Title: *Coxiella burnetii* isolates cause genogroup-specific virulence in mouse and guinea pig models of acute Q fever

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Running Title: *Coxiella burnetii* isolate virulence

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ABSTRACT

Q fever is a zoonotic disease of worldwide significance caused by the obligate intracellular bacterium *Coxiella burnetii*. Humans with Q fever may experience an acute flu-like illness and pneumonia and/or chronic hepatitis or endocarditis. Various markers demonstrate significant phylogenetic separation between and clustering among isolates from acute and chronic human disease. The clinical and pathologic responses to infection with the following phase I *C. burnetii* isolates from four genomic groups were evaluated in immunocompetent and immunocompromised mice and in guinea pig infection models: group I (Nine Mile, African, and Ohio), group IV (Priscilla and P), group V (G and S), and group VI (Dugway). Isolates from all groups produced disease in the SCID mouse model, and genogroup-consistent trends were noted in cytokine production in response to infection in the immunocompetent mouse model. Guinea pigs developed severe acute disease when aerosol-challenged with group I isolates, mild to moderate acute disease in response to group V isolates, and no acute disease when infected with group IV and VI isolates. *C. burnetii* isolates have a range of disease potentials; isolates within the same genomic group cause similar pathologic responses, and there is a clear distinction in strain virulence between these genomic groups.
INTRODUCTION

Coxiella burnetii, the etiologic agent of acute and chronic Q fever, is an obligate intracellular bacterium of worldwide distribution with a diverse host range. Livestock serve as the organism’s primary reservoir and may be asymptomatic carriers or exhibit reproductive disorders. Ticks are important in the maintenance of the disease in nature and have been shown to transmit the infection transovarially (37). Humans are most often infected through inhalation of the bacterium in fine-particle aerosols, though transmission may also occur through ingestion of the organism from contaminated, unpasteurized, dairy products (22, 27). Although a high percentage of infections may result in subclinical or asymptomatic infection, humans can become ill from exposure to as few as 10 organisms (6) and may display signs of: 1) an acute flu-like illness with or without pneumonia and/or hepatitis (30, 31); or 2) a chronic disease manifesting most frequently as endocarditis and/or hepatitis (40, 41).

C. burnetii isolates have been obtained from natural Q fever infections in humans and other animals. Several theories have been proposed to explain the dichotomy in development of acute or chronic Q fever. Unique sequence differences between genomic groups correlate with the clinical expression of Q fever (44). Biochemical markers have grouped C. burnetii isolates from chronic disease patients separately from acute disease/arthropod/domestic animal isolates, but whether these groupings predict virulence potential and acute/chronic disease outcomes has not yet been fully resolved (20). Samuel et al. were the first to separate these isolates and their resulting diseases based on plasmid patterns (44). Hackstadt used variations in LPS banding patterns to divide isolates of C. burnetii into three groups, and group distinction was noted in correlation with acute or
chronic disease (16). Hendrix et al. separated *C. burnetii* isolates into six genomic groups (20). Group I-III isolates have a QpH1 plasmid and have been isolated from ticks, acute human Q fever cases, cow’s milk, and livestock abortions. Groups IV and V have a QpRS plasmid or no plasmid (plasmid related sequences integrated into chromosome), respectively, and have been associated with livestock abortions and human chronic endocarditis or hepatitis. Group VI isolates were collected from wild rodents in Dugway, Utah, and were infectious but avirulent in rodent models of disease (47, 48). Jager et al. used restriction fragment length polymorphism (RFLP) to differentiate 80 *C. burnetii* isolates and reproduced distinguishable patterns for reference isolates in groups I, IV, V, and VI (23). More recently, multiple locus variable nucleotide tandem repeat (VNTR) analyses (49) have validated these groupings. Infrequent restriction site-PCR (IRS-PCR) of 14 livestock and tick isolates resulted in six groups; subsequent MLVA typing of 42 isolates revealed 36 genotypes (2). Glazunova et al. used multispacer sequence typing (MST) to analyze 173 isolates, a majority of which were acquired from chronic disease patients, and identified thirty genotypes in three monophyletic groups; an association between plasmid type, some genotypes, and the nature of disease was observed (15). These monophyletic groups supported the early RFLP groups and placed group I, II, and III in one monophyletic group, group IV in the second monophyletic group, and group V in the third monophyletic group. A comprehensive microarray-based whole genome comparison by Beare et al. confirmed the relatedness of RFLP-grouped isolates and added two more genomic groups, VII and VIII (4). Differences in novel gene content and psuedogenes may be factors in the variations seen in virulence among group I, IV, V, and VI isolates (5). It has been shown in an intraperitoneal (IP) challenge guinea pig model
that $10^1$ organisms of the acute-disease-associated group I isolate Nine Mile RSA493 (NM) caused fever, but $10^6$ chronic-disease-associated group IV isolate MSU Goat Q177 (Priscilla) organisms were required to induce fever (36).

In opposition to the theory of genotype/pathotype correlation, Stein and Raoult evaluated 28 human isolates and found that isolates bearing the QpH1 plasmid were present in both acute and chronic Q fever patients in France, and isolates without the QpH1 plasmid were able to cause acute disease (46). QpH1 plasmid-containing isolates have also been isolated from chronic endocarditis patients (50). Several groups have speculated that host factors are primarily responsible for the outcome of infection with \textit{C. burnetii}. Individual differences in immune function lead to varying sensitivity to infection and disease development. In this model, acute and chronic disease could be caused by organisms from the same isolate group, and chronic disease could develop because of compromised resistance of the host rather than as a consequence of a specific property of the pathogen. For example, HIV infection is a risk factor for the development of chronic Q fever endocarditis (9, 29). Deficiencies in the host specific cell-mediated immune response in Q fever patients have been associated with the suppression of monocyte and macrophage activity (25), and monocytes from chronic Q fever patients have been shown to be defective in phagosome maturation and have impaired \textit{C. burnetii} killing potential, regulated in part by elevated interleukin-10 expression (14). There is strong clinical evidence to support the role of increased host production of IL-10 in the development of both Q fever endocarditis and chronic fatigue syndrome (11, 12, 21, 39). A recent study suggests that chronic Q fever endocarditis may be associated with an
atypical M2 polarization and stimulation of bacterial replication (7), but the pathogenic
process that mediates this polarization was undefined.

Route of infection may also be an important determining factor in the
manifestations of acute and chronic Q fever. LaScola and Marrie demonstrated that the
route of infection and size of inoculum affect clinical illness and pathology associated
with infection in mouse and guinea pig models (26, 33). Differences in the geographic
distribution of disease have also been noted (32); in Nova Scotia, for example, the
primary manifestation of acute Q fever is pneumonia (34), but in France it is hepatitis,
possibly due to ingestion of raw milk and unpasteurized cheeses (51).

The pathogenicity of \textit{C. burnetii} has been evaluated using guinea pigs, mice and
chicken embryos. Febrile response, splenomegaly and mortality in guinea pigs,
splenomegaly and mortality in mice, and mortality in chicken embryos are indicators of
virulence of \textit{C. burnetii}. The establishment of an aerosol model of \textit{C. burnetii} infection in
guinea pigs (43) provides a relevant model in which to test isolate virulence.

Additionally, severe combined immunodeficient (SCID) mice are highly sensitive to the
\textit{C. burnetii} prototype (NM isolate) (1), and the LD$_{50}$ of NM in SCID mice was at least
$10^8$ times less than in wild type mice. We speculated that with these highly sensitive
rodent models it may be possible to observe intra- and inter-group pathogenicity
differences of \textit{C. burnetii} isolates. To confirm whether SCID mice could be used to
model isolate-specific virulence, we gave multiple infectious doses of a group IV Q fever
isolate to immune competent CB-17 and SCID mice (on the same background) to
compare with previously reported group I isolate (NM) infections (1). Eight isolates from
four genomic groups (Table 1) were then evaluated for their ability to cause acute disease
in SCID mouse intraperitoneal (IP)-challenge and guinea pig aerosol-challenge models. We hypothesized that isolates within the same genotypic group would cause similar disease and that there would be a distinct difference in disease manifestation between isolate groups. Finally, we evaluated the potential protection of a vaccine composed of one *C. burnetii* isolate in protecting guinea pigs against infection with an isolate from another group since cross-protection between disparate isolate groups is a further indication of antigenic relatedness.

**MATERIALS AND METHODS**

**Animals.** Female, six to seven week old CB-17/Icr-scid/scid (SCID) and wild type CB-17/Icr-+/+ (CB-17) mice used in Japan were purchased from Japan CLEA (Tokyo, Japan); A/J mice were purchased from Japan SLC (Shizuoka, Japan). A/J mice were used because they are considered more susceptible to *C. burnetii* than other inbred mouse strains (45). Female six to eight week old SCID and wild type CB-17 mice used in the US were purchased from Taconic (Hudson, NY). Female Hartley guinea pigs weighing approximately 350-450g were purchased from Charles River Laboratories (Wilmington, MA).

All infected animals were housed in approved ABSL-3 facilities and immunodeficient mice were housed under sterile conditions. All animals used in this study were acclimated to the facility and assessment procedures during the week prior to infection to decrease stress-related abnormalities. Animal health was assessed daily by a veterinarian.

Mouse experiments performed in Japan adhered to the guidelines for animal experiments at Gifu University. Texas A&M University (TAMU) Laboratory Animal
Care Committee reviewed and approved the mouse and guinea pig research at TAMU, and experiments were carried out in AAALAC approved facilities in accordance with university and federal regulations.

*C. burnetii*. Eight isolates from four genomic groups (Table 1) were used. For the initial dose-effect experiment in Japan, *C. burnetii* MSU Goat Q177 (Priscilla), obtained from Dr. J. Kazar, Institute of Virology, Bratislava, Slovakia, was maintained in mice by passage in spleen homogenates at Gifu University. The spleen homogenate was stored at -80°C until use. Absence of contamination of other pathogens was confirmed by direct staining (Giménez and Gram staining), detection of Mycoplasma DNA using PCR Mycoplasma detection set (Takara; Shiga, Japan), and inoculation of the spleen homogenate to cell culture and SCID mice (independent experimental-infection from the study described in this paper). Bacterial dose was evaluated as 50% tissue culture infectious dose (TCID$_{50}$) in BGM cells (buffalo green monkey fibroblast), 50% infectious dose (ID$_{50}$) in CB-17 mice, and 50% lethal dose (LD$_{50}$) in SCID mice. TCID$_{50}$ was determined by detecting the bacteria 6 days after infection using immunofluorescence staining with anti-*C. burnetii* rabbit antiserum. ID$_{50}$ was determined by detecting seroconversion (IgG, >1:16) using indirect microimmunofluorescence. LD$_{50}$ was determined as reported previously (1).

For all subsequent experiments, all of the *C. burnetii* isolates were maintained at Texas A&M Health Science Center. *C. burnetii* isolates were cultivated in embryonated chicken eggs, purified by gradient centrifugation as previously reported (19, 44), and stored at -80°C until use. Absence of contamination by other pathogens was confirmed as described above. *C. burnetii* was quantified by optical density (OD) (38), direct viable
particle count using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes; Eugene, OR), and quantitative real-time PCR (qPCR) using primers amplifying the com1 gene (8) (Table S1). The bacterial dose used for mouse infections was determined by qPCR; guinea pig doses were calculated using OD.

Experimental infection in mice. (i) Dose/effect experiment with Priscilla isolate. Six mice/group were used. SCID, C.B-17, and A/J mice were inoculated IP with serial 10-fold dilutions of Priscilla (10^2 to 10^-7 TCID50 per animal) or sterile phosphate buffered saline (PBS) (sham infection). SCID mice were observed for 112 days (16 weeks), and C.B-17 and A/J mice were observed for 30 days. (ii) Genomic group comparison. Four mice per group were used. Each of eight C. burnetii isolates described in Table 1 (10^5 genome copies/animal) or PBS were administered IP to SCID and C.B-17 mice. Two independent infections were performed; mice were observed for 28 days (for all the C. burnetii isolates in SCID and C.B-17 mice) or until death (four representative C. burnetii isolates in SCID mice).

Clinical signs were evaluated every 2 days by visual observation (ruffled fur, hunched back appearance and lethargy) and body weight measurement. Body weight changes were evaluated using a body weight index (BWI). BWI = relative body weight / mean relative body weight of the control group; relative body weight = body weight of day “x” of infection/ body weight of the day of infection. Cachexia was diagnosed when a mouse was lethargic and had a BWI less than 0.85. At necropsy, spleen weight was measured as an indicator of C. burnetii infection (54) and tissues were collected. To quantify growth of C. burnetii, DNA was extracted from spleen tissue and C. burnetii com1 gene copies were detected by qPCR as previously described (8). Heart, lung, liver,
spleen, kidney and femur were formalin-fixed, paraffin-embedded and sliced, then prepared by hematoxylin-eosin staining and immunocytochemistry, as described previously (1, 8) to evaluate histopathologic changes and bacterial distribution in tissues. The degree of inflammation present in each tissue sample was scored numerically by the following system: 0, none; 1, mild; 2, moderate; 3, marked; 4, severe. IgG titers for phase I and II C. burnetii in the serum of C.B-17 mice were measured by micro-immunofluorescence as described elsewhere (1). For cytokine assays, blood was collected from the lateral saphenous vein at 3, 7, 10, 14 and 21 days post infection (p.i) and via cardiac puncture at 28 days p.i. after euthanasia, and the group-pooled sera were stored at -80°C until use. Sixteen cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12p40, IL-12p70, IL-10, GM-CSF, IFN-γ, KC, MIP-1α, RANTES and TNF-α) were measured using the Bio-Plex cytokine assay system (Bio-Rad; Hercules, CA) following manufacture’s protocol. The cytokine quantification assay was performed in duplicate for each sample. Cytokine levels of infected sera were evaluated as fold induction as compared to values of uninfected sera.

Experimental infection in guinea pigs. A chamber specially designed to deliver droplet nuclei directly to the alveolar spaces (College of Engineering Shops, University of Wisconsin, Madison, WI), allowing the infection of multiple guinea pigs simultaneously and ensuring uniform infection within each challenge group (35, 43, 52), was used for all guinea pig infection studies. 1) Three guinea pigs per group were infected with low (10^5), mid (10^4), or high (10^3) doses of one of the phase I C. burnetii isolates described in Table 1. Four negative control animals were sham-infected with sterile PBS. Body weight, rectal temperature, and behavioral attitude were recorded along
with any abnormalities noted on thoracic auscultation and abdominal palpation. A rectal temperature ≥39.5°C was defined as fever. Guinea pigs were observed for 28 days post infection (p.i.). Spleen and liver were weighed at necropsy. Tissues were collected and formalin fixed for histopathologic evaluation. Serum was obtained from each animal for serologic testing. 2) In a separate experiment, three guinea pigs per group were exposed to PBS or $2 \times 10^6$ NM, P, G, or Dugway. Daily assessment of these animals was performed as described above, and organs were weighed at necropsy 14 days p.i. to detect splenomegaly and/or hepatomegaly. 3) In the heterologous protection study, guinea pigs were vaccinated with 40μg of formalin-inactivated group I C. burnetii (NM) or group V (S) in Freund’s incomplete adjuvant or adjuvant alone twice, with two week intervals between vaccinations and infection. Animals were then infected with high doses of either NM or S. Three animals per group were separated into the following six groups: i) non-vaccinated, NM infected; ii) non-vaccinated, S infected; iii) NM vaccinated, NM infected; iv) S vaccinated, S infected; v) NM vaccinated, S infected; and vi) S vaccinated, NM infected. Guinea pigs were monitored for 14 days p.i. for development of fever and other clinical signs of illness.

Histopathologic samples were prepared by hematoxylin and eosin staining or by immunohistochemistry using Vectastain® ABC kit and Vector NovaRED substrate kit (Vector Laboratories; Burlingame, CA) and in-house generated rabbit anti-Nine Mile C. burnetii (3) and counterstaining with hematoxylin. All slides were evaluated in a blinded fashion. Serum samples collected at necropsy were tested by ELISA for IgG titers against phase I NM C. burnetii antigen as previously described (43). Sera from uninfected guinea pigs were used as negative controls.
Statistical analyses. Results were expressed as means for each group and were compared using one- and two-way ANOVAs or student’s t-tests, as appropriate. Differences were considered significant at p<0.05.

RESULTS

*C. burnetii* Priscilla is infective and exhibits delayed virulence in SCID mice.

A detailed analysis of dose-effect in an immunocompromised mouse model supported the previous study by Moos and Hackstadt that evaluated the ability of the Priscilla isolate to cause fever in IP-challenged guinea pigs (36). The infectious titer of the Priscilla isolate in the splenic homogenate used for the multiple dose infection was $2 \times 10^4$ TCID$_{50}$/ml in BGM cells, $2 \times 10^9.3$ ID$_{50}$/ml in C.B-17 mice and $2 \times 10^{10}$ LD$_{50}$/ml in SCID mice. (One TCID$_{50}$ corresponded to $10^{5.3}$ ID$_{50}$/ml in C.B-17 mice and to $10^6$ LD$_{50}$/ml in SCID mice.) The LD$_{50}$ in C.B-17 mice could not be determined because no C.B-17 mice died with any infectious dose used in this study, and the ID$_{50}$ in SCID mice could not be determined due to lack of antibody production. The ID$_{50}$ in C.B-17 mice and the LD$_{50}$ in SCID mice were similar, suggesting that SCID mice could be lethally infected with very few viable organisms.

Multiple dose infection of SCID mice with Priscilla isolate resulted in slow, progressive and long-term persistent disease. Clinical signs included ruffled fur, extremely distended abdomens, and death. Body weight loss, inactivity, and cachexia were not observed until a few days prior to death. Survival time ranged from 55 to 109 days p.i. Progression of clinical signs and survival time were dose dependent, with shorter times corresponding to higher infectious doses (Table S2). Similar lesions were
found in all SCID mice that died, most notably severe hepatosplenomegaly, and all organs had cellular infiltration, primarily macrophages containing bacteria. The severity of the lesions in infected SCID mice was not dependent on the *C. burnetii* challenge dose. On the other hand, C.B-17 and A/J mice displayed transitory clinical signs only at infection with the highest dose of Priscilla. Both mouse strains showed ruffled fur from 4 to 13 days p.i., but only A/J mice demonstrated transient body weight loss (data not shown). No other clinical signs were observed. At 28 days p.i. C.B-17 and A/J mice had mild splenomegaly and seroconversion as evidence of infection (data not shown). Small granulomas were present in spleen and liver, but bacterial antigen was not detectable by immunohistochemistry.

**Genomic group-specific virulence in mice.** It is important to establish whether the results of infection seen with the Priscilla isolate and those previously noted with the NM isolate are genomic group-specific (24). To determine this, the pathogenicities of multiple isolates were compared by delivering a single dose of eight *C. burnetii* isolates from four genomic groups (Table 1) to mice by IP injection. Infection was initially compared in SCID and C.B-17 mice sacrificed at 28 days p.i.

All *C. burnetii* isolates caused disease in SCID mice with varying clinical courses. There was no mortality during the 28 day infection period. Clinical signs including significant body weight loss (p<0.05) and cachexia, summarized in Figures 1A and S1A, were most apparent in mice infected with group I isolates, followed by those given group V, IV, then VI isolates, respectively. In C.B-17 mice, only mild transient disease was noted with minimal loss of body weight in response to all isolates and noticeably ruffled fur with group I isolate infection (FIG. 1B).
Splenomegaly in response to infection was more severe in SCID than in C.B-17 mice (FIG. 2A). The number of bacteria in the spleens was determined by qPCR (FIG. 2B), and consistently higher numbers of comI genes were detected in SCID compared to C.B-17 mice. SCID mice showed phylogenetic group-characteristic spleen size and growth of bacteria. Splenomegaly was greatest in SCID mice with mild clinical disease infected with bacteria from groups IV and VI. However, the number of organisms in the spleen was greater in mice with severe clinical disease following infection with phylogenetic groups I and V. In C.B-17 mice, splenic enlargement and number of bacteria increased with the severity of clinical disease. C.B-17 mice displayed differences between infection with the acute *C. burnetii* isolates (phylogenetic group I) and chronic *C. burnetii* isolates (phylogenetic groups IV and V), but there was no difference between groups infected with chronic isolates. All infected mice except those infected with group IV isolates, which developed significant splenomegaly and but had significantly fewer splenic bacteria compared to group I, V and VI mice (p<0.05).

Evaluation of histopathology at 28 days p.i. revealed more lesions in SCID mice than in C.B-17 mice (Table S3). SCID mice showed histopathologic changes in all organs investigated. Group I isolates caused the most inflammation, followed by groups V, IV, and VI, respectively. The inflammatory cell population was similar in all groups and consisted of few neutrophils and numerous macrophages containing abundant intracytoplasmic bacteria. *C. burnetii* antigen was diffusely distributed in all organs examined. C.B-17 mice had mild histopathologic changes in some organs, but even in the tissues with an inflammatory response, *C. burnetii* antigen was rarely detected.
Circulating cytokines are altered in *C. burnetii* infected CB-17 mice.

Variations in pathology and inflammation associated with these isolate groups infections suggest a difference in the immune response. To expand on this observation, the serum levels of 16 cytokines and chemokines were measured. In C.B-17 mice, serum cytokine levels differed between mice infected with group I isolates and those given isolates from other groups. Group I isolates induced persistently high cytokine secretion throughout the 28 day experiment; group IV and V isolates caused moderate cytokine secretion at the peak of clinical disease (7 to 14 days p.i.) (FIG. 3). After 14 days p.i., group I isolates induced higher secretion of IL-3, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IFN-γ, TNF-α, MIP-1α, and RANTES when compared with other groups. The KC and G-CSF levels of mice infected with group I isolates were higher than in mice infected with other groups prior to 14 days p.i. Serum levels of IL-1α, IL-1β, IL-2, IL-5, and eotaxin secretion were not increased during the infection period (data not shown).

**Lethal potential of all genomic groups in SCID mice.** The lethal potential of representative isolates from each phylogenetic group was investigated in SCID mice, and it was determined that all of the isolates evaluated could eventually lead to clinical illness and death in the immunodeficient model (FIG. S1B). Isolates that caused a long period of cachexia led to severe body weight loss in infected mice (FIG. S2). Group I isolate (NM) induced the earliest and longest period of cachexia and correspondingly the most severe body weight loss. Mice infected with isolates from groups V (G) and VI (Dugway) had similar survival times, but those given group V isolates had longer periods of cachexia and more severe body weight loss than group VI-infected mice. Infection with group IV isolates (Priscilla and P) resulted in the shortest period of cachexia, and body
weight loss was not observed until the terminal stage of infection. Survival time was shortest in mice challenged with group I isolates (32.0 ± 0.8 days), followed by those infected with groups V (36.0 ± 0.0 days) and VI (35.5 ± 1.0 days), and IV (47.5 ± 0.6 days for P and 77.3 ± 2.8 days for Priscilla). Probable cause of death was multiple organ failure due to massive systemic infection.

The pathologic changes in SCID mice at mortality were more advanced than those observed at 28 days p.i. (data not shown). The severity of inflammatory changes in the liver and spleen were similar in all groups of infected mice, but animals given group I isolates exhibited a greater degree of inflammation in the heart and lungs than those given group IV, V, and VI isolates. The extent of splenomegaly changed with survival time; however, the number of bacteria in the spleen was similar in all groups, suggesting that the detected amount of bacteria ($10^{10}$ genome copy/spleen) is the saturation point in SCID mice. *C. burnetii* antigen was diffusely distributed in all tissue sections.

**Genomic group specific outcome of acute Q fever pneumonia in the guinea pig aerosol model.** Aerosol-challenge in the guinea pig provides a physiologically relevant model that simulates both the natural route of infection and common clinical presentations associated with human acute Q fever infection, making this a choice model for evaluating the comparative virulence of different *C. burnetii* isolates and was thus used in the logical progression of experiments after differing virulence were observed in mouse models of infection. Guinea pigs challenged with group I and V isolates developed a significant fever in response to infection (p<0.01), whereas those given isolates from groups IV and VI were afebrile even at the highest challenge dose (FIG. 4).
Fever response, weight loss, and other clinical signs displayed a dose-dependent relationship in guinea pigs infected with group I C. burnetii isolates African and Ohio as has been described for the reference isolate in this group, NM (43). All animals receiving high-dose African or Ohio organisms died within 7-9 days post infection, as did 2/3 receiving NM; lower infectious doses were not lethal. Gross lung consolidation and overall lack of normal body fat were noted on necropsy at 7-9 days p.i. in guinea pigs infected with the highest dose of organisms. Histologically, these animals had severe panleukocytic bronchointerstitial pneumonia with bronchial and alveolar exudates. Lung tissues from the surviving NM-infected guinea pig and those given the mid-dose group I organisms were evaluated at 28 days p.i. for comparison to animals infected with other isolates evaluated at this time-point and exhibited moderate multifocal lymphohistiocytic pneumonia with granuloma formation.

No significant fever or other overt clinical signs were noted in guinea pigs infected with group IV isolates. Mild lymphohistiocytic pneumonia was seen histologically at 28 days p.i. in animals delivered the highest dose of organism.

Group V isolate infected guinea pigs all developed fever when delivered the highest challenge dose, and dose-dependent temperature increase and other clinical signs were again noted, with no fever development in those animals receiving the lowest dose of organism. Though auscultation confirmed respiratory compromise, none of the infections were lethal. At 28 days p.i., the lungs had a mild to moderate lymphohistiocytic interstitial pneumonia and a few small granulomas.

No major clinical or pathologic changes were noted in guinea pigs infected with the group VI isolate or in negative control animals. Table S4 compares the severity of
histopathologic changes in guinea pigs infected with high dose *C. burnetii* isolates from each group at 28 days p.i. Immunohistochemistry confirmed the presence of *C. burnetii* organisms, primarily in macrophages, in the lungs, liver, and spleen of infected animals. Experimental guinea pigs in all dose groups for each isolate seroconverted by the time of euthanasia with the exception of high-infected NM, African, and Ohio infected animals necropsied at one week p.i. and low-dose Dugway infected guinea pigs. The degree of seroconversion was dose dependent and varied among isolates (data not shown). No PBS control animals seroconverted.

**Genomic group specific severity of hepatitis and splenomegaly in guinea pigs.** The doughnut granulomas common in human acute Q fever hepatitis (31) have not been previously described in animals experimentally infected with *C. burnetii* and were similarly not seen in the guinea pigs in this study. Mild hepatitis and severe hepatic lipidosis were noted at death 7 days p.i. in guinea pigs challenged with high-dose group I isolates, as has been previously reported for NM aerosol-infected guinea pigs (43). Tissue sections from the remaining NM-infected guinea pig and those infected with the mid-dose group I organisms were evaluated for comparison with animals infected with other isolates at 28 days p.i. and revealed vacuolization and degeneration of centrilobular hepatocytes, lymphocyte infiltration in periportal regions, and multiple small granulomas. Group IV infected guinea pigs also had periportal lymphocytic infiltration as well as multiple granulomas of varying sizes. The granulomas in Priscilla- and P-infected guinea pigs were more defined, with more histiocytic involvement than was seen in guinea pigs infected with group I isolates. Subjectively, of all animals necropsied from
each isolate group, hepatic granulomas from those infected with P were the greatest in
size and number.

The livers of group V isolate G- and S-infected guinea pigs contained few small
granulomas and mild to moderate infiltration of lymphocytes along portal tracts. The
hepatic changes observed in guinea pigs infected with group V isolates suggested that
isolates from this group are less hepatovirulent than group IV isolates but more than
group I isolates.

No hepatic granulomas or other significant pathologic changes were noted in
guinea pigs infected with the group VI isolate Dugway. Liver weights did not vary
significantly within or between genomic groups.

There were no significant differences in spleen weight at 28 days p.i. within or
between genomic or dose groups. Animals infected with all isolates examined at 14 days
p.i. (NM, P, G, and Dugway) had significantly larger spleens than PBS control animals,
and spleens from NM- and G-infected guinea pigs were significantly larger (p<0.01 and
p<0.05, respectively) than those of P- and Dugway-infected animals (FIG. S3).

Pathologic findings included multiple small granulomas in the spleens of group I-infected
guinea pigs; fewer small granulomas were occasionally noted in animals infected with
group IV and V isolates.

**Heterologous protection of cross vaccination and challenge in guinea pigs.**

The infection studies described in this paper illustrate that there is pathotype diversity
between *C. burnetii* isolates from different genogroups and are consistent with
phylogenetic studies cataloging distinct gene content(4). We therefore strove to
determine whether this diversity was great enough to affect the ability of vaccines to
protect against infection. Guinea pigs were given group I (NM) or group IV (S) vaccines and cross-challenged to evaluate potential heterologous protection against high-dose infection. Non-vaccinated guinea pigs developed a noticeable fever response by day 5 p.i., and infection was lethal in 3/3 NM and 1/3 S challenged animals. Guinea pigs vaccinated with either formalin-killed NM or S were completely protected against fever development and death when challenged with either NM or S (FIG. 5).

**DISCUSSION**

The potential for genomic group-specific pathogenicity of *C. burnetii* was evaluated using immunocompetent mice and guinea pigs and immunodeficient mice. The hypotheses that isolates belonging to the same genomic group would cause similar disease and that there would be distinctions in disease manifestations between isolate groups were supported by the findings presented here. A detailed analysis of Priscilla isolate dose-effect in SCID mice revealed differences in virulence of *C. burnetii* isolates. Disease development after Priscilla infection was progressive but slower compared to the disease caused by NM previously reported in SCID mice (1); the survival time of SCID mice infected with Priscilla was longer with the same LD$_{50}$ dose. This result supports the previous study by Moos and Hackstadt that evaluated the lower ability of the Priscilla isolate to cause fever in IP-challenged guinea pigs (36). Interestingly, the mice infected with Priscilla did not exhibit cachexia until the terminal stages of infection when they had extremely severe hepatosplenomegaly. Although the disease caused by Priscilla was milder than that associated with NM, all mice that developed clinical illness died. This result confirms a
high infectivity and lethal potential of *C. burnetii* that is not restricted to acute isolates and suggests that the SCID mouse model can be useful for evaluation of *C. burnetii* virulence.

The virulence of *C. burnetii* isolates tested in SCID mice was determined to be genomic group-specific. Acute Q fever associated group I isolates caused the most rapidly progressing disease and the most severe pathologic changes. Groups IV and V, isolates associated with chronic Q fever, caused a slower progression of disease. Overall, pathologic changes in mice infected with group IV and V isolates were milder than those of group I infected mice. The number of bacteria in the spleen at 28 days p.i. was greater in mice with severe disease from infection with group I isolates; however, bacterial load at the time of death was similar in all infected mice. This suggests that the rate of proliferation of *C. burnetii in vivo* may be virulence-related. An *in vitro* comparison of infection in L929 cells using NM, Priscilla, and S isolates showed that all isolates can persistently infect, but Priscilla requires a greater period of time to establish an infection (42), and it has been shown that inclusion forming units produced by NM and Priscilla isolates were similar in Vero cells (36). However, because of developmental differences in clinical signs and pathologic changes, replication rate does not seem to be the only virulence factor involved since clinical signs then would be similar with differences in disease progression only. At both time points, 28 days p.i. and at time of death due to infection, heart and lung lesions caused by group IV, V, and VI isolates were milder than those produced by infection with group I isolates. This observation seems to conflict with the hypothesis that chronic isolates cause chronic Q fever, including heart disease. However, our observation is consistent with the report that isolates from heart lesions of...
chronic Q fever patients have similar genetic characteristics to acute isolates (46). The hypothesis that acute isolates do not cause endocarditis has been supported by two other research groups (17, 24). The correlation between virulence and phylogeny has been controversial because of a lack of comprehensive studies. One study detected acute isolate specific genes in isolates from chronic Q fever patients and concluded that isolates were not disease-specific (46). The isolates used in the study were isolated by cell culture, and though the cell culture system is highly effective for isolation, acute isolates are known to infect cultured cells more efficiently than chronic isolates, so there remains a potential that the study collected only cell culture-adapted isolates. Several in vivo studies have reported isolate-specific virulence using guinea pig and mouse models (17, 24, 36); however, the number of isolates used in these studies was limited, making it difficult to conclude genomic group-specific virulence. The present study using eight isolates from four phylogenetic groups strongly supports the variation in virulence among C. burnetii isolate groups.

In the absence of functional T and B cells, cytokine profiles showed no group-specific differences. In immunocompetent mice group I isolates caused a stronger immune response with high levels of multiple cytokines over a longer time period than other groups. Interestingly, Dugway (group VI) induced the least change in C.B-17 mice. The inflammatory cytokine changes in immunocompetent mice in this study were similar to those of humans with acute Q fever (10): TNF-α and IL-6 were upregulated, but IL-1β was not. IFN-γ increased in C.B-17 mice infected with group I isolates, and is associated with the control of bacterial growth, stimulates phagosome-lysosome fusion, and may enable monocytes/macrophages to kill C. burnetii (13, 14). A difference in vacuole
formation between isolates has also been shown, with NM and S developing within single
large vacuoles while Priscilla occupied several smaller vacuoles per cell (18). This in
vitro study suggested a difference in isolate ecology within host cells, which may
correlate with their virulence in vivo.

The ability to cause fever and respiratory illness was isolate and dose dependent
in the guinea pig aerosol-challenge model, with isolates from groups I and V causing
disease consistent with human acute Q fever. Isolates within the same genomic group
produced similar clinical illness, strongly supporting the mouse experiments
demonstrating that genomic differences in the bacterial isolates do play a role in
virulence. It was shown here that isolates associated with chronic disease, G and S, have
the ability to cause acute disease in the guinea pig model. Our study confirmed and
expanded the observations by Kazar et. al. as to the greater virulence of NM and S
isolates compared to that of Priscilla.

Lesny et al. compared the cross-immunity of whole-cell and soluble Q fever
vaccines made from phase I Nine Mile, S, Priscilla, and Luga isolates. They found that
vaccines from Nine Mile and Priscilla afforded a higher degree of protection than S and
Luga vaccines and that whole-cell vaccines were more effective than soluble vaccines
(28). In the guinea pig challenge study presented here, killed whole cell vaccines made
from isolates differing in LPS banding pattern (16), plasmid type (44), and genomic
group (20), specifically isolates from groups I and V, conferred heterologous protection
against virulent high dose challenge in accordance with previous studies (28). This
suggests that although the manifestation of disease and genomic content differ among
various isolate groups, the antigenic properties of whole cell vaccines are shared enough
that cross protection is possible. Such information is valuable for new vaccine design and could be of utmost importance in offering reliable protection in the event of an outbreak.

The differences in perceived infectious dose noted when OD, particle count, and genome copy enumeration were compared underline the importance of using multiple quantitation methods to compare studies with earlier observation. Some of the differences in disease manifestation seen in guinea pigs in this study could be due to slight differences in infectious dose delivered. For instance, Priscilla and P both induced hepatic changes, though guinea pigs infected with P appeared to induce more severe lesions than Priscilla, which had a lower infectious dose by OD and qPCR. The difference in infectious dose as determined by genome copy number could account for this variation. However, G and S both caused fever, and although guinea pigs infected with G did not attain the same degree of febrile response as S infected animals, quantitation by particle count and real-time PCR showed infectious doses of S to be over a log lower than G. It could be argued that Priscilla infected guinea pigs did not develop fever because fewer bacteria were present in the aerosol challenge; however, the group IV isolates did not induce fever at any of the challenge doses while group I isolates induced fever even at the lowest dose. We believe that despite the variation in infectious dose depending on enumeration technique, the significant differences noted among genotypic groups are valid.

Phase variation is the only well characterized phenotypic difference that relates to virulence in *C. burnetii* (50). Although LPS may be a major virulence determinant, and isolate LPS banding patterns have been correlated with acute or chronic disease (16), other components alone or in association with LPS may be responsible for differences in
mortality in SCID mice and fever development in aerosol-challenged guinea pigs. It has been hypothesized that differences in the lipid A component are responsible for the variations in virulence, but lipid structural information indicates they are similar. The combination of a variety of factors expressed by phase I bacteria likely govern the ability of *C. burnetii* to infect cells and maintain continuous growth within the phagolysosome. Indeed, the combination of pathotype variation of disease in infected guinea pigs and cross-protection of different isolates suggest conserved predominant antigenic components and with virulence determinant specificity.

A recent report compared all open reading frames (ORF) of NM phase I to African, Ohio, P, G, S, and Dugway, among others (4), and a majority of the ORFs deleted from Nine Mile in the other isolates were either hypothetical or nonfunctional; however, a few were associated with assorted cellular functions. Beare et al. compared the complete genome sequences of NM, K, G, and Dugway and found distinct collections of psuedogenes and unique gene content that may contribute to pathotype-specific virulence including type II and type IV secreted effector molecules (5). Integrating our *in vivo* data with these molecular details, as well as with other *in vitro* studies, may reveal the critical virulence determinants of *C. burnetii* and ultimately identify targets for vaccine and therapeutic intervention.

Isolates of phase I *C. burnetii* have the potential to cause a range of clinical signs, including fever, pneumonia, hepatitis, and splenomegaly. Isolates from one human chronic disease group induced mild to moderate acute disease in the physiologically relevant guinea pig aerosol-challenge model while a separate isolate group representing several chronic disease isolates caused no acute disease. All isolates examined were
capable of producing disease in the immunocompromised SCID mouse model, and
genogroup-consistent trends were noted in cytokine production in response to infection in
the immunocompetent mouse model. In these studies, isolates within the same genomic
group caused similar pathologic responses, with a distinction in strain virulence between
established genogroups, sustaining the theory that genetic differences in the bacterial
isolates affect their virulence.

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REFERENCES


**TABLE 1.** Isolates evaluated for virulence

<table>
<thead>
<tr>
<th>Genomic Group</th>
<th>Isolate</th>
<th>Notation in this study</th>
<th>Sample</th>
<th>Year</th>
<th>Location</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Nine Mile RSA493</td>
<td>NM</td>
<td>Tick</td>
<td>1935</td>
<td>Montana, US</td>
<td>n/a (acute, flu-like in humans)</td>
</tr>
<tr>
<td></td>
<td>Ohio RSA270</td>
<td>Ohio</td>
<td>Cow’s Milk</td>
<td>1956</td>
<td>Ohio, US</td>
<td>Persistent</td>
</tr>
<tr>
<td>IV</td>
<td>MSU Goat Q177</td>
<td>Priscilla</td>
<td>Goat Cotyledon</td>
<td>1980</td>
<td>Montana, US</td>
<td>Abortion</td>
</tr>
<tr>
<td></td>
<td>P Q173</td>
<td>P</td>
<td>Human Heart Valve</td>
<td>1979</td>
<td>California, US</td>
<td>Endocarditis</td>
</tr>
<tr>
<td>V</td>
<td>G Q212</td>
<td>G</td>
<td>Human Heart Valve</td>
<td>1981</td>
<td>Nova Scotia, Canada</td>
<td>Endocarditis</td>
</tr>
<tr>
<td></td>
<td>S Q217</td>
<td>S</td>
<td>Human Liver Biopsy</td>
<td>1981</td>
<td>Montana, US</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>VI</td>
<td>Dugway 5F106-111</td>
<td>Dugway</td>
<td>Rodents</td>
<td>1958</td>
<td>Utah, US</td>
<td>n/a</td>
</tr>
</tbody>
</table>
FIG 1. Average body weight changes in SCID mice (A) and CB-17 mice (B) infected with *C. burnetii* isolates during 28 days of infection. Body weights were significantly lower in SCID mice throughout the infection period and transiently in CB-17 mice infected with all isolates except Priscilla when compared to PBS controls (p<0.05).
FIG 2. Splenomegaly (A) and splenic bacterial load (B) in mice at 28 days p.i. A) All infected animals developed significant splenomegaly compared to controls, and infected SCID mice had significantly larger spleens than CB-17 mice (p<0.05). B) Mice infected with group IV, V, and VI isolates had significantly fewer bacteria than those infected with group I isolates (p<0.05). Black bars, SCID mice; white bars, C.B-17 mice.

* = p<0.05
FIG 3. Mean circulating cytokine levels in response to infection in CB-17 mice with different \textit{C. burnetii} isolates. Isolates from genomic group I induced persistently high cytokine secretion with increased levels of IL-6, TNF-\(\alpha\), IL-12p40, IL-12p70, IFN-\(\gamma\), IL-3, IL-4, IL-10, MIP-1\(\alpha\), and RANTES when compared with other genogroups (\(p<0.05\)).

(\(\blacklozenge\) - PBS, \(\square\) - NM, \(\triangle\) - African, \(\diamond\) - Ohio, \(\bullet\) - Priscilla, \(\blacklozenge\) - P, \(\blacksquare\) - G, \(\blacktriangle\) - S, \(\bigcirc\) - Dugway)
FIG 4. Fever response of guinea pigs to infection with high dose *C. burnetii* isolates.

Mean daily temperatures +/- SEM (n=3) of animals infected with $2 \times 10^6$ of each *C. burnetii* isolate. Temperatures $\geq 39.5^\circ C$ (black bar) were considered fever. Arrows indicate days on which death occurred in NM, African, and Ohio infected groups.
FIG 5. Heterologous vaccination and challenge in guinea pigs. Average daily temperatures of animals vaccinated with NM (dotted line), S (dashed line), or adjuvant alone (solid line) and challenged with high dose NM (♦) or S (□). Temperatures ≥ 39.5°C were considered fever.