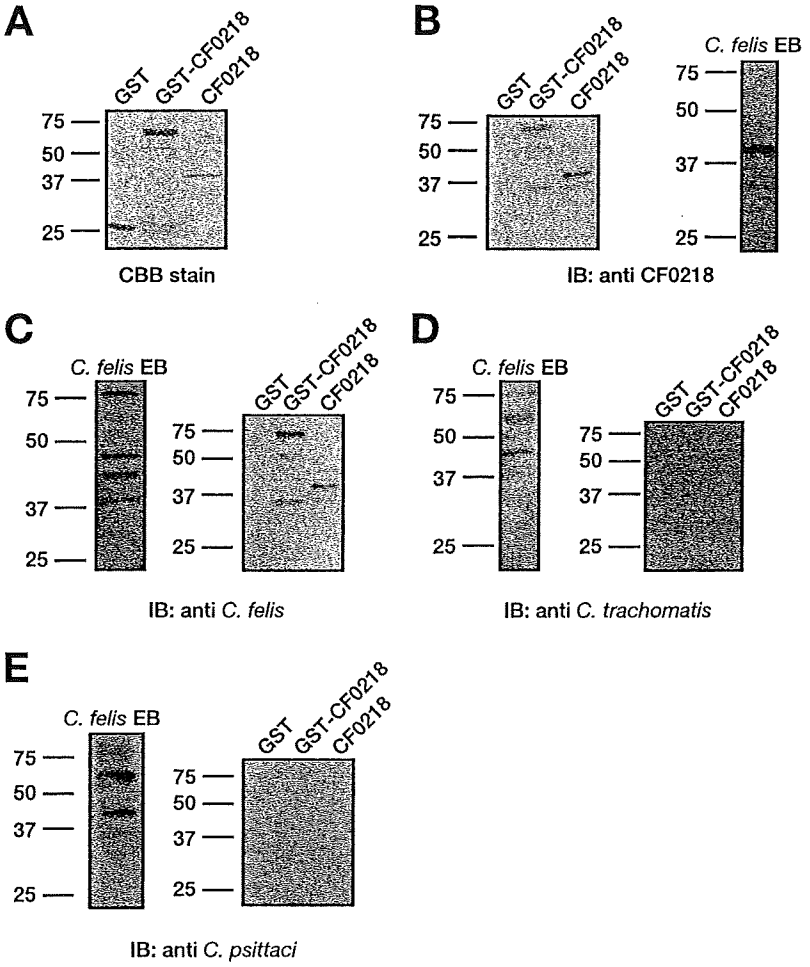

FIG. 3. Production of recombinant CF0218 and its immunogenicity. (A) Purified GST alone, GST-CF0218, and GST-cleaved CF0218 were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB). (B to E) Equal amounts of recombinant CF0218 shown in panel A and purified *C. felis* EB were separated by SDS-PAGE and analyzed by immunoblotting using rabbit antiserum raised against CF0218 (B), *C. felis*-hyperimmunized antiserum (C), *C. trachomatis*-hyperimmunized antiserum (D), and *C. psittaci*-hyperimmunized antiserum (E). Molecular mass standards are indicated in kilodaltons on the left sides of panels.

Antibody response against CF0218 in experimentally vaccinated and *C. felis*-infected cats. As mentioned in the introduction, the modified live and inactivated vaccines against *C. felis* have been used in several countries including Japan (40). It is difficult to differentiate vaccinated and infected cats since both vaccinated cats and infected cats are assessed as positive by means of current serological tests. Therefore, to assess the potential of CF0218 for diagnostic use, antibody responses against CF0218 in vaccinated and *C. felis*-infected cats were examined. Specific-pathogen-free cats were inoculated with the *C. felis* inactivated vaccine twice (at 0 and 3 weeks) and subsequently challenged with *C. felis* at 5 weeks (2 weeks after the second vaccination; for details of the experimental design, see Materials and Methods). Sera were collected at each time point, and antibody responses against *C. felis* EB and the recombinant CF0218 were measured by ELISA (Fig. 6). As shown in Fig. 6A, antibody response against *C. felis* EB was elevated following the *C. felis* vaccination from 4 weeks after the first vaccination in the vaccinated group (closed squares) but not in the nonvaccinated group (closed circles). Thereafter, both groups were challenged with *C. felis* (at 5 weeks), and antibody

FIG. 2. Comparative analysis of the predicted amino acid sequences of CF0218, CAB764, CAB766, and CCA797. (A) Multiple alignment of CF0218, CAB764, CAB766, and CCA797. Boxes indicate identical residues. (B) Comparison of hydropathy profiles of CF0218, CAB764, CAB766, and CCA797. Profiles were determined using the algorithm developed by Kyte and Doolittle (18) with a window size of 11 amino acids. The relative hydrophobicity of each protein is shown on the vertical axis. Negative numbers indicate relative hydrophilicities. The bilobed hydrophobic region present in each protein is circled.

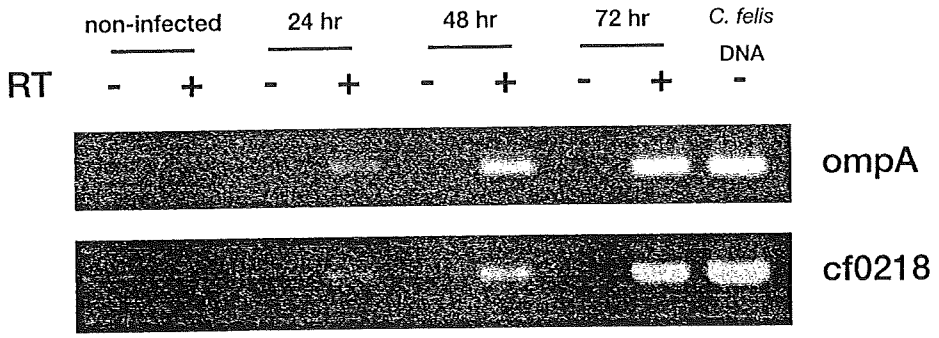

FIG. 4. RT-PCR analysis of cf0218 expression in infected cells. Specific messages for *ompA* and cf0218 were detected from total RNA of HeLa cells infected with *C. felis* at the times indicated (including noninfected cells as negative control). DNase I-treated total RNA was applied to reactions in the absence (−) or presence (+) of reverse transcriptase (RT). Message for *ompA* was amplified as a control for chlamydial infection. *C. felis* genomic DNA was amplified as a PCR control.

against EB was elevated from 7 weeks (2 weeks after the challenge) in both groups. These results confirmed that antibody response due to the vaccination and the infection occurred correctly in the experiments. Antibody response against CF0218 in the same samples was measured by ELISA using the recombinant CF0218 as an antigen (Fig. 6B). The level of antibody against CF0218 was not increased until 5 weeks later in the vaccinated group (Fig. 6B, closed squares and solid line), although the level of antibody against EB was elevated at this time point in the vaccinated

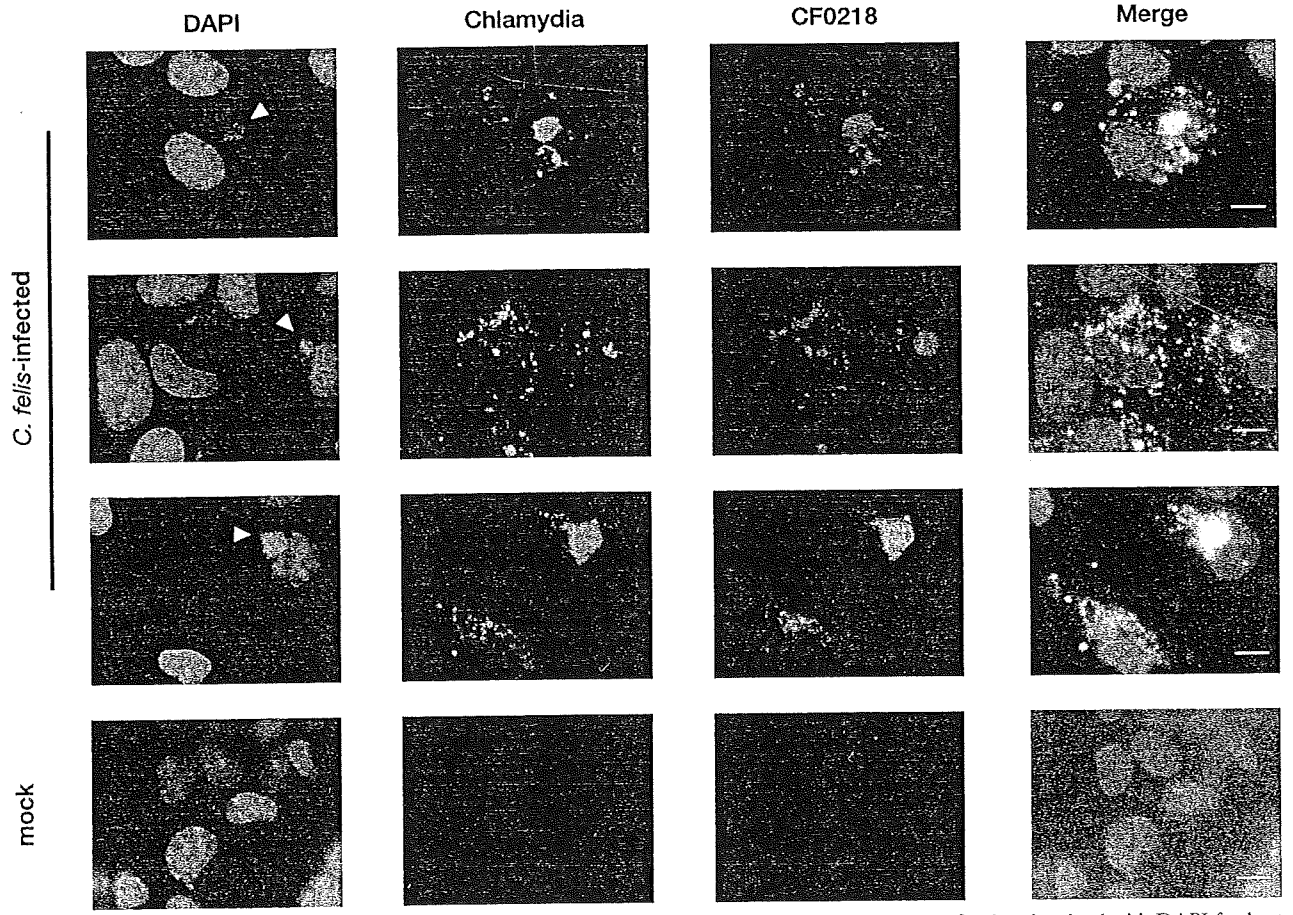

FIG. 5. Localization of CF0218 in *C. felis*-infected cells. HeLa cells infected with *C. felis* for 72 h were fixed and stained with DAPI for host cell nuclei and apparent chlamydial inclusion bodies (blue; white arrowheads show large chlamydial inclusion bodies), anti-chlamydial LPS (red), and anti-CF0218 (green). The rightmost columns show merged triple fluorescence images. Bars, 10 μm.

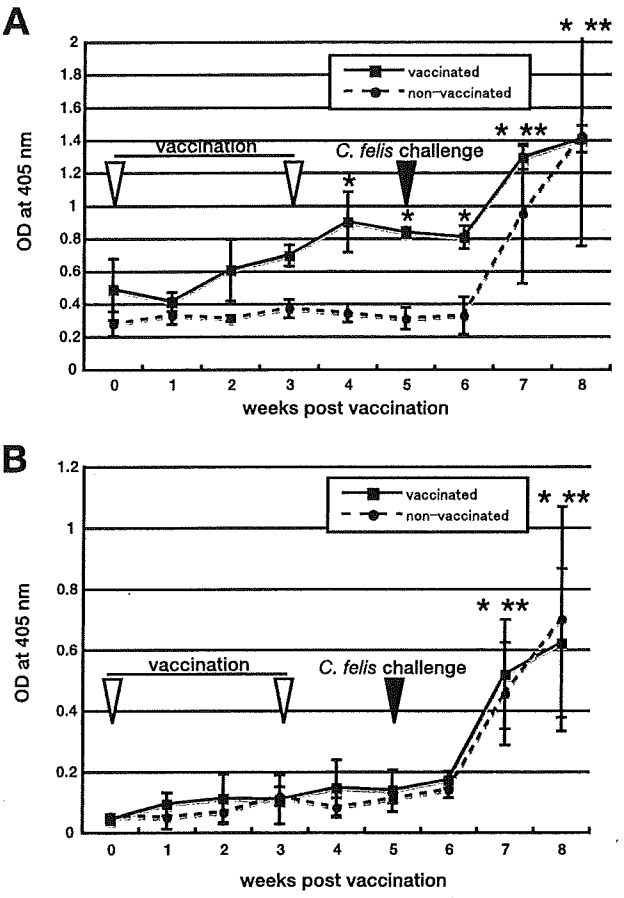

FIG. 6. Antibody response against EB and CF0218 in experimentally vaccinated and *C. felis*-infected cats. Female specific-pathogen-free cats were inoculated with the vaccine containing formalin-inactivated *C. felis* EB (closed squares, solid line) or with saline (closed circles, dashed line) via the intramuscular route twice (at 0 and 3 weeks; open triangles). Subsequently, 5 weeks after the first vaccination (black triangles), all cats were inoculated with 10^4 50% embryo infectious doses of *C. felis* via the mucosal (conjunctival, oral, and nasal) route. To assess antibody responses against *C. felis* EB (A) and CF0218 (B) from sera at each time point, ELISAs were performed using detergent-inactivated *C. felis* EB and the recombinant CF0218 as antigens. Each sample was measured in duplicate. Data represent the mean OD value ± standard deviation. Significant differences ($P < 0.01$) between control serum (at 0 weeks) and at each time point are indicated as asterisks (single for the vaccinated cats and double for the nonvaccinated cats).

group (Fig. 6A). In contrast, the level of antibody against CF0218 was increased following *C. felis* infection from 7 weeks onward (2 weeks after the challenge) in the vaccinated group and even in the nonvaccinated group (Fig. 6B, closed circles and dashed line). These results suggest that the level of antibody against CF0218 was not elevated by the vaccination but only by live *C. felis* infection.

DISCUSSION

In this study, we describe the cloning and molecular characterization of *C. felis* CF0218 as a new diagnostic antigen. Our results show the following: (i) CF0218 was obtained by immunoscreening of a *C. felis* genomic library with the *C. felis*-immunized cat serum; (ii) the location of cf0218 in the *C. felis* genome is the syntenic region of the TMH locus in the *C. abortus* genome, which was not present in the genome of *C. trachomatis* or *C. pneumoniae*; (iii) recombinant CF0218 was recognized by serum against *C. felis* but not by serum against *C. trachomatis* and *C. psittaci*; (iv) CF0218 was expressed in *C. felis*-infected HeLa cells and was colocalized in *C. felis*-formed inclusion bodies; (v) the level of antibody against CF0218 was elevated by *C. felis* infection but not by vaccination in experimentally vaccinated and infected cats.

The TMH locus is a newly identified gene cluster showing limited distribution among chlamydial species. Thomson et al. reported that this region is present in *C. abortus* and *C. caviae*, but the syntenic regions in *C. trachomatis*, *C. pneumoniae*, and *C. muridarum* revealed significant levels of variation in gene content (41). The cf0218 obtained in this study by immunoscreening is present in the putative TMH locus in the *C. felis* genome (Fig. 1). The TMH locus is characterized by several ORFs encoding paired N-terminal transmembrane motifs with various lengths, which are termed TMH family proteins. CF0218 shows similarity with the *C. abortus* and *C. caviae* TMH family proteins CAB764, CAB766, and CCA797, especially at the N terminus, and possesses a bilobed hydrophobic motif at the N terminus as do CAB764, CAB766, and CCA797 (Fig. 2), suggesting that CF0218 is a TMH family protein of *C. felis*.

At this time, there has been no report describing the role of the TMH family proteins during the course of chlamydia infection. However, the presence of paired N-terminal transmembrane helices suggests that TMH family proteins may belong to the Inc family of proteins (41). Inc family proteins play a major role in the formation of chlamydial inclusion membranes and may participate in the chlamydial developmental process including growth and survival within the host cells (35). TMH family proteins may play a role similar to that of Inc family proteins in the chlamydial developmental process.

In *C. felis*-infected HeLa cells, CF0218 is distributed throughout the chlamydial inclusion bodies (Fig. 5). In contrast, other researchers showed that a large number of chlamydial Inc proteins are localized to the inclusion membranes (3, 35). In addition, unexpectedly, CF0218 was also detected by immunoblotting in the purified *C. felis* EB (Fig. 3B), although several Inc proteins of *C. caviae* (formerly *C. psittaci* GPIC) and *C. trachomatis* were not reported to be detected in purified EBs (4, 36). On the other hand, *C. trachomatis* IncA was detected in purified EB to an even lesser extent (36) and CopN (a component of the type III secretion system) was also detected in purified EB (10). Both IncA and CopN play pivotal roles in the chlamydial infection process. It remains to be elucidated whether CF0218 is a structural component of EB or acts as an Inc-like protein in infected cells during the *C. felis* infection process.

In general, serological diagnosis of chlamydia is exclusively performed by using EB or chlamydial cell surface components (LPS, MOMP, and PMPs) as antigens (see the introduction), while other chlamydial products represent immunogenicity in infected animals or human patients. For example, *C. caviae* IncA and IncC were initially identified by sera from *C. caviae*-infected guinea pigs (4, 34). Additionally, several recombinant

putative Inc proteins are recognized by sera from *C. trachomatis*- and *C. pneumoniae*-infected patients (3). These reports indicate that chlamydial products other than LPS, MOMP, and PMPs are immunogenic and can be used as diagnostic antigens. CF0218 is highly conserved at the amino acid level among different *C. felis* isolates. The antigenicity of CF0218 was examined by using serum against *C. felis* and was confirmed (Fig. 3C). Furthermore, levels of antibody against CF0218 were increased in experimentally *C. felis*-infected cats (Fig. 6B). These results indicate that CF0218 is immunogenic, with potential as a diagnostic antigen of *C. felis*.

Interestingly, the TMH locus has been identified in the *C. abortus* and *C. caviae* (41) and *C. felis* (this study) genomes but not in the *C. trachomatis* and *C. pneumoniae* genomes, and orthologues of CF0218 have not been identified in the *C. trachomatis* and *C. pneumoniae* genomes (1), suggesting that the TMH family proteins may be specific for nonhuman chlamydophila. There is a possibility of cross-reactivity of CF0218 with closely related non-*C. felis* chlamydiae possessing TMH family proteins, since *C. abortus* CAB764 and CAB766 and *C. caviae* CCA797 exhibited 25 to 35% identity with CF0218. Since we do not have antisera against *C. abortus* and *C. caviae*, we examined the cross-reactivity of CF0218 with antiserum against *C. psittaci* Prk/daruma, and this serum did not react with CF0218 (Fig. 3E). Currently the genome sequence of *C. psittaci* is unavailable; however, *C. psittaci* Prk/daruma is genetically closely related to *C. abortus* rather than to other *C. psittaci* strains (11). This result may suggest that CF0218 has potential as a diagnostic antigen specific for *C. felis*. As far as we know, there is no report that *C. abortus* and *C. caviae* infect cats. However, von Bomhard et al. reported that *Neochlamydia hartmannellae* can be a causative agent for feline chlamydiosis (42). Therefore, further study is needed to examine the cross-reactivity of CF0218 with *N. hartmannellae*. As described in the introduction, *C. felis* is a suspected zoonotic agent (20). The fact that the recombinant CF0218 was not recognized by the serum against *C. trachomatis* (Fig. 3D) raises the possibility of using CF0218 to clarify whether *C. felis* infection in humans is the cause of non-*C. trachomatis* conjunctivitis.

Finally, we examined antibody responses against CF0218 in experimentally vaccinated cats since the vaccine against *C. felis* leads to difficulty in distinguishing vaccinated and infected cats by means of the current serodiagnostic methods (see the introduction). Our result (shown in Fig. 6B) suggests that it is possible to differentiate the vaccinated and the infected cats by measuring levels of antibody against CF0218.

It is noteworthy that CF0218 was detected by immunoblotting in the purified *C. felis* EB, which as a component of the vaccine was in a formalin-inactivated form (Fig. 3B). The reason why the level of antibody against CF0218 is not elevated by vaccination is unclear. However, Shewen et al. reported that formalin-inactivated *C. felis* vaccines did not induce the complement-fixing antibodies in experimentally vaccinated cats but the vaccines reduced the clinical severity of subsequent *C. felis* infections (37). Like complement-fixing antibodies, the level of antibody against CF0218 might not be increased by vaccination. In addition, although the vaccine used in this study contains formalin-inactivated *C. felis* Cello EB, the level of antibody against CF0218 was elevated only after challenge with live *C. felis* Cello strain. In this case, it may be that the recognition of antigen (CF0218) by the host immune system requires infection by a live organism. It is noteworthy that modified live vaccines for *C. felis*, although used in other countries (22, 40), are not approved in Japan, and so we did not test them in this study. According to our marketing research (S. Ishiguro, unpublished data), the inactivated *C. felis* vaccine as a percentage of total sales of *C. felis* vaccine in the United States is around 50% (50% in 2005 and 47% in 2006). Further studies are needed to determine whether modified live vaccines induce an antibody response to CF0218.

In conclusion, we identified CF0218, a novel TMH family protein of *C. felis*, which can be used as a diagnostic antigen specific for *C. felis* infection. The precise role of CF0218 during the course of *C. felis* infection should be explored. This is the first report to describe the molecular characteristics of a *C. felis* TMH family protein. We are currently determining the seroprevalence of CF0218 in Japanese cats.

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Coxiella burnetii Isolates Cause Genogroup-Specific Virulence in Mouse and Guinea Pig Models of Acute Q Fever▽†

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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 pathological responses to infection with phase I *C. burnetii* isolates from the following 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 of the 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 pathological responses, and there is a clear distinction in strain virulence between these genomic groups.

Coxiella burnetii, the etiologic agent of acute and chronic Q fever, is an obligate intracellular bacterium with worldwide distribution and 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 (i) an acute flu-like illness with or without pneumonia and/or hepatitis (30, 31) or (ii) 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 and chronic Q fever. Unique sequence differences between genomic groups are correlated 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 lipopolysaccharide (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 to 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 (with plasmid-related sequences integrated into the 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, UT, 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 analyses (49) have validated these groupings. Infrequent-restriction-site PCR of 14 livestock and tick isolates resulted in six groups; subsequent multiple-locus variable-number tandem repeat analysis typing of 42 isolates revealed 36 genotypes (2). Glazunova et al. used multispacer sequence typing to analyze 173 isolates, a majority of which were acquired from chronic-

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TABLE 1. Isolates evaluated for virulence

Genomic group	Isolate	Notation in this study	Original source			
			Sample	Yr	Location	Disease
I	Nine Mile RSA493	NM	Tick	1935	Montana, US	NA[a] (acute; flu-like in humans)
	African RSA334	African	Human blood	1949	Central Africa	Acute; Congolese Red Fever
	Ohio 314 RSA270	Ohio	Cow's milk	1956	Ohio, US	Persistent
IV	MSU Goat Q177	Priscilla	Goat Cotyledon	1980	Montana, US	Abortion
	P Q173	P	Human heart valve	1979	California, US	Endocarditis
V	G Q212	G	Human heart valve	1981	Nova Scotia, Canada	Endocarditis
	S Q217	S	Human liver biopsy specimen	1981	Montana, US	Hepatitis
VI	Dugway 5J108-111	Dugway	Rodents	1958	Utah, US	NA

[a] NA, not applicable.

disease patients, and identified 30 genotypes in three monophyletic groups; an association between the plasmid type, some genotypes, and the nature of disease was observed (15). These monophyletic groups supported the early RFLP groups and placed groups 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 contents and pseudogenes may be factors in the variations in virulence seen among group I, IV, V, and VI isolates (5). It has been shown in an intraperitoneal (i.p.)-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 that 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 *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, human immunodeficiency virus 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 activities (25), and monocytes from chronic-Q fever patients have been shown to be defective in phagosome maturation and to have impaired *C. burnetii*-killing potential, regulated in part by elevated interleukin-10 (IL-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 suggested that chronic Q fever endocarditis may be associated with atypical M2 polarization and stimulation of bacterial replication (7), but the pathogenic process that mediates this polarization was undefined.

The route of infection may also be an important determining factor in the manifestation of acute and chronic Q fever. La Scola et al. and Marrie et al. demonstrated that the route of infection and the size of the inoculum affected clinical illness and pathology associated with infection in mouse and guinea pig models (26, 33). Differences in the geographic distributions of the diseases 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 *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 for *C. burnetii*. The establishment of an aerosol model of *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 *C. burnetii* prototype (NM isolate) (1), and the 50% lethal dose (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 intergroup pathogenicity differences of *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 them with previously reported group I isolate (NM) infections (1). Eight isolates from four genomic groups (Table 1) were then evaluated for the ability to cause acute disease in SCID mouse i.p.-challenge and guinea pig aerosol challenge models. We hypothesized that isolates within the same genotypic group would cause similar diseases and that there would be a distinct difference in disease manifestations between isolate groups. Finally, we evaluated the potential of a vaccine composed of one *C. burnetii* isolate to protect 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. The female 6- to 7-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). The female 6- to 8-week-old SCID and wild-type CB-17 mice used in the United States were purchased from Taconic (Hudson, NY). Female Hartley guinea pigs weighing approximately 350 to 450 g were purchased from Charles River Laboratories (Wilmington, MA).

All infected animals were housed in approved animal biosafety level 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. The Texas A&M University Laboratory Animal Care Committee reviewed and approved the mouse and guinea pig research at Texas A&M University, and experiments were carried out in AAALAC-approved facilities in accordance with university and federal regulations.

C. burnetii. Eight *C. burnetii* 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 J. Kazar, Institute of Virology, Brastislava, Slovakia, was maintained in mice by passage in spleen homogenates at Gifu University. The spleen homogenates were stored at −80°C until they were used. The absence of contamination with other pathogens was confirmed by direct staining (Giménez and Gram staining), detection of *Mycoplasma* DNA using a PCR *Mycoplasma* detection set (Takara, Shiga, Japan), and inoculation of the spleen homogenate into cell culture and SCID mice (independent experimental infection from the study described here). The bacterial dose was evaluated as the 50% tissue culture infectious dose ($TCID_{50}$) in BGM cells (buffalo green monkey fibroblasts), the 50% infectious dose (ID_{50}) in CB-17 mice, and the LD_{50} in SCID mice. The $TCID_{50}$ was determined by detecting the bacteria 6 days after infection using immunofluorescence staining with anti-*C. burnetii* rabbit antiserum. The ID_{50} was determined by detecting seroconversion (immunoglobulin G [IgG], >1:16) using indirect microimmunofluorescence. The LD_{50} was determined as reported previously (1).

For all subsequent experiments, all of the *C. burnetii* isolates were maintained at the Texas A&M Health Science Center. The *C. burnetii* isolates were cultivated in embryonated chicken eggs, purified by gradient centrifugation as previously reported (19, 44, 53), and stored at −80°C until they were used. The absence of contamination by other pathogens was confirmed as described above. *C. burnetii* was quantified by optical density (OD) (53), 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) (see Table S1 in the supplemental material). The bacterial dose used for mouse infections was determined by qPCR; guinea pig doses were calculated using the OD.

Experimental infection in mice. (i) Dose/effect experiment with the Priscilla isolate. Six mice per group were used for the dose/effect experiment. SCID, CB-17, and A/J mice were inoculated i.p. with serial 10-fold dilutions of Priscilla (10^2 to 10^{-7} $TCID_{50}$ per animal) or sterile phosphate-buffered saline (PBS) (sham infection). SCID mice were observed for 112 days (16 weeks), and CB-17 and A/J mice were observed for 30 days.

(ii) Genomic group comparison. Four mice per group were used for the genomic group comparison. Each of eight *C. burnetii* isolates described in Table 1 (10^5 genome copies/animal) or PBS was administered i.p. to SCID and CB-17 mice. Two independent infections were performed, and the mice were observed for 28 days (for all of the *C. burnetii* isolates in SCID and CB-17 mice) or until death (for 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) derived as follows: BWI = relative body weight/mean relative body weight of the control group; relative body weight = body weight on day "*x*" of infection/body weight on the day of infection. Cachexia was diagnosed when a mouse was lethargic and had a BWI of less than 0.85. At necropsy, the spleen weight was measured as an indicator of *C. burnetii* infection (54), and tissues were collected. To quantify the growth of *C. burnetii*, DNA was extracted from spleen tissue and *C. burnetii com1* gene copies were detected by qPCR as previously described (8). The heart, lung, liver, spleen, kidney, and femur were formalin fixed, embedded in paraffin, sliced, and 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 sera of CB-17 mice were measured by microimmunofluorescence as described elsewhere (1).

For cytokine assays, blood was collected from the lateral saphenous vein at 3, 7, 10, 14, and 21 days postinfection (p.i.) and via cardiac puncture at 28 days p.i. after euthanasia, and the group pooled sera were stored at −80°C until they were used. Sixteen cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12p40, IL-12p70, IL-10, granulocyte-macrophage colony-stimulating factor, gamma interferon [IFN-γ], KC, macrophage inflammatory protein 1α [MIP-1α], RANTES, and tumor necrosis factor alpha [TNF-α]) were measured using the Bio-Plex cytokine assay system (Bio-Rad, Hercules, CA) following the manufacturer's protocol. The cytokine quantification assay was performed in duplicate for each sample. The cytokine levels of infected sera were evaluated as the induction values compared to the 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), 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. (i) Three guinea pigs per group were infected with low (10^2), mid-level (10^4), or high (10^6) 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 of ≥39.5°C was defined as fever. The guinea pigs were observed for 28 days p.i. The spleens and livers were weighed at necropsy. Tissues were collected and formalin fixed for histopathologic evaluation. Serum was obtained from each animal for serologic testing. (ii) In a separate experiment, three guinea pigs per group were exposed to PBS or 2×10^6 particle equivalents of NM, P, G, or Dugway. Daily assessment of these animals was performed as described above, and the organs were weighed at necropsy 14 days p.i. to detect splenomegaly and/or hepatomegaly. (iii) In the heterologous-protection study, guinea pigs were vaccinated twice with 40 μg of formalin-inactivated group I (NM) or group V (S) *C. burnetii* in Freund's incomplete adjuvant or with adjuvant alone, with 2-week intervals between the vaccinations and infection. The animals were then infected with high doses of either NM or S. Three animals per group were separated into the following six groups: (a) nonvaccinated, NM infected; (b) nonvaccinated, S infected; (c) NM vaccinated, NM infected; (d) S vaccinated, S infected; (e) NM vaccinated, S infected; and (f) S vaccinated, NM infected. The 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 a Vectastain ABC kit and a Vector NovaRed substrate kit (Vector Laboratories, Burlingame, CA) and in-house-generated rabbit anti-*C. burnetii* NM (3) and by counterstaining them with hematoxylin. All slides were evaluated in a blinded fashion. Serum samples collected at necropsy were tested by enzyme-linked immunosorbent assay for IgG titers against phase I *C. burnetii* NM antigen as previously described (43). Sera from uninfected guinea pigs were used as negative controls.

Statistical analyses. The results were expressed as means for each group and were compared using one- and two-way analysis of variance or Student's *t* test, as appropriate. Differences were considered significant at a P value of <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 i.p.-challenged guinea pigs (36). The infectious titer of the Priscilla isolate in the splenic homogenate used for the multiple-dose infection was 2×10^4 $TCID_{50}$/ml in BGM cells, $2 \times 10^{9.3}$ ID_{50}/ml in CB-17 mice, and 2×10^{10} LD_{50}/ml in SCID mice (1 $TCID_{50}$ corresponded to $10^{5.3}$ ID_{50} in CB-17 mice and to 10^6 LD_{50} in SCID mice). The LD_{50} in CB-17 mice could not be determined because no CB-17 mice died from 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 CB-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 the Priscilla iso-

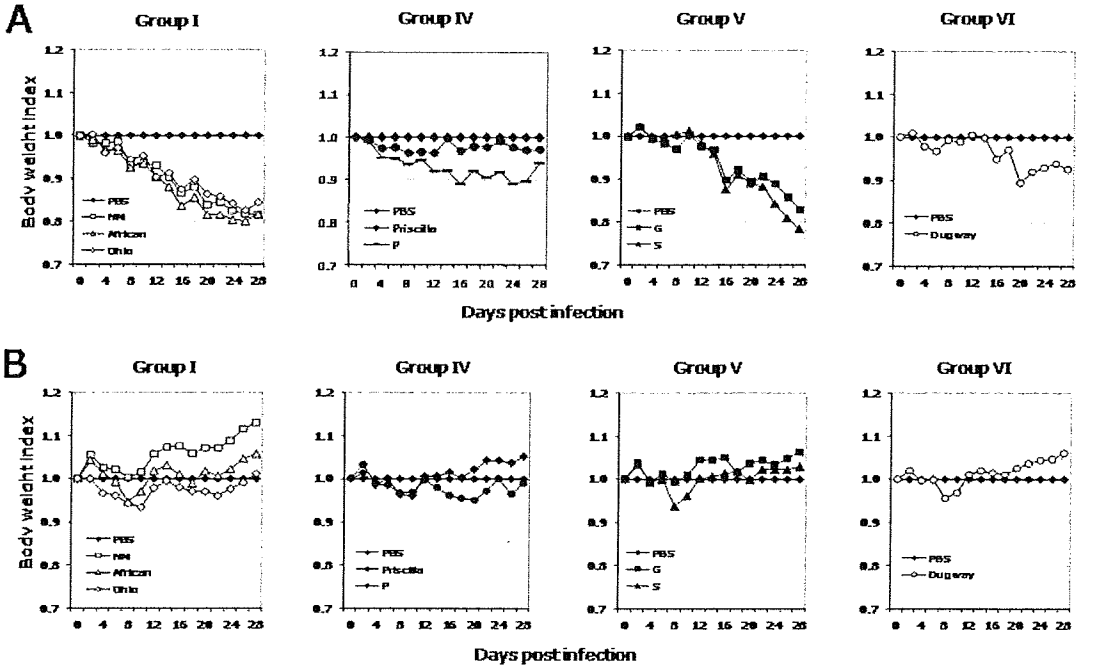

FIG. 1. Average body weight changes in SCID mice (A) and CB-17 mice (B) infected with *C. burnetii* isolates during 28 days of infection. Body weights were significantly lower in SCID mice throughout the infection period and transiently in CB-17 mice infected with all isolates except Priscilla compared to PBS-injected controls ($P < 0.05$).

late 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 times were dose dependent, with shorter times corresponding to higher infectious doses (see Table S2 in the supplemental material). Similar lesions were found in all of the 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, CB-17 and A/J mice displayed transitory clinical signs only after 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., CB-17 and A/J mice had mild splenomegaly and seroconversion as evidence of infection (data not shown). Small granulomas were present in the spleen and liver, but bacterial antigen was not detectable by immunohistochemistry.

Genomic-group-specific virulence in mice. It was important to establish whether the results of infection seen with the Priscilla isolate and those previously noted with the NM isolate were 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 i.p. injection. The infections were initially compared in SCID and CB-17 mice sacrificed at 28 days p.i.

All *C. burnetii* isolates caused disease in SCID mice, with various 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 Fig. 1A and in Fig. S1A in the supplemental material, were most apparent in mice infected with group I isolates, followed by those given group V, IV, and VI isolates. In CB-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 CB-17 mice (Fig. 2A). The number of bacteria in the spleens was determined by qPCR (Fig. 2B), and consistently higher numbers of *com1* genes were detected in SCID than in CB-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 CB-17 mice, splenic enlargement and numbers of bacteria increased with the severity of clinical disease. CB-17 mice displayed differences between infection with the *C. burnetii* isolates that caused acute disease (phylogenetic group I) and infection with the *C. burnetii* isolates that caused chronic disease (phylogenetic groups IV and V), but there was no difference between groups infected with isolates that caused chronic disease. All infected mice developed significant splenomegaly,

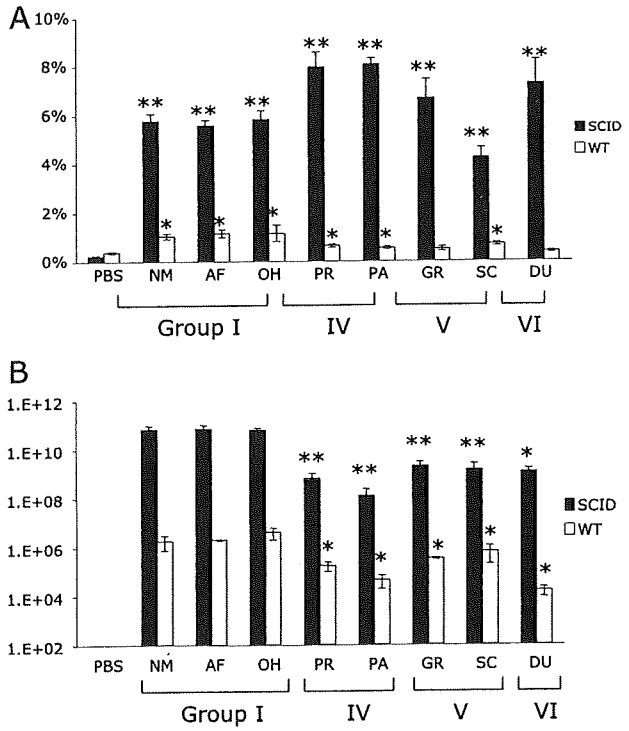

FIG. 2. Splenomegaly (A) and splenic bacterial loads (B) in mice at 28 days p.i. (A) All infected animals developed significant splenomegaly compared to controls, and infected SCID mice had significantly larger spleens than CB-17 mice ($P < 0.05$). (B) Mice infected with group IV, V, and VI isolates had significantly fewer bacteria than those infected with group I isolates ($P < 0.05$). *, $P < 0.05$. The error bars indicate standard deviations.

but mice infected with group IV, V, and VI isolates had significantly fewer splenic bacteria than mice infected with group I isolates ($P < 0.05$).

Evaluation of histopathology at 28 days p.i. revealed more lesions in SCID mice than in CB-17 mice (see Table S3 in the supplemental material). SCID mice showed histopathologic changes in all organs investigated. Group I isolates caused the most inflammation, followed by groups V, IV, and VI. The inflammatory-cell populations were 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. CB-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. The variations in pathology and inflammation associated with these isolate group infections suggest differences in the immune responses. To expand on this observation, the serum levels of 16 cytokines and chemokines were measured. In CB-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 than other groups. The KC and granulocyte-macrophage colony-stimulating factor levels of mice infected with group I isolates were higher than those in mice infected with other groups prior to 14 days p.i. Serum IL-1α, IL-1β, IL-2, and IL-5 levels and eotaxin secretion were not increased during the infection period (data not shown).

Lethal potentials of all genomic groups in SCID mice. The lethal potentials of representative isolates from each phylogenetic group were 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 (see Fig. S1B in the supplemental material). Isolates that caused a long period of cachexia led to severe body weight loss in infected mice (see Fig. S2 in the supplemental material). A 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. The 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), VI (35.5 ± 1.0 days), and IV (47.5 ± 0.6 days for P and 77.3 ± 2.8 days for Priscilla). The probable cause of death was multiple-organ failure due to massive systemic infection.

The pathological 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 was 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 numbers of bacteria in the spleen were similar in all groups, suggesting that the number of bacteria (10^{10} genome copies/spleen) detected 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, making this a choice model for evaluating the comparative levels of virulence of different *C. burnetii* isolates, and thus, it was used in the logical progression of experiments after different levels of virulence were observed in mouse models of infection. Guinea pigs challenged with group I and V isolates developed 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 the group I *C. burnetii* isolates African and Ohio, as has been described for the reference isolate in this group, NM (43). All animals that received African or Ohio organisms at a high

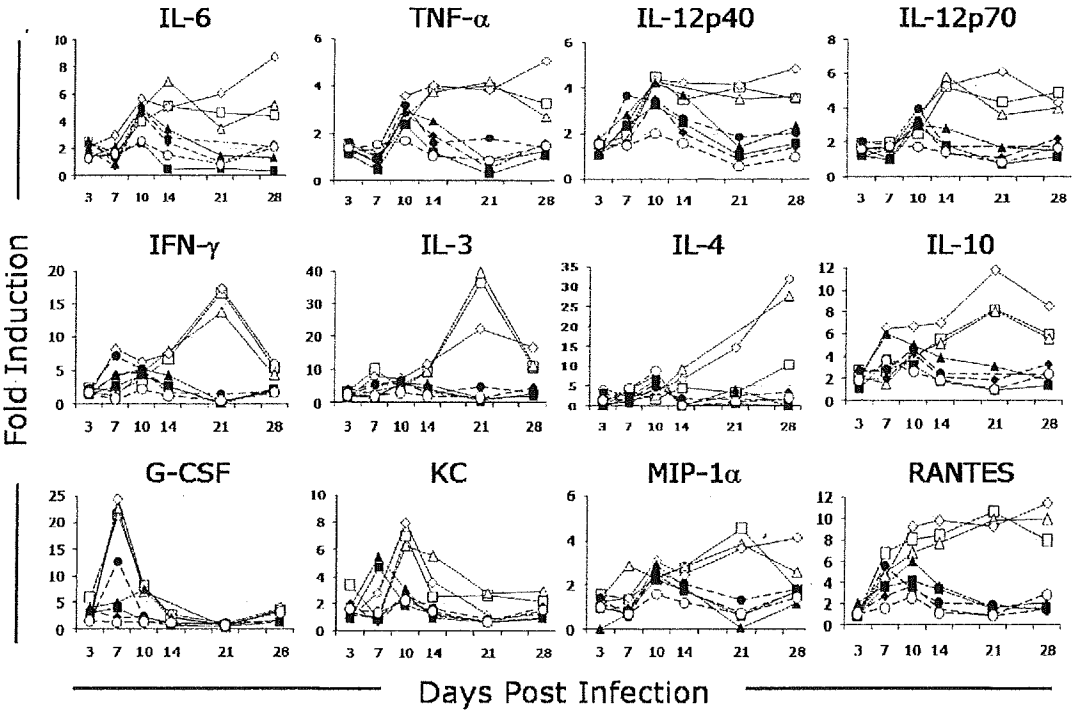

FIG. 3. Mean circulating cytokine levels in response to infection in CB-17 mice with different *C. burnetii* isolates. Isolates from genomic group I induced persistently high cytokine secretion with increased levels of IL-6, TNF-α, IL-12p40, IL-12p70, IFN-γ, IL-3, IL-4, IL-10, MIP-1α, and RANTES compared with other genogroups ($P < 0.05$). ◆, PBS; □, NM; △, African; ◇, Ohio; ●, Priscilla; ◆, P; ■, G; ▲, S; ○, Dugway.

dose died within 7 to 9 days p.i., as did two of three that received NM; lower infectious doses were not lethal. Gross lung consolidation and overall lack of normal body fat were noted on necropsy at 7 to 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-level dose of group I organisms were evaluated at 28 days p.i. for comparison to animals infected with other isolates evaluated at this time, and they 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 given the highest dose of organisms.

Group V isolate-infected guinea pigs all developed fever when given the highest challenge dose, and dose-dependent temperature increases 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 mild to moderate lymphohistiocytic interstitial pneumonia and a few small granulomas.

No major clinical or pathological changes were noted in guinea pigs infected with the group VI isolate or in negative control animals. Table S4 in the supplemental material compares the severity of histopathologic changes in guinea pigs infected with high doses of *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, livers, and spleens of infected animals.

Experimental guinea pigs in all dose groups for each isolate seroconverted by the time of euthanasia, with the exception of animals infected with high doses of NM, African, and Ohio necropsied at 1 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-injected 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) had not been previously described in animals experimentally infected with *C. burnetii* and were also 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 doses of group I isolates, as had been previously reported for NM aerosol-infected guinea pigs (43). Tissue sections from the remaining NM-infected guinea pig and those infected with mid-level doses of the 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 various sizes. The granulomas in Priscilla- and P-infected guinea pigs were more defined, with more histiocytic involvement than was

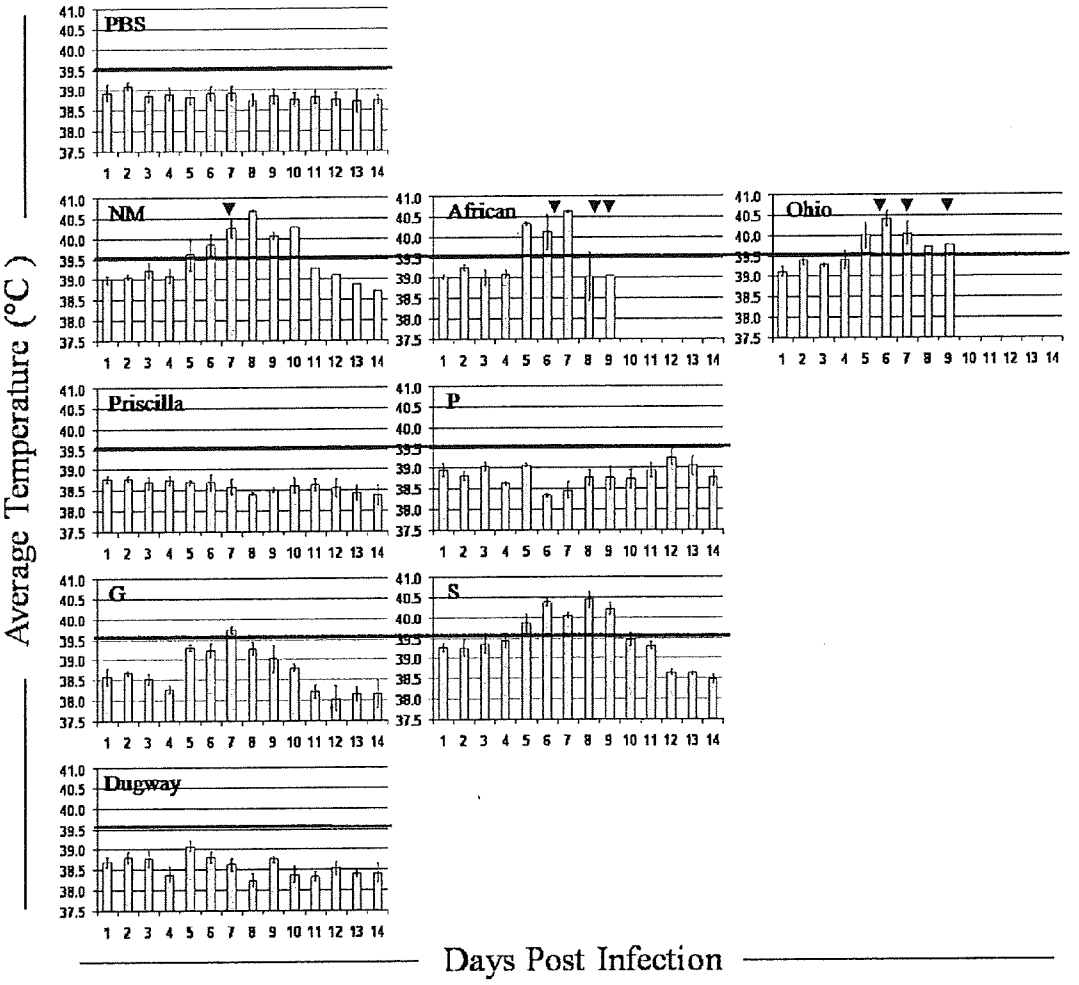

FIG. 4. Fever responses of guinea pigs to infection with high doses of *C. burnetii* isolates. The mean daily temperatures ± standard errors of the mean ($n = 3$) of animals infected with 2×10^6 bacteria of each *C. burnetii* isolate. Temperatures of ≥39.5°C (black lines) were considered fever. The arrows indicate days on which death occurred in NM-, African-, and Ohio-infected groups.

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 guinea pigs infected with the group V isolates G and S contained a 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 so than group I isolates.

No hepatic granulomas or other significant pathological 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 weights 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-injected 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 (see Fig. S3 in the supplemental material). Pathological 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 here illustrate that there is pathotype diversity between *C. burnetii* isolates from different genogroups, and they are consistent with phylogenetic studies cataloging distinct gene contents (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) vaccine and cross-challenged to evaluate potential heterologous protection against high-dose infection. Nonvaccinated guinea pigs developed a noticeable fever response by day 5 p.i.,

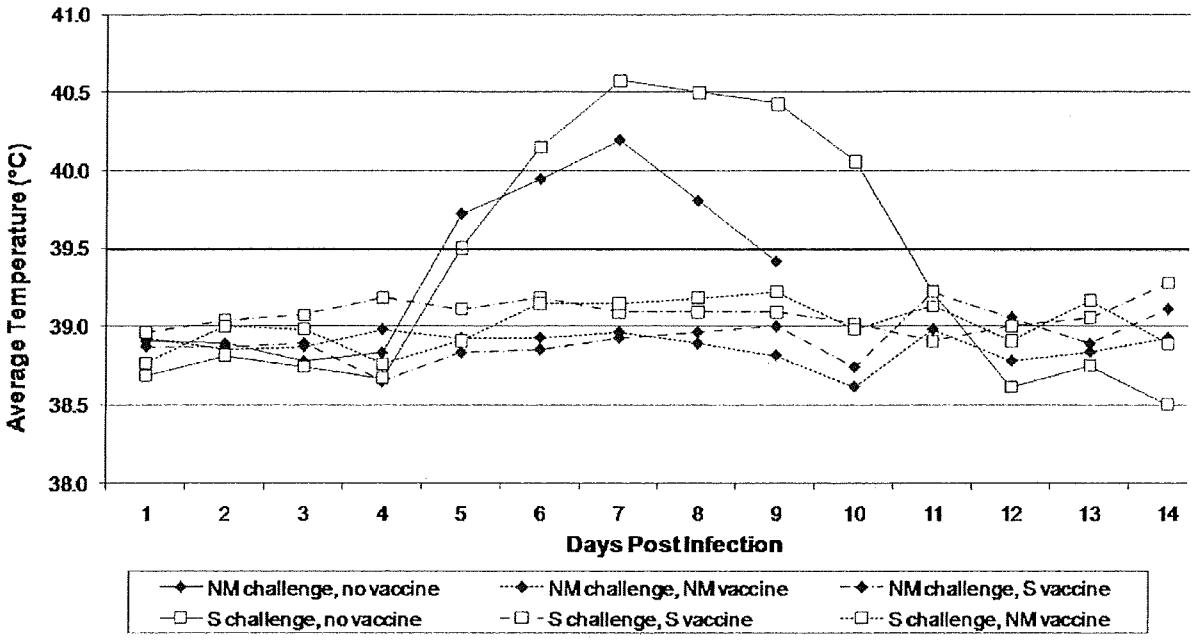

FIG. 5. Heterologous vaccination and challenge in guinea pigs. Shown are average daily temperatures of animals vaccinated with NM (dashed and dotted line), S (dashed line), or adjuvant alone (solid line) and challenged with high doses of NM (◆) or S (□). Temperatures of ≥39.5°C were considered fever.

and infection was lethal in three of three NM- and one of three 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 the Priscilla isolate dose-effect in SCID mice revealed differences in virulence of *C. burnetii* isolates. Disease development after Priscilla infection was progressive but slower than the development of 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}. This result supports the previous study by Moos and Hackstadt that evaluated the lesser ability of the Priscilla isolate to cause fever in i.p.-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 the high infectivity and lethal potential of *C. burnetii*, which is not restricted to isolates that cause acute disease, 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 pathological changes. Groups IV and V, isolates associated with chronic Q fever, caused a slower progression of disease. Overall, pathological 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, the bacterial loads at the time of death were 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 of the isolates could persistently infect, but Priscilla required 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 pathological changes, the replication rate does not seem to be the only virulence factor involved, since clinical signs would then be similar with differences only in disease progression. At both time points, 28 days p.i. and the 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 isolates from chronic disease 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 genetic characteristics similar to those of isolates from acute disease (46). The hypothesis that isolates from acute disease 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 genes specific to isolates from acute disease in isolates from chronic Q fever patients and concluded that the isolates were not disease specific (46). The isolates used in the study were isolated by cell culture, and although the cell culture system is highly effective for isolation, isolates from acute disease are known to infect cultured cells more efficiently than isolates from chronic disease, 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 that there was 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 than other groups. Interestingly, Dugway (group VI) induced the least change in CB-17 mice. The inflammatory-cytokine changes in immunocompetent mice in this study were similar to those in humans with acute Q fever (10): TNF-α and IL-6 were upregulated, but IL-1β was not. IFN-γ increased in CB-17 mice infected with group I isolates, and it 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 be correlated 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 illnesses, 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 of Kazar et al. that the virulence of NM and S isolates was greater than that of Priscilla.

Lesny et al. compared the cross-immunity of whole-cell and soluble Q fever vaccines made from phase I NM, S, Priscilla, and Luga isolates. They found that vaccines from NM 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 manifestations of disease and genomic contents 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 the design of new vaccines and could be of the utmost importance in offering reliable protection in the event of an outbreak.

The differences in perceived infectious doses noted when ODs, particle counts, and genome copy enumerations were compared underline the importance of using multiple quantitation methods to compare studies with earlier observations. Some of the differences in disease manifestations seen in guinea pigs in this study could be due to slight differences in the infectious doses delivered. For instance, Priscilla and P both induced hepatic changes, although guinea pigs infected with P appeared to develop more severe lesions than those infected with Priscilla, which had a lower infectious dose by OD and qPCR. The difference in infectious dose as determined by the 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 unit lower than those of 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 the infectious dose depending on the enumeration technique, the significant differences noted among genotypic groups are valid.

Phase variation is the only well-characterized phenotypic difference that is related 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 governs the ability of *C. burnetii* to infect cells and to maintain continuous growth within the phagolysosome. Indeed, the combination of pathotype variation of disease in infected guinea pigs and cross-protection of different isolates suggests conserved predominant antigenic components with virulence determinant specificity.

A recent report compared all open reading frames of NM phase I to those of African, Ohio, P, G, S, and Dugway, among others (4), and a majority of the open reading frames deleted from NM 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 pseudogenes and unique gene contents 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 pathological 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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Human leptospirosis cases and the prevalence of rats harbouring *Leptospira interrogans* in urban areas of Tokyo, Japan

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Thirteen patients with leptospirosis were identified, as confirmed by laboratory analysis during the last 5 years in our laboratory, who came from urban areas of Tokyo, Japan. All of the patients came into contact with rats before the onset of illness. Seventeen per cent of Norway rats captured in the inner cities of Tokyo carried leptospires in their kidneys. Most of these rat isolates were *Leptospira interrogans* serovar Copenhageni/Icterohaemorrhagiae. Antibodies against these serovars and their DNA were detected in the patients. This suggests that rats are important reservoirs of leptospirosis, and that rat-borne leptospires occur in urban areas of Tokyo.

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INTRODUCTION

Leptospirosis is caused by infection with pathogenic *Leptospira*. It is a globally important zoonotic disease that affects humans in rural and urban settings, in both industrialized and developing countries (Bharti *et al.*, 2003; Levett, 2001; McBride *et al.*, 2005). Transmission of *Leptospira* pathogens to humans occurs mainly through indirect contact with water or soil contaminated by the urine of infected animals (Faine *et al.*, 1999). Leptospirosis has become an important public health problem in Asia and Latin America. In these tropical areas, large outbreaks of leptospirosis are most likely to occur after floods, hurricanes or other disasters. Leptospirosis has also become an urban problem in developing countries. Outbreaks occur in poor urban slum communities during seasonal periods of heavy rainfall (Johnson *et al.*, 2004; Ko *et al.*, 1999; LaRocque *et al.*, 2005). The risk of infection in urban inhabitants is not limited to developing countries because the importance of urban leptospirosis has already been recognized in inner-city populations of the USA (Vinetz *et al.*, 1996). In the present study, we report the presence of leptospirosis and rat reservoirs of leptospires in urban areas of Tokyo, Japan.

METHODS

Serodiagnosis of patients with clinically suspected leptospirosis. The microscopic agglutination test (MAT) for detection of anti-*Leptospira* antibodies in patient serum samples was performed (Faine *et al.*, 1999) using a battery of reference strains described previously (Koizumi *et al.*, 2008). These reference strains were cultivated in liquid modified Korthof's medium with 10% rabbit serum at 30 °C (Faine *et al.*, 1999). Detection of IgM was also carried out by IgM dot enzyme-linked immunoassay (*Dip-S-Ticks*; PanBio) for cases 6 and 8 (Supplementary Table S1 available with the online journal).

Isolation of leptospires from rats. Norway rats (*Rattus norvegicus*) were captured using live traps at 14 locations in urban areas of Tokyo from 2002 to 2007. For the isolation of leptospires, rat kidneys were inoculated into medium and cultivated as described above.

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Abbreviation: MAT, microscopic agglutination test.

The GenBank/EMBL/DDBJ accession numbers for the *flaB* sequences of rat isolates and patient samples are AB454100–AB454125.

A table of detection test data and a figure of PFGE results are available as supplementary data with the online version of this paper.

PCR. DNA was extracted from *Leptospira* isolates, and the blood and urine samples of patients, using a DNeasy tissue kit (Qiagen). Extracted DNAs were subjected to PCR to detect the *Leptospira flaB* gene (*flaB*-PCR; Kawabata *et al.*, 2001; Koizumi *et al.*, 2003). Sequencing of amplicons was performed by the dideoxynucleotide chain-termination method using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems).

Identification of serogroups of rat isolates. The serogroups of the isolates were identified by MAT using a panel of anti-*Leptospira* rabbit sera for serovars Australis, Autumnalis, Canicola, Copenhageni, Hebdomadis and Icterohaemorrhagiae, which are present in the main island of Japan.

PFGE. PFGE of rat isolates was carried out as described previously (Koizumi *et al.*, 2009).

RESULTS AND DISCUSSION

Human leptospirosis cases in urban areas of Tokyo

In the last 5 years (from the first case on 4 September 2003 to the last on 18 September 2008), we carried out laboratory examinations for leptospirosis for 55 cases. According to their physicians in Tokyo, the symptoms in these patients matched those of leptospirosis. A total of 16 cases were revealed to be positive for leptospirosis during the period of the study; 13 were from Tokyo (Table 1, Supplementary Table S1 available with the online journal) and the other 3 cases were from Bali (Indonesia), Borneo (Malaysia) and Fiji (data not shown). Among the 13 cases in Tokyo, 12 patients were definitively diagnosed by MAT (fourfold increase in antibody titre between acute and convalescent serum samples or reciprocal MAT titre of at least 400 in a single serum sample), including 4 patients who were also positive for the leptospiral *flaB* gene by PCR using urine or blood specimens. One probable case was demonstrated by anti-leptospiral IgM and a MAT titre of 1 : 160. All patients were hospitalized with severe manifestations, such as acute renal failure and jaundice, indicating that they had contracted Weil's disease (a severe type of leptospirosis). All patients declared that they had come into contact with rats (Table 1). Two patients (nos 1 and 2) worked at a place where they came in contact with rats, and rats were frequently found in the houses or stores of other patients (nos 3–13). The patients neither performed agricultural work nor undertook recreational activity in an endemic area, nor were they exposed to possible reservoir animals other than rats, which are generally considered as high risk behaviours. Among the 39 leptospirosis-negative cases, only 4 patients came in contact with rats (1 patient was a construction worker, and rats were seen at the houses of the other 3 patients). This indicated that the ratio of contact with rats among leptospirosis-positive cases was significantly higher than that among leptospirosis-negative cases in urban areas of Tokyo (Fisher's exact test, $P<0.01$). Since leptospirosis became a reportable disease in Japan (from November 2003), another laboratory-confirmed case other than the 13 cases mentioned above was reported from a regional medical centre in Tokyo in September 2006. The patient saw rats at his restaurant (T. Iida, personal communication).

Isolation and characterization of *Leptospira interrogans* from Norway rats captured in urban areas of Tokyo

We captured 127 Norway rats (*R. norvegicus*) at 14 locations in urban areas of Tokyo from 2002 to 2007. Leptospires were isolated from 22 of the 127 rats from 6 of

Table 1. Human leptospirosis in urban areas of Tokyo diagnosed in our laboratory from 2003 to 2008

Patient no.	Year	Sex	Age (years)	Occupation	Association with rats
1	2003	M	66	Construction worker	Probable environmental contamination with rat urine
2	2004	M	35	Sewer worker	Probable environmental contamination with rat urine
3	2005	F	53	Housewife	Rats appeared frequently in patient's house
4	2005	M	65	Butcher	Patient cleaned the urine and faeces of rats in his store without wearing gloves
5	2006	M	51	Fish dealer	Rats appeared frequently in patient's store
6	2006	M	62	Unknown	Rats appeared frequently in patient's house
7	2006	M	54	Fish dealer	Patient cleaned the urine and faeces of rats in his store without wearing gloves
8	2007	M	51	Day worker	Rats appeared frequently in patient's house (he had been bitten by a rat at his house)
9	2007	F	57	Restaurant worker	Rats appeared frequently in patient's restaurant
10	2008	M	57	Supermarket salesman	Patient was involved in killing rats captured at his store
11	2008	M	56	Fish market worker	Rats appeared frequently in the fish market
12	2008	M	58	Unknown	Patient had contact with rat urine in his house
13	2008	M	68	Restaurant chef	Patient had been bitten by a rat in his restaurant

F, Female; M, male.

the 14 places (Table 2). Nucleotide sequences of the partial *flaB* gene from 18 rats captured at locations F, G, H, K and M were identical (GenBank accession numbers AB454100–AB454117) and those from 4 rats at location A (the *flaB* sequences were identical among the four; GenBank accession numbers AB454118–AB454121) were different in six bases from those described above. The sequences from the 18 rats were identical to those of the reference strains of *L. interrogans* serovar Copenhageni and Icterohaemorrhagiae, suggesting that all the isolates were *L. interrogans*. These isolates reacted equally with anti-Copenhageni and anti-Icterohaemorrhagiae sera, but not with the other sera (data not shown). The *Not*I restriction patterns of the genomes of the isolates from the 18 rats were identical on PFGE not only to each other, but also to the reference strains of serovars Copenhageni and Icterohaemorrhagiae (Supplementary Fig. S1 available with the online journal). It has been determined by PFGE that the genomes of leptospiral serovars have been remarkably conserved over time and across a wide geographical distribution (Herrmann *et al.*, 1991, 1992). Most (but not all) serovars give unique patterns on PFGE carried out using the restriction enzyme *Not*I, although the *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae are indistinguishable. These two serovars are also very difficult to distinguish by serological methods (Kobayashi *et al.*, 1984). These results indicate that isolates from the 18 rats belonged to *L. interrogans* serovar Copenhageni or Icterohaemorrhagiae, which are known to frequently cause Weil's disease in Japan and other countries. We could not carry out MAT for serogroup identification and PFGE on the four isolates at location A due to poor growth.

Table 2. Isolation of leptospires from rats captured in urban areas of Tokyo

Location*	No. of rats captured	No. of rats from which *Leptospira* was isolated (%)
A – park	15	4 (27)
B – park	2	0
C – street	4	0
D – street	8	0
E – building	12	0
F – street	29	12 (41)
G – street	1	1 (100)
H – garden (house)	4	3 (75)
I – park	1	0
J – street	12	0
K – street	13	1 (8)
L – street	5	0
M – store	2	1 (50)
N – street	19	0
Total	127	22 (17)

*Location M was a store in which patient 7 used to work; other locations are not related to the putative exposure sites of the patients.

Conclusion

In 5 of the 13 human leptospirosis cases (cases 1–3, 6 and 7), the patients were infected with serovar Copenhageni or Icterohaemorrhagiae as shown by serological and PCR-based evidence (Supplementary Table S1 available with the online journal). Cross-agglutination and even paradoxical reactions are observed in MAT, but the existence of antibodies against serovars Copenhageni and Icterohaemorrhagiae in all other serum samples suggests the possibility of infection with these serovars (Supplementary Table S1 available with the online journal). Nucleotide sequences of the partial *flaB* gene from urine and blood (patients 1, 2, 3 and 7; GenBank accession numbers AB454122–AB454125) were identical to those from the rat isolates. In particular, *Leptospira* was isolated from a rat captured at the store where patient 7 previously worked. Although there is a possibility of recall bias, all the patients said they had rat contact (Table 1). Dogs and cats may also serve as reservoir hosts in urban settings. We attempted to isolate leptospires from the kidney tissues of stray or abandoned dogs and cats in Tokyo (304 dogs and 77 cats), but *Leptospira* was not obtained. These results strongly suggest that the patients contracted leptospirosis (Weil's disease) from rats in urban areas of Tokyo, though the possibility of involvement of other reservoir animals cannot be excluded.

Outbreaks of leptospirosis are recognized through occupational exposure, such as rice farming and other agricultural activities in rural areas of the tropics (Tangkanakul *et al.*, 2000). Leptospirosis has also become a health problem in urban slums in developing countries (Johnson *et al.*, 2004; Ko *et al.*, 1999; LaRocque *et al.*, 2005). In 'developed countries', recreational activities have recently been identified as a significant risk factor for leptospirosis (Bharti *et al.*, 2003; Levett, 2001; McBride *et al.*, 2005). The present study suggests that humans could contract leptospirosis through occupational exposure or exposure during activities of daily life in environments contaminated with rat urine containing leptospires in urban areas in Tokyo. Leptospirosis constitutes one of the neglected diseases in Japan except for Okinawa Prefecture (Nakamura *et al.*, 2006; Narita *et al.*, 2005). This is one of the reasons why few cases have been identified over the 5 year period despite the high carriage of leptospires in rats in Tokyo. A high prevalence of *Leptospira* spp. in Norway rats from urban settings has also been reported from temperate and tropical endemic countries, but human leptospirosis in urban areas is underreported even in an endemic country (Ariyapruchya *et al.*, 2003; Demers *et al.*, 1985; Doungchawee *et al.*, 2005; Easterbrook *et al.*, 2007; Krøjgaard *et al.*, 2009). Physicians and public health authorities should, therefore, be aware of the severe risk of contracting leptospirosis associated with rats in urban areas.

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