wild type (α-LPSpso) or the pso variants HK2 (α-LPSHK2) and HK15 (α-LPSHK15) were examined for antibodies against WT, HK2, and HK15 LPS and compared with naive rabbit serum. (E) Silver-stained polyacrylamide gel of affinity-purified LPS from P. vulgaris OX2 and OX19 and P. mirabilis OXK. (F) Immunoblot of P. vulgaris OX2 and OX19 and P. mirabilis OXK LPS with α-RcLPS or α-OtLPS. (G) α-LPSWT, α-LPSHK2, or α-LPSHK15 was examined for antibodies cross-reactive against P. vulgaris OX2 and OX19 and P. mirabilis OXK LPS.

Defects in O-antigen synthesis and autotransporter display in R. conorii HK2 were also associated with reduced attachment to Vero cells (WT, 4.0 log10 PFU; HK2, 2.4 log10 PFU, P < 0.0001 at 1-h postinoculation; Fig. 3C). In pairwise comparisons of rickettsial replication at timed intervals, wild-type and HK15 R. conorii expanded at similar rates (WT, 7.5 log10 PFU; HK15, 7.1 log10 PFU at 6 d postinoculation, P > 0.05; Fig. 3C). In contrast, R. conorii HK2 replicated at a slower rate (HK2, 5.8 log10 PFU at 6 d postinoculation; WT vs. HK2, P < 0.0001; Fig. 3C). The defects in host cell attachment and intracellular growth were restored when R. conorii HK2 was transformed with pHTRL8 [WT, 7.3 log10 PFU; HK2 (pHTRL7), 5.8 log10 PFU; HK2 (pHTRL8), 7.0 log10 PFU at 6 d postinoculation; WT vs. HK2 (pHTRL7), P < 0.0001; WT vs. HK2 (pHTRL8), P > 0.05; SI Appendix, Fig. S3]. In addition to the attachment defect, the HK2 mutant produced a significantly reduced cytopathic area on Vero cell cultures after 3 d of infection (WT, 7.5 ± 0.6 × 104 μm2; HK2, 0.8 ± 0.1 × 104 μm2; HK15, 4.0 ± 0.3 × 104 μm2; WT vs. HK2, P < 0.001; WT vs. HK15, P < 0.001; Fig. 3D and E). Electron microscopy analysis of cytopathic Vero cells identified R. conorii, mostly within the cytoplasm of host cells, without significant changes in bacterial size and shape (Fig. 3F and SI Appendix, Fig. S4).

R. conorii O-Antigen Synthesis Is Required for Spotted Fever Pathogenesis. To investigate whether R. conorii HK2 or HK15 exhibits virulence defects in the mouse model for acute disease, cohorts of mice were inoculated i.v. with 1 × 106 PFU wild-type R. conorii or its HK2 or HK15 variants. Animals infected with wild-type R. conorii exhibited disseminated vascular disease with...
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3) (infection < locus is conserved lesions. Even R. conorii < variants at day 3 HK2 did HK2 abolishes the synthesis of the O-antigen repeats α HK2 or HK15 Rickettsia parkeri Rickettsia 0.05; ** 23 for HK15). Data are B 0.0001) during the first 7 d of infection followed by a postinfection (Fig. 3C). After 14 d, infection with wild-type R. conorii (α-WT) elicited IgG antibody responses against rickettsial LPS that were not observed in animals infected with R. conorii HK2 or HK15 (Fig. 4D). However, preexisting antibodies recognizing OX2 and OX19 LPS in naive mice prevented further correlation between R. conorii infection and positive Weil–Felix serology (Fig. 4E). Importantly, α-WT, but neither α-HK2 nor α-HK15, promoted complement-mediated killing of R. conorii in mouse plasma (Fig. 4F).

Discussion

Our results demonstrate that the R. conorii pso locus is responsible for O-antigen biosynthesis, contributes to the pathogenesis, and is essential for the development of bactericidal Weil–Felix antibodies. In light of these findings, bactericidal Weil–Felix antibodies can be assigned a role in protective immunity, supporting earlier clinical observations associating increased Weil–Felix antibodies with survival and convalescence and bactericidal Weil–Felix antibodies with protective immunity in individuals receiving whole-cell Rocky Mountain spotted fever or epidemic typhus vaccines (27, 28). Although the pso locus is conserved among spotted fever- or typhus-causing Rickettsia, there exist variations in pso gene content among the 2 groups. We hypothesize that the genetic differences of the pso locus are responsible for differences in Weil–Felix serology as, for example, spotted fever agents, but not typhus agents, elicit antibodies that are cross-reactive with P. vulgaris OX2 (29). Nevertheless, similar to carbohydrate-specific bactericidal antibodies in established bacterial vaccines (5), the discovery of the pso locus may be exploited to generate O-antigen specific subunit vaccines against spotted fever and typhus agents whose bactericidal antibodies are likely to provide a correlate for protective immunity against the corresponding rickettsial diseases.

We show here that chloramphenicol is a suitable antibiotic to select for R. conorii variants with insertional kkaebi lesions. Even if the insertional lesion disrupts a gene that contributes to tissue cell invasion or intracellular replication, some of these mutants will exert partial phenotypes of delayed replication, diminished invasion, or reduced cell-to-cell spread that should allow their isolation and phenotypic characterization. Thus, a library of several thousand R. conorii mutants with mapped insertional lesions may be tremendously useful for the field of rickettsial biology in assigning function to the more than 800 genes that remain as of yet uncharacterized. Others have initiated similar insertional mutagenesis studies focusing on Rickettsia parkeri, Rickettsia rickettsii, and R. prowazekii (30–33). Together, the results from all of these studies should enable comparative genetic analyses of rickettsial species in the near future, which would be a tremendous boost for a field that for many years was hampered by the lack of tools for genetic studies.

We think it is likely that the insertional disruption of Rc0457 in R. conorii HK2 abolishes the synthesis of the O-antigen repeats within LPS, allowing the mutant to synthesize a rudimentary lipid A core molecule that cannot be further modified. LPS is a major outer-membrane component essential for the growth of many gram-negative bacteria. The biosynthesis and transport of LPS are tightly controlled and coupled to the synthesis and assembly of other cell envelope components, such as peptidoglycan and S-layer proteins, to prevent loss of outer-membrane integrity (34, 35). We envision that the altered composition of the outer
membrane, notably increased surface proteins rOmpA and rOmpB, contributes to the survival of the HK2 variant under high osmolarity pressure. It would be interesting to learn the detailed structure of \textit{R. conorii} O antigen and to compare its structure with relevant O antigens from spotted fever- and typhus-Rickettsia as well as \textit{P. vulgaris} and \textit{V. cholerae}. Such analyses, combined with the study of antibodies that cross-react with specific LPS molecules, as is provided here, should resolve remaining questions on how certain bacteria elicit antibodies cross-reactive to rickettsial LPS, where these antibodies bind, and how they may be exploited for the design of immune therapeutics and vaccines against \textit{Rickettsia}.

Materials and Methods

Detailed information describing materials and methods is provided in SI Appendix, Materials and Methods. \textit{kkaebi} Transposon Mutagenesis. The \textit{kkaebi} mini-transposon DNA was PCR amplified (PCRf, 5′-AAAGACGGTGTCTTTAGCATACACACTC-3′; PCRe, 5′-GACAGGCTGCTATTACACACCACTC-3′; PcrF, 5′-GACAGGCTGCTTTAGCATACACACTC-3′), digested with PshAI, purified, and incubated with Tn5 transposase (Lucigen; 10 μg \textit{kkaebi} mixed with 2 units Tn5) for 24 h at room temperature to generate transpososome complexes. The transpososome complexes were dialyzed against 250 mM sucrose prior to electroporation (3 kV cm⁻¹, 200 Ω, 25 μF, 5.0 ms; Gene Pulser Xcell, Bio-Rad) into electrocompetent \textit{R. conorii} prepared by washing 3 times in cold 250-mM sucrose. \textit{kkaebi} variants were immediately recovered with DMEM supplemented with 5% HI-FBS and incubated on well tissue culture plates of confluent Vero cells. After 1 h incubation at 34 °C in 5% CO₂ atmosphere, 6-well plates were overlaid with 0.5% agarose in DMEM supplemented with 5% HI-FBS. Infected cells were incubated at 34 °C, 5% CO₂ for an additional 5 h and treated with chloramphenicol (MP Biologicals) at a final concentration of 0.3 μg ml⁻¹ to select for \textit{kkaebi} variants. Mutants were isolated via plaque formation after 4 to 15 d of incubation in Vero cell cultures. To expand mutant strains, isolated plaques were resuspended in 2 mL DMEM with 5% HI-FBS and inoculated on each well of 6-well plates of confluent Vero cells. After 1 h incubation at 34 °C in 5% CO₂ humidified chamber, medium was aspirated and replaced with fresh DMEM with 5% HI-FBS and 0.29 μg ml⁻¹ chloramphenicol. At 4 d postinfection, when monolayers of Vero cells were fully infected, Vero cells were mechanically disrupted with 3-mm glass beads, releasing intracellular \textit{R. conorii}. After host cell debris was removed by centrifugation (1,000 × g, 4 °C, 5 min), the supernatant containing \textit{R. conorii} was transferred to 225-cm² flasks of confluent Vero cells to expand at 34 °C, 5% CO₂ for 4 d. \textit{Rickettsiae} were purified from Vero cells by differential centrifugation through 33% MD-76R solution (21,000 × g, 4 °C, 20 min), washed in Sucrose-Phosphate-Glutamate (SPG) buffer (218 mM sucrose, 3.8 mM KH₂PO₄, 7.2 mM KH₂PO₄, 4.9 mM L-glutamate, pH 7.2), and suspended in 1 mL SPG buffer. A₀₂₀₀ was measured with bacterial samples diluted in SPG buffer.

Nucleotide Sequence Analysis. Blogo sequence analysis was conducted with type 2 logos and base representation calculated from the 9-bp nucleotide sequences flanking the \textit{kkaebi} insertion sites (36). The background frequencies of A, C, G, and T used for the Blogo analysis were 0.35, 0.19, 0.17, and 0.29, respectively.

Outer-Membrane Fractionation. Outer-membrane fractionation was conducted based on a previously published protocol (37). The centrifugation sediments of \textit{R. conorii} wild-type and \textit{pos} variants or overnight cultures of \textit{P. vulgaris} were suspended in 500 μL of buffer A (200 mM Tris HCl, 1 M sucrose, 1 mM EDTA, pH 8.0) and mixed with 100 μL of lysozyme (5 mg ml⁻¹ in \textit{dH}_{₂}O). After 5 min incubation at room temperature, 2 mL of \textit{dH}_{₂}O was added and incubated for 20 min at room temperature. Then, 3 mL buffer B (50 mM Tris HCl, 2% Triton X-100, 10 mM MgCl₂, pH 8.0) and 50 μL of DNase I (1 mg ml⁻¹ in \textit{dH}_{₂}O; Sigma) were added and incubated for 20 min at room temperature. The mixture was ultracentrifuged at 160,000 × g for 60 min at 4 °C. The sediment was suspended in 500 μL of buffer C (200 mM Tris HCl, 2% SDS, 10 mM EDTA, pH 8.8) and used for subsequent analyses.

Affinity Purification of Lipopolysaccharide. LPS was affinity purified using polymyxin B-agarose (38). Specifically, a fractionated outer-membrane sample was diaлизed (2-kDa molecular weight cutoff; Thermo Scientific) twice against 4 L \textit{dH}_{₂}O at room temperature. This solution was brought to 50 mM Tris HCl, pH 7.5, mixed with 20 μL of protease K (10 mg ml⁻¹ in \textit{dH}_{₂}O; Sigma), and incubated at 55 °C for 5 h. The crude polysaccharide solution was diaлизed (2-kDa molecular weight cutoff; Thermo Scientific) against 4 L \textit{dH}_{₂}O at 4 °C overnight and brought to 100 mM \textit{NaHCO₃}, pH 8.0, 0.9% NaCl. The crude polysaccharide sample was applied to a 2-mL poly- myxin B-agarose (Sigma) column and incubated at 4 °C for 16 h, followed by washing with 10 mL of wash buffer (100 mM \textit{NaHCO₃}, pH 8.0). LPS was eluted from the column with 10 mL of elution buffer (1% deoxycholic acid in

Fig. 4. \textit{R. conorii} O-antigen synthesis is required for pathogenesis. (A and B) Kaplan–Meier analysis for survival (A) and body-weight analysis (B) of C3H/HeN mice (n = 10) infected with 1 × 10⁶ PFU \textit{R. conorii} WT or the \textit{pos} mutants HK2 and HK15 or mock infected (Mock). (C) Body-weight analysis of C3H/HeN mice (n = 10) infected with 1 × 10⁶ PFU of \textit{R. conorii} WT and \textit{pos} mutant strains. Data are representative of 2 independent experiments. The proportion of survival was analyzed using the 2-tailed log-rank test. Two-way ANOVA with Bonferroni posttests were performed to analyze the statistical significance of body-weight change. (D) Immunoblotting of affinity-purified LPS from \textit{R. conorii} WT, HK2, or HK15 with α-\textit{WT}, α-HK2, or α-HK15 mouse immune serum. (E) Immunoblotting of affinity-purified LPS from \textit{P. vulgaris} OX2 or OX19 with α-\textit{WT} or naïve mouse sera. (F) Survival of \textit{R. conorii} in mouse plasma mixed with naïve, α-\textit{WT}, α-HK2, or α-HK15 mouse immune serum (n = 3). Data are the mean (±SEM) of 3 independent determinations. Statistically significant differences were analyzed with 1-way ANOVA with Dunnett’s posttest. *P < 0.05; **P < 0.001; ***P < 0.0001.
100 mM NaH2HCO3, pH 8.0) and extensively dialyzed against 4 L of deoxycholic acid removal buffer (4 mM Tris-HCl, pH 8.0, 0.25% NaCl, 10% ETOH), followed by dialysis against 250 mL. LPS samples were concentrated using a Speed-Vac and stored at 4 °C.

SDS-PAGE and Immunoblotting. Samples were mixed with sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and boiled at 95 °C for 10 min. Samples were separated on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue R-250 for detection of outer-membrane proteins (Amsbio). A silver-staining kit was used for detection of LPS (Bio-Rad). For immunoblot analyses, samples were electrophoretically transferred from the gel onto a 0.22-μm PVDF membrane (GE Healthcare). The membrane was immersed in blocking buffer (TBS-T [100 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween-20] with 5% milk) for 1 h at room temperature. The membrane was washed and incubated in a solution containing primary antibodies or antisera for 1 h at room temperature. OmpA-specific mouse monoclonal antibody 1:10,000 (against R. conorii) or 1:1,000 (against R. vulgaris) dilution in TBS-T. Mouse hyperimmune sera collected from R. conorii-infected mice were used at a 1:1,000 dilution (against R. conorii) or 1:625 (against P. vulgaris) dilution in TBS-T. Human antisera collected from patients with confirmed diagnoses of R. conorii or O. tsutsugamushi infections were used at a 1:1,000 dilution in TBS-T (kindly provided by Ranjan Premaratna, University of Kelaniya) (41). Of note, controls to store and use human antisera for research and diagnosis purposes were obtained at the time of sample collection. This study was approved by the ethics review committee, Faculty of Medicine, University of Kelaniya (IRB reference no. P/106/04/2018). The membrane was washed 3 times and incubated with peroxidase-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG [Cell Signaling] and anti-human IgG [Abcam]) at a 1:10,000 dilution in TBS-T for 1 h at room temperature. After a final wash, the membrane was developed using SuperSignal West Pico PLUS (Thermo Scientific) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

R. conorii Survival in Mouse Plasma. Whole blood was collected by cardiac puncture of C3H/HeN mice (Charles River Laboratories) and antiocoagulated with 10 μg/mL desirudin (Marathon Pharmaceuticals). Plasma was generated by centrifugation of desirudin-treated blood (1,000 × g for 5 min at 4 °C, followed by 10,000 × g for 3 min at 4 °C) for removal of blood cells. The hyperimmune sera samples were heat inactivated at 56 °C for 30 min, followed by incubation on ice for 5 min. Aliquots (50 μL) of 5 × 108 PFU R. conorii were opsonized with 50 μL of hyperimmune sera on ice for 10 min and then heated to 37 °C for 10 min to prepare or heat-inactivated plasma (200 μL). The infected samples were incubated at 37 °C with rotation for 60 min, at which time all plasma samples were incubated on ice and brought to 1 mL volume with ice-cold DMEM with 5% HI-FBS. Infectious R. conorii titers were determined by plaque assay. R. conorii survival was calculated as the percentage of the average R. conorii initial inoculum at 60 min.

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