ズーノーシスハンドブック

医療関係者・獣医療関係者のための診断・治療ガイド

監修

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媒介動物	保有動物	主な感染様式
	トリ，一部の哺乳類	吸入（感染動物の糞便，分泌物）

この疾患について

オウム病（Psittacosis）は，オウム病クラミジア*Chlamydophila psittaci*によって引き起こされる疾患である．年間40例程度の報告がある．単発例や家族発症が多いが，展示施設での集団発生もみられている．春から初夏の繁殖時期にはトリにストレスがかかって排菌量が増える傾向があり，患者発生も多い．

感染経路　図1

感染したトリの排泄物中の*C. psittaci*を吸入する飛沫感染が主体である．口移しの給餌や，噛まれて感染することもある．

潜伏期間

*C. psittaci*を吸入し，1～2週間の潜伏期間の後，発症する．

症　状

高熱で突然発症し，頭痛，全身倦怠感，筋肉痛，関節痛などがみられ，比較的徐脈，肝障害を示すことが多い．乾性あるいは湿性の咳がみられ，血痰，チアノーゼを認める重症例もある．治療が遅れて急性呼吸窮迫症候群（ARDS）や重症肺炎に至った場合，髄膜炎，多臓器障害，播種性血管内凝固症候群（DIC）さらにショック症状を呈し致死的な経過をとることもある．

検査・診断

早期診断には，トリとの接触歴，飼育歴についての詳細な問診が重要である．一般検査では白血球数は正常で，CRPや赤沈は亢進し，中等度の肝機能異常をきたすことが多い．特殊検査としては，咽頭スワブや痰からの分離培養，抗原検出法，遺伝子検出法などで，通常はPCR法が行われる．血清診断にはmicro-IF法など，種の特定が可能な検査法を用いる．補体結合反応（CF）は届け出基準の対象検査から除かれている．

予防・治療

テトラサイクリン系抗菌薬が第一選択薬で，マクロライド系#，ニューキノロン系#がこれに次ぐ．投与期間については，約2週間と長めの投与がよい．β-ラクタム系，アミノ配糖体などの抗菌薬は無効である．

一般の飼育者の予防としては，乾燥糞を吸わないように注意する，口移しの給餌など過度な接触を避ける，などである．トリを扱った後は手洗いをし，死亡したトリの取り扱いには特に注意する．

#：保険適応外

動物での状況　図2

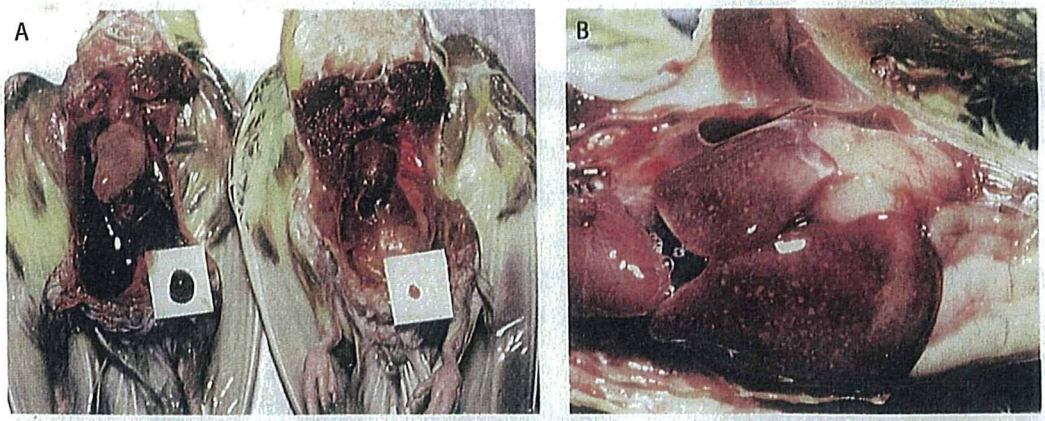
症　　状　*C. psittaci*は，鳥類ではオウム目を含む18目145種から報告されており，保有率は約5%である．通常，元気消失，食欲不振，鼻腔からの漿液性滲出物や緑白色下痢を認める．急性例では，無症状のまま死亡することもある．不顕性感染のトリの発症は，飼育環境の悪化・輸送などによるストレスが誘因となる．

検査・診断　生前診断では，臨床所見および排泄物からの病原体検出を行う．病原体の検出は，検査材料の発育鶏卵・培養細胞への接種，PCR法による．発症したトリの剖検所見では，脾臓・肝臓の腫大が認められ，蛍光抗体法により細胞質内封入体が確認される．実験室内感染防止のため，安全キャビネット内で作業を行うか，検査機関に検査を依頼する．

予防・治療　現行では有効なワクチンはない．飼育環境の改善，不顕性感染のトリの摘発・治療により集団発生を予防する．新規にトリを導入する場合には，数週間の検疫および病原体検査が有効である．治療には，ドキシサイクリンなどのテトラサイクリン系抗菌薬の給餌・飲水投与が効果的であり，感染したトリには40日程度の連続投与が推奨されている．治療は同一飼育群すべてに行うべきである．β-ラクタム系抗菌薬は使用すべきではない．アミノ配糖体系抗菌薬には感受性がない．

治療予後　抗菌薬の投与期間中は，健康状態に注意し，適宜，病原体の検査を行う．抗菌薬の長期投与により菌の保有が認められなければ，一般的に予後は良好である．

【図1】オウム病の感染経路



【図2】オウム病クラミジアに感染したトリ（セキセイインコ）に認められる，典型的な剖検所見（平井原図）
脾臓の腫大（A）と肝臓の灰白色壊死巣（B）．

「オウム病」著：大屋 賢司・岸本 寿男・福士 秀人

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Original Article

Epidemiology of *Chlamydophila caviae*-like *Chlamydia* Isolated from Urethra and Uterine Cervix

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In 2000, chlamydial strains OK133 and OK135 were isolated from 2 female patients with cervicitis. These strains were unresponsive to commercially available PCR and LCR test kits for the diagnosis of *Chlamydia trachomatis* infection, and their phenotypic characteristics were very similar. The OK135 nucleotide sequence in MOMP-VD2 gene closely resembled that of *Chlamydophila caviae* GPIC. A similar strain was isolated in 2003 from a male patient OKM2 with urethritis, from which the strain SC10-6 was cloned by the plaque purification method. The nucleotide sequence of the entire MOMP gene of SC10-6 was exactly the same as that of OK135. Thus, the strains OK135 and SC10-6, together with OK133, have been called *C. caviae*-like *Chlamydia*. We designed primers for nested PCR assay, the product of which showed a single-band 311-bp fragment, to detect *C. caviae*-like *Chlamydia*. Of swab specimens obtained from 202 patients from 2003 to 2006 (119 male and 83 female patients), 18 specimens (8.9%) from 14 male and 4 female patients were positive, suggesting that *C. caviae*-like *Chlamydia* infection is rather common. Thus far, it has not been determined whether *C. caviae*-like *Chlamydia* is pathogenic for humans.

Key words: *Chlamydophila caviae*-like *Chlamydia*, urethra, uterine cervix, epidemiology, sexually transmitted infection

Species of the family *Chlamydiaceae* are obligate intracellular prokaryotic parasites of various types of eukaryotic cells including human and animal cells. A unique developmental cycle, in which chlamydial organisms alternate between an infectious elementary body (EB) and the vegetative reticulate body (RB), distinguishes chlamydial organisms from other bacteria [1]. The family *Chlamydiaceae* includes 2 genera *Chlamydia* and *Chlamydophila*, and nine species, *Chlamydia trachomatis*, *Chlamydia muridarum*, *Chlamydia suis*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Chlamydophila pecorum*, *Chlamydophila abortus*, *Chlamydophila caviae* and *Chlamydophila felis* [2]. *C. pneumoniae* and *C. psittaci* are well known as pathogens of the respiratory tract. *C. trachomatis*, which was originally associated with the ocular disease trachoma,

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is recognized as the most common pathogen of sexually transmitted infections (STIs) including urethritis and epididymitis in men, cervicitis, urethritis and upper genital tract infections in women, and conjunctivitis and pneumonia in newborns. Complications arising from *C. trachomatis* infection include pelvic inflammatory diseases such as ectopic pregnancy and infertility in women. Efforts to reduce the prevalence of infection with *C. trachomatis* in both men and women may be hampered by the relatively high frequency of asymptomatic patients in both sexes [3]. Due to the diversification of sexual cultures and behaviors, the increase in STI has become a social problem that cannot be ignored in Japan [4].

A commercially available PCR test kit and a ligase chain reaction (LCR) test kit, both targeting the 7.5-kb cryptic plasmid common to all members of *C. trachomatis*, have been widely used in the diagnosis of urogenital *C. trachomatis* infection in Japan. The results of laboratory experiments have shown that the detection limit for both PCR and LCR test kits is just two EBs, and these test kits are highly sensitive and specific [5, 6]. However, using these test kits alone, the biological characteristics of etiologic *C. trachomatis* strains cannot be analyzed because the isolation and propagation of *C. trachomatis* strains are not required in the diagnosis. Furthermore, studies have reported the presence of *C. trachomatis* lacking the plasmid [7] and STI with plasmid-free *C. trachomatis* [8–10]. Moreover, *C. trachomatis* strains missing part of the nucleic acid sequence in the plasmid were currently reported [11]. Hence, when using only the PCR or LCR test kit, infection caused by *C. trachomatis* lacking the plasmid and/or missing a plasmid sequence would be overlooked.

In a preliminary survey of *C. trachomatis* urogenital infection in 2000, we isolated strains OK133 and OK135, which were unresponsive to PCR and LCR test kits, from female patients with severe cervicitis. Their phenotypic characteristics were very similar. Genetic analysis of OK135 revealed that the nucleotide sequence of the MOMP-VD2 gene closely resembled that of *C. caviae* GPIC. A strain similar to OK135 was more recently isolated from a male patient OKM2 with urethritis and cloned as strain SC10-6 by the plaque purification method [7]. Analysis of the entire MOMP gene showed that SC10-6 and OK135 were identical and closely resembled *C. caviae* GPIC (hereinafter referred to as *C. caviae*-like *Chlamydia*). In the present paper, we report an epidemiological study of *C. caviae*-like *Chlamydia* detected in swab specimens collected from the urethra and uterine cervix, using nested PCR with primers especially designed in our laboratory.

Materials and Methods

Patient specimens and cell cultures for isolation of C. caviae-like Chlamydia. The specimens were obtained from patients with symptoms consistent with STI including OK133, OK135 and OKM2 from 2000 to 2006. Several specimens were obtained from female patients who were asymptomatic, but anxious to undergo STI examinations. For female genital specimens, a cotton swab was inserted into the endocervical canal and was gently rotated. For male urethral specimens, a swab was inserted 3 to 4 cm into the urethra and rotated. Each swab was placed in a test tube containing 0.5 ml of sucrose-phosphate-glutamate (SPG) buffer and 0.5 g of glass beads (0.5-mm in diameter), and then stored at −70℃ until testing. After quickly thawing at 37℃, a Vortex mixer was used to vigorously stir each test tube to release the chlamydial organisms from the cotton swab with about 1 ml of SPG buffer (0.5 ml in the tube and another 0.5 ml to wash the beads). After centrifuging at 300 × g for 3 min at room temperature, the supernatant (0.25 ml/well) was placed on McCoy cell confluent monolayers in a 24-well culture plate (Corning Costar Corp., Corning, NY, USA), followed by centrifugation (860 × g, 25℃, 60 min) using a Hitachi himac CR21E centrifuge (Hitachi Koki Co. Ltd., Tokyo, Japan). The inoculated cells were then incubated at 37℃ in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) containing 1 μg/ml of cycloheximide, 10 μg/ml of kanamycin, 10 μg/ml of vancomycin, 10 μg/ml of amphotericin B and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Life Technologies Inc., Grand Island, NY, USA). Under a phase-contrast microscope, cell conditions were monitored at appropriate intervals, and once a cytopathic effect was seen, the cells were suspended in SPG buffer (1 ml/well) and stored at −70℃.

Fluorescent and iodine staining. McCoy

cell monolayers prepared on cover slips (14mm in diameter) were inoculated with chlamydial isolates by centrifugation and incubated at 37℃. At 24 to 30h post-inoculation, the cells were fixed with ethanol and stained with fluorescein-conjugated monoclonal antibody directed against the genus-specific antigen (*Chlamydia* FA Seiken [DFA stain]; Denka Seiken, Tokyo, Japan) to observe chlamydial inclusions. To check for glycogen accumulation in inclusions, the cover slips harvested at 30h post-inoculation were dried and fixed with methanol, and then subjected to iodine staining according to the method of Matsumoto *et al.* [12].

Plaque purification. Plaque purification was carried out according to the method of Matsumoto *et al.* [7]. Briefly, 100μl of chlamydial suspensions prepared in a series of 10-fold dilutions was directly added to each well containing 2ml culture medium. After sufficient stirring and centrifugation at 860 × g for 60min, the cells were overlaid with agarose medium consisting of 10% FBS-DMEM containing 1μg/ml of cycloheximide and 0.5% agarose (SeaKem ME agarose; FMC BioProducts, Rockland, ME, USA). After solidification, the liquid medium, which was prepared by omitting agarose from the agarose medium, was added and incubated at 37℃ in a 5% CO_2 incubator. The liquid medium was exchanged every 4 to 5 days. At an appropriate time after infection, the liquid medium was removed, and agarose medium containing 0.03% (final concentration) of neutral red was loaded on the agarose medium. After incubation at 37℃ for 12 to 15h, single plaques with sufficient separation from each other were recovered with agar-well punchers commonly used to make holes in the Ouchterlony immunodiffusion test. Each agarose plug placed in 1ml of SPG buffer was sonicated and centrifuged at 300 × g for 5min, and the supernatant was inoculated onto McCoy cells as described above. To purify each strain, this procedure was repeated three times.

In vitro drug susceptibility. The antimicrobial agents tested were clarithromycin (Taisho Pharmaceutical, Tokyo, Japan), minocycline (Wyeth Lederle, Tokyo, Japan) and tosufloxacin (Toyama Chemical, Tokyo, Japan). The agents were dissolved by the master dilution method. MICs of chlamydial strains obtained by plaque purification were determined by the standard method of the Japan Society of Chemotherapy [13]. Briefly, HeLa 229 cell confluent monolayers prepared on cover slips placed in 24-well culture plate were inoculated with chlamydial suspension at 1,000 IFU/well by centrifugation (860 × g, 60min). After centrifugation, 1ml of culture medium, consisting of Eagle's MEM, 10% heat-inactivated FBS, and cycloheximide, at a final concentration of 1μg/ml was applied. The medium also contained one concentration of the antimicrobial agents. Then, the plates were incubated at 37℃ in an atmosphere of 5% CO_2 for 48h. After ethanol fixation, the infected cells were stained with DFA and the inclusions were observed. The MIC was defined as the lowest concentration at which inclusion formation was completely inhibited.

Purification of EBs. EBs of purified strains and laboratory strains, such as *C. caviae* GPIC (VR-813, obtained from the American Type Culture Collection, Manassas, VA, USA), *C. trachomatis* biovars D and L2, and *C. psittaci* Cal 10 were prepared by the method reported previously [7]. Infected McCoy cells with well-developed inclusions were sonicated to facilitate the release of chlamydial organisms from the host cells. After brief centrifugation at 300 × g for 10min to remove large debris, the supernatant was subjected to 25% sucrose-cushioning centrifugation (8,000 × g, 4℃, 60min), and then the suspension was incubated with DNase (20μg/ml) and RNase (20μg/ml) in a water bath at 37℃ for 60min, followed by treatment with trypsin (10μg/ml) at 37℃ for 60min. Similarly, EBs of *C. pneumoniae* TW183 were prepared from HEp2 cells having well-grown inclusions. After sonication to disperse large aggregates, the suspension was subjected to 25% sucrose-cushioning centrifugation again. The sediment was suspended in SPG buffer and used for the extraction of genomic DNA.

MOMP gene sequencing. The Puregene DNA purification kit (Gentra System, Minneapolis, MN, USA) was used for genomic DNA extraction from purified EBs of each strain. PCR was performed under the conditions shown in Table 1 using genomic DNA as a template and the primers reported by Kaltenboeck *et al.* [14] to amplify the entire MOMP gene of strains, namely OK135 and SC10-6, obtained through plaque purification. The resulting amplification products were cleaned using MagExtractor (Toyobo, Osaka, Japan), and the BigDye terminator

cycle sequencing FS ready reaction kit (Applied Biosystems Japan, Tokyo, Japan) was used to make sequencing templates. The ABI 310 Genetic Analyzer (Applied Biosystems Japan) was used to determine the MOMP gene of each strain, and a BLAST search was used to investigate the homology of the sequence in the MOMP genes with that of the standard strain, *C. caviae* GPIC [15].

Molecular diagnosis of clinical specimens. Based on the nucleotide sequence data of the MOMP gene of the OK135 clone, primers (2 pairs) specific to the *ompA* gene of the same strain were designed (Table 1). The expected amplification product of nested PCR using these primer sets was a 311-bp DNA fragment. The genomic DNA of each strain was prepared from purified EBs with the QIAamp DNA stool mini kit (QIAGEN, Tokyo, Japan). Under the conditions shown in Table 1, 5μl of template and 45μl of reaction solution were added (10 $\times$ buffer, 0.2mM dNTPs, 0.625U *Taq* polymerase (TaKaRa, Shiga, Japan) and 0.2μM primer) for a total volume of 50μl to perform nested PCR using Astec PC 801 thermal cycler. The resulting PCR product was subjected to electrophoresis using 1.2% agarose gel in relation to a 100-bp DNA ladder (New England Biolabs Japan, Tokyo, Japan) as a molecular standard, and the 311-bp band was examined.

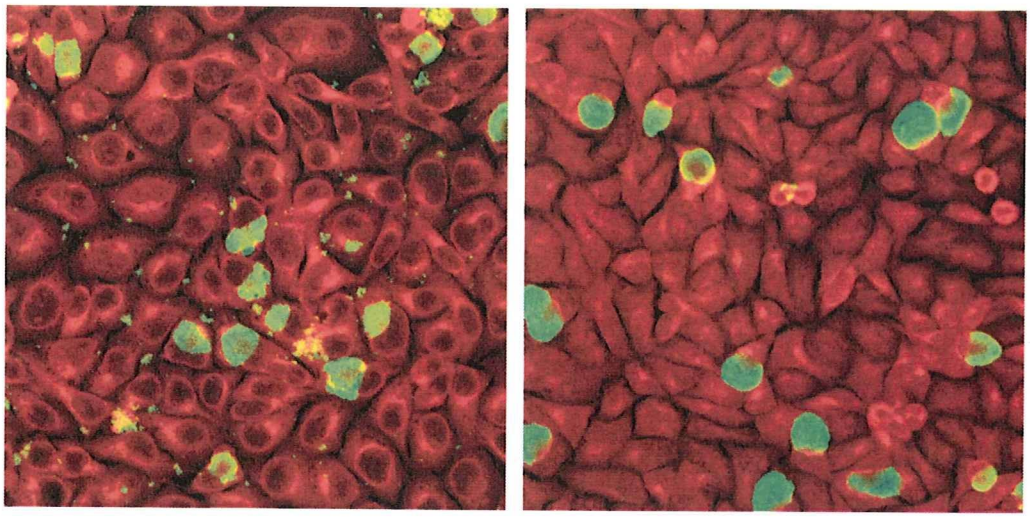
In addition to the chlamydial culture, all swab specimens, from 119 male and 83 female subjects, were examined with the commercially available PCR test kit (AMPLICOR *Chlamydia trachomatis*: Roche Diagnostics K.K., Tokyo, Japan) for *C. trachomatis* and the PCR test kit (AMPLICOR *Neisseria gonorrhoeae*; Roche Diagnostic K.K.) for *N. gonorrhoeae* or gonococcal cultures. When the microscopic examinations were positive but the PCR for *C. trachomatis* was undetermined, nested PCR, for which 2 pairs of primers specific to the *ompA* gene of OK135 were especially designed, was carried out (Table 1). For the male samples, *Mycoplasma genitarium*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* were also examined with the multiplex PCR test kit (Mitsubishi Chemical Medience Corp., Tokyo, Japan) to confirm a solo infection with the chlamydial strain identical with OK135. Such tests were, however, not subjected to the female samples because of their heavier contamination with other microorganisms.

Results

Morphology of chlamydial strains obtained by the plaque purification. In the preliminary DFA test, the inclusions formed from swab specimens of 3 patients, OKM2, OK133 and OK135 were noted their morphology. Therefore, these isolates were examined with staining methods in detail. It was suggested strongly that the swab specimen of the OKM2 patient contained, at least, 2 different *Chlamydia* species. To separate and purify each strain, the plaque formation was carried out successively, and 2 strains, SC10-6 and ST13-6-1, were obtained. The inclusions of ST13-6-1 were round or oval in shape (Fig. 1B) while those of SC10-6 were irregular (Fig. 1A). Simultaneously, many small particles showing specific stainability with DFA were scattered on the cell layers, suggesting that the particles were chlamydial bodies resulted from an inclusion burst. Consequently, it was likely that the strain SC10-6 grew rapidly. Fig. 2 shows the iodine-stained images of both SC10-6 and ST13-6-1 strains. The ST13-6-1 inclusions at 48h post-inoculation were intensely stained (Fig. 2B), indicating glycogen accumulation. The stainability, together with the round-shaped inclusion morphology in the DFA test, indicated that the ST13-6-1 strain was undoubtedly a member of the *C. trachomatis* species. By contrast, the SC10-6 inclusions were not stained even after 48h post-inoculation

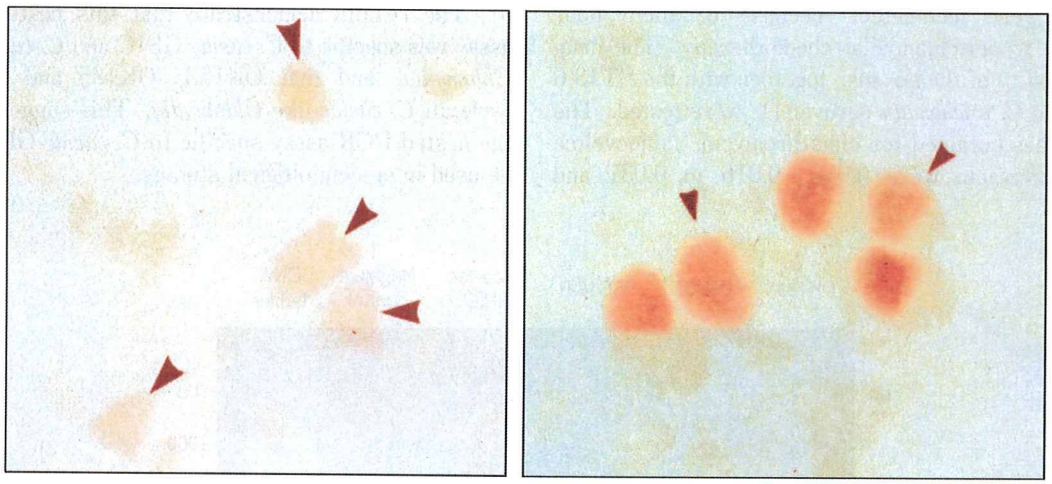
Table 1 PCR primers and conditions used in this study

Primer specificity		Primer sequences	Product length [bp]	PCR conditions				Reference
				Initial denaturation	Cycling	Cycle	Final extention	
ompA of *Chlamydophila*		F: 5'-ACGCATGCAAGACACTCCTCAAAGCC-3' R: 5'-ACGAATTCCTAGGTTCTGATAGCGGGAC-3'	1,400	3min, 94°C	10min, 96°C; 1min, 69°C; 1min, 72°C 11min, 96°C; 1min, 59°C; 1min, 72°C	10 25	10min, 72°C	14
ompA of OK135	1st PCR	F: 5'-CCTTGTGATCCTTGCGCTACTT-3' R: 5'-GTGAGCAGCTCTTTCGTTGAT-3	951	3min, 94°C	30sec, 94°C; 20sec, 55°C; 25sec, 72°C	30	5min, 72°C	this study
	2nd PCR	F: 5'-CCGTTGCAGACAGGAATAAC-3' R: 5'-GCACAACCACATTCCCATAAAG-3'	311		30sec, 94°C; 20sec, 55°C; 25sec, 72°C	30	5min, 72°C	



A : SC10-6 B : ST13-6-1

Fig. 1 Fluorescent images of SC10-6 (A) and ST13-6-1 (B), 2 clones from OKM2 (a male patient with urethritis). DFA staining with genus-specific fluorescein-conjugated monoclonal antibody was used. Inclusions of ST13-6-1 were of the typical round shape, indicating *C. trachomatis*, but those of SC10-6 had irregular borders, and numerous lysed cells were seen at 48h post-inoculation.



A : SC10-6 B : ST13-6-1

Fig. 2 Iodine staining for SC10-6 (A) and ST13-6-1 (B) infected cells. Inclusions of ST13-6-1 stained brown, indicating glycogen accumulation associated with *C. trachomatis*. However, inclusions of SC10-6 were not stained, and no glycogen accumulation was confirmed.

(Fig. 2A). This result, together with the inclusion morphology and the presence of scattered chlamydial bodies in the DFA test, indicated that the SC10-6 strain was different from *C. trachomatis*. Additionally, the ST13-6-1 strain reacted positively with the PCR test kit while SC10-6 did not.

Determination of the species of SC10-6 and OK135 strains. To determine the species of SC10-6 and OK135 strains, the *ompA* gene was amplified from purified EBs of each strain and its nucle-

otide sequence was analyzed. The results revealed that the nucleotide sequences of *ompA* gene of both strains, SC10-6 and OK135, were exactly the same, that their *ompA* genes were highly homologous to the nucleotide sequence (99.0%) of the *ompA* gene in *C. caviae* GPIC, and that their putative 389-amino-acid sequences were exactly the same as that of OmpA in *C. caviae* GPIC. Therefore, it was concluded that OKM2 and OK135 patients were infected with 2 different species of chlamydiae; one was a typical *C. trachomatis* strain and the other was an unusual chlamydial species in human urogenital infections, *C. caviae*-like *Chlamydia*. Although the analysis of the *ompA* gene was not done for OK133, its phenotypic characteristics were quite similar to those of other two *C. caviae*-like *Chlamydia* strains, and the result obtained in nested PCR confirmed that OK133 was also *C. caviae*-like *Chlamydia* (see below).

Drug susceptibility of C. caviae-like Chlamydia. Determination of drug susceptibility of the strains SC10-6 and OK135 was requested by physicians at the clinics where the patients OKM2 and OK135 were medicated, because of their poor response to antichlamydial chemotherapy. The drug susceptibility of the strains, together with the ST13-6 strain and *C. trachomatis* serover D, were tested. The MIC values obtained for clarithromycin, minocycline and tosufloxacin were 0.016, 0.016 to 0.031 and 0.25μg/ml, respectively; no difference in the MIC value was detected between the 2 strains. Additionally, there was no marked difference from the standard *C. trachomatis* serovar D. Based on the results, it was very likely that the patient's poor response to chemotherapy was not a result of the chlamydial properties, but might have been due to *in vivo* drug circulation into histopathological regions in the patients.

Epidemiological analysis. The fact that the patients were dually infected with either SC10-6 or OK135 and *C. trachomatis* strains impelled us to investigate whether *C. caviae*-like *Chlamydia* was a pathogenic agent of human STI, and if so, whether *C. caviae*-like *Chlamydia* is widespread. Genomic DNA was extracted from purified EBs of OK133, OK135, SC10-6, *C. caviae* GPIC, *C. trachomatis* serovar D and L2, *C. psittaci* Cal 10 and *C. pneumoniae* TW183. Using extracted DNA, the nested PCR assay, designed on the basis of the *ompA* sequence of the OK135 strain, was performed as shown in Table 1, and a 311-bp amplification product was observed for OK133, OK135, SC10-6 and *C. caviae* GPIC (Fig. 3). The results demonstrate that this nested PCR assay was specific to *C. caviae* GPIC and *C. caviae*-like *Chlamydia*, and that OK133, OK135 and SC10-6 were all *C. caviae*-like *Chlamydia*. This suggests that the nested PCR assay specific to *C. caviae* GPIC can be used in epidemiological studies.

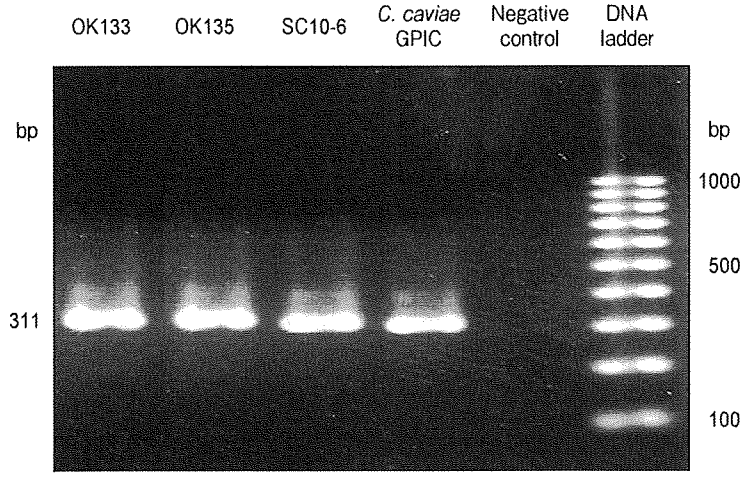


Fig. 3 Electrophoresis of amplification products obtained by nested PCR assay which was performed using two pairs of primers specific to the *ompA* gene of *C. caviae*-like *Chlamydia* OK135 (Table 1). Electrophoresis was conducted using 1.2% agarose gel and a 100-bp DNA ladder as a molecular standard. The expected amplification product was a 311-bp DNA fragment.

Based on the results, the nested PCR assay was performed on swab specimens obtained from a total of 202 patients, including 119 male patients with urethritis and 83 female patients with either cervicitis or suspected STI from July, 2003 to December, 2006. In each patient, the presence or absence of *N. gonorrhoeae* (117 male and 83 female patients tested), *C. trachomatis* (114 male and 83 female patients tested) and *C. caviae*-like *Chlamydia* (all 202 patients tested) was determined (Fig. 4). Of the 119 male patients with urethritis, 34 had *N. gonorrhoeae*, 33 had *C. trachomatis*, and 8 had both *N. gonorrhoeae* and *C. trachomatis*. With regard to the 83 female patients, 45 asymptomatic patients underwent tests for sexually transmitted diseases after becoming pregnant or changing partners, and the other 38 patients had cervicitis-related symptoms, such as increased discharge, abnormal color and vaginal erosion. Of the 83 female patients, 2 had *N. gonorrhoeae*, 15 had *C. trachomatis*, and 1 patient had both *N. gonorrhoeae* and *C. trachomatis*. To determine the infection of *C. caviae*-like *Chlamydia*, the nested PCR assay was carried out as mentioned above; the results are summarized in Fig. 4. Among 14 male patients, including OKM2, who were positive for *C. caviae*-like *Chlamydia*, 7 had *C. trachomatis*, 4 had *N. gonorrhoeae*, and 1 had both *N. gonorrhoeae* and *C. trachomatis*. On the other hand, among the 4 female patients positive for *C. caviae*-like *Chlamydia*, 2 had *C. trachomatis*. In other words, *C. caviae*-like *Chlamydia*, with neither *C. trachomatis* nor *N. gonorrhoeae*, was detected in a total of 6 patients (4 male and 2 female patients). Table 2 summarizes the clinical backgrounds of the 20 patients who were positive for *C. caviae*-like *Chlamydia*, including OK133 and OK135. Eleven of the 14 male patients had urethritis-related symptoms, such as pain on urination and pus discharge, and 2 of the 6 female patients had cervicitis-related symptoms. In most patients, symptoms improved after chemotherapy. However, in case OK135, symptoms did not improve and intractable cervicitis developed, and in cases 9, 13 and 14, symptoms such as pain on urination continued even after chemotherapy. *M. genitalium* was detected in the male patients (cases 9, 10, 13 and 14) who were positive for *C. caviae*-like *Chlamydia* and negative for *C. trachomatis* and *N. gonorrhoeae*. In the female cases 11 and 12, the presence or absence of *Mycoplasma* and *Ureaplasma* spp. was not tested.

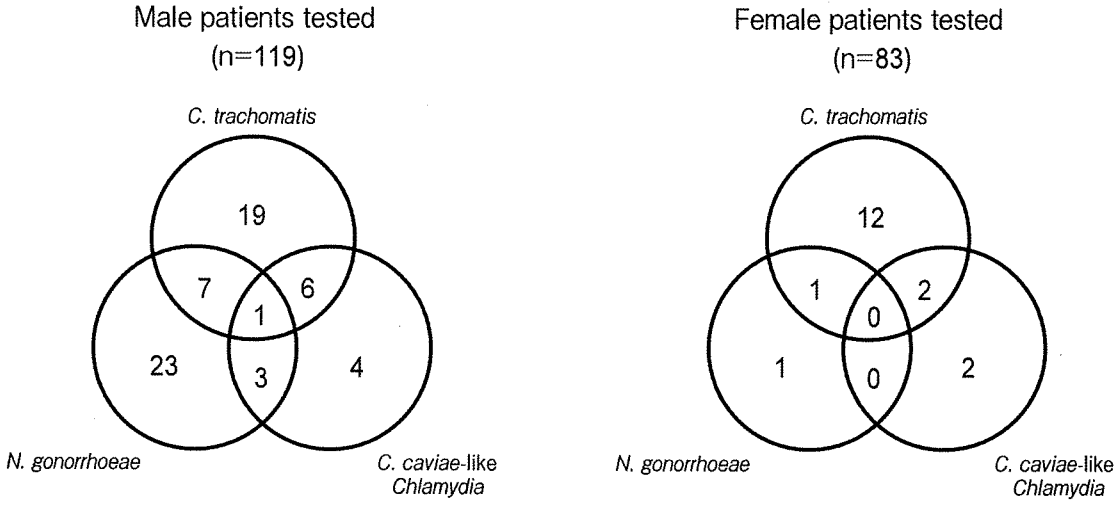


Fig. 4 *C. trachomatis*, *N. gonorrhoeae* and *C. caviae*-like *Chlamydia* infections are summarized for the 119 male and 83 female patients tested. In each patient, the presence or absence of *N. gonorrhoeae* (117 male and 83 female patients tested), *C. trachomatis* (114 male and 83 female patients tested) and *C. caviae*-like *Chlamydia* (all 202 patients tested) was determined. *C. caviae*-like *Chlamydia* was detected in 14 male and 4 female patients, including OK133 and OK135, which were isolated in 2000; a total of 20 *C. caviae*-like *Chlamydia* strains were isolated from 2000 to 2006.

Table 2 Clinical analysis of STI patients with *C. caviae*-like *Chlamydia*

No. of cases	Patient identification number	Date of the first medical examination	Sex	Age	Symptoms	*C. trachomatis*	*N. gonorrhoeae*	PCR for OK135 clone	Strain identification number of *C. caviae*-like *Chlamydia*
	OK133	2000	female	20	nothing special	+	−	+	OK133
	OK135	2000	female	27	leukorrhea	+	−	+	OK135
1	OKM2	2003. 07. 01	male	34	pus discharge	+	−	+	SC10-6
2	OKM10	2004. 01. 08	male	20	pain on urination	+	−	+	no cloning
3	OKM11	2004. 01. 26	male	29	inguinal pain	+	−	+	no cloning
4	OKM13	2004. 02. 17	male	29	nothing special	+	−	+	no cloning
5	OKM52	2005. 06. 18	female	33	leukorrhea	+	−	+	no cloning
6	OKM59	2005. 08. 08	male	44	residual urine	+	−	+	no cloning
7	OKM75	2005. 10. 14	male	21	pain on urination	−	+	+	no cloning
8	OKM88	2005. 11. 30	female	20	nothing special	+	−	+	no cloning
9	OKM96	2006. 02. 13	male	36	pain on urination, pus discharge	−	−	+	no cloning
10	OKM98	2005. 12. 16	male	44	NR	−	−	+	no cloning
11	OKM104	2006. 02. 15	female	NR	check for STI	−	−	+	no cloning
12	OKM109	2006. 01. 05	female	NR	check for STI	−	−	+	no cloning
13	OKM112	2006. 03. 02	male	21	pain on urination	−	−	+	no cloning
14	OKM116	2006. 03. 13	male	NR	pain on urination, pyuria	−	−	+	no cloning
15	OKM136	2006. 06. 24	male	NR	pain on urination, pus discharge	+	−	+	no cloning
16	OKM147	2006. 08. 18	male	30	pain on urination, pus discharge	+	+	+	no cloning
17	OKM185	2006. 10. 31	male	43	pus discharge	−	+	+	no cloning
18	OKM202	2006. 12. 09	male	24	pus discharge	−	+	+	no cloning

NR: no records

Discussion

The results obtained in the present study revealed the presence of novel chlamydial strains that were undetectable with the PCR or LCR commonly used for diagnosis of *C. trachomatis* infection and that closely resembled *C. caviae*. *C. caviae* GPIC is well known as an etiologic agent in guinea pigs, but to the best of our knowledge, there have been no reports of isolated *C. caviae* or similar chlamydial strain from humans. How then was *C. caviae*-like *Chlamydia* isolated from the male urethra and cervical canal? No conclusive evidence on this point has been obtained. At the moment, it is speculated that *C. caviae*-like *Chlamydia* has been transmitted and colonized in the human pharynx or sexual organs through close contact with guinea pigs, and that sexual acts, including oral sex, can spread the organism from human to human. Such a speculation seems to be supported by the facts that other *Chlamydophila*, such as *C. abortus* and *C. felis*, regarded for a long time as animal- restricted pathogens, were revealed to be the causes of chlamydial abortion [16, 17] and conjunctivitis [18–20] in humans who might have been infected through close contact with carrier and/or infected animals. During transmission and colonization of *C. caviae*-like *Chlamydia*, genetic variations appeared to occur, because the homology of the *ompA* gene of this strain to the GPIC strain was very high but not identical. This fact leads us to speculate that several genes, not only the gene encoding MOMP but also other genes encoding functional proteins, have evolved to be stable during their transmission and multiplication in humans. Thus, it will be necessary to sequence the whole genome of *C. caviae*-like *Chlamydia* in the future.

Using the nested PCR assay specific to *C. caviae*-like *Chlamydia*, the organism was detected in a total of 18 patients. Of these patients, *C. trachomatis* and *N. gonorrhoeae* were detected in 9 and 4 patients, respectively, with one patient having both. Thus, *C. caviae*-like *Chlamydia*, with neither *C. trachomatis* nor *N. gonorrhoeae*, was detected in a total of 6 patients (4 male and 2 female patients). However, additional diagnostic tests detected *M. genitalium* in the 4 male patients. The samples of the 2 female patients were not tested because of possible genital contamination by microorganisms. Therefore, we cannot state conclusively that *C. caviae*-like *Chlamydia* is pathogenic for humans; the organism appeared to be rather common (positive percentage was 8.9%).

In general, chlamydial isolations should be done prior to antibiotic medication; fortunately, we man-

aged to isolate strains OK135 and SC10-6 through the McCoy cell after anti-chlamydial medication. In spite of the poor response *in vivo* and the rapid growth *in vitro*, the purified strains of *C. caviae*-like *Chlamydia* were susceptible to drugs such as clarithromycin, minocycline and tosufloxacin at similar levels as other chlamydiae. It is therefore concluded that the failure of chemotherapy was due to reduced drug circulation into histopathological regions in the patients. This finding should be a warning that the drug susceptibility of chlamydiae should be determined using organisms isolated from unresponsive patients; otherwise, the drug efficacy can be misunderstood.

Recently, we have started to collect pairs of samples: from the throat and urethra for men and from the throat and uterine cervix for women. These studies are expected to clarify the prevalence of infection with *C. caviae*-like *Chlamydia* as an STI, as well as its pathogenicity. Because urethritis or cervicitis caused solely by *C. caviae*-like *Chlamydia* has not been reported, further investigations are needed to determine its pathogenicity in humans. It will also be necessary to investigate the possibility of *C. caviae*-like *Chlamydia* causing nongonococcal/nonchlamydial urethritis or cervicitis, intrapelvic peritonitis, or infertility.

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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-

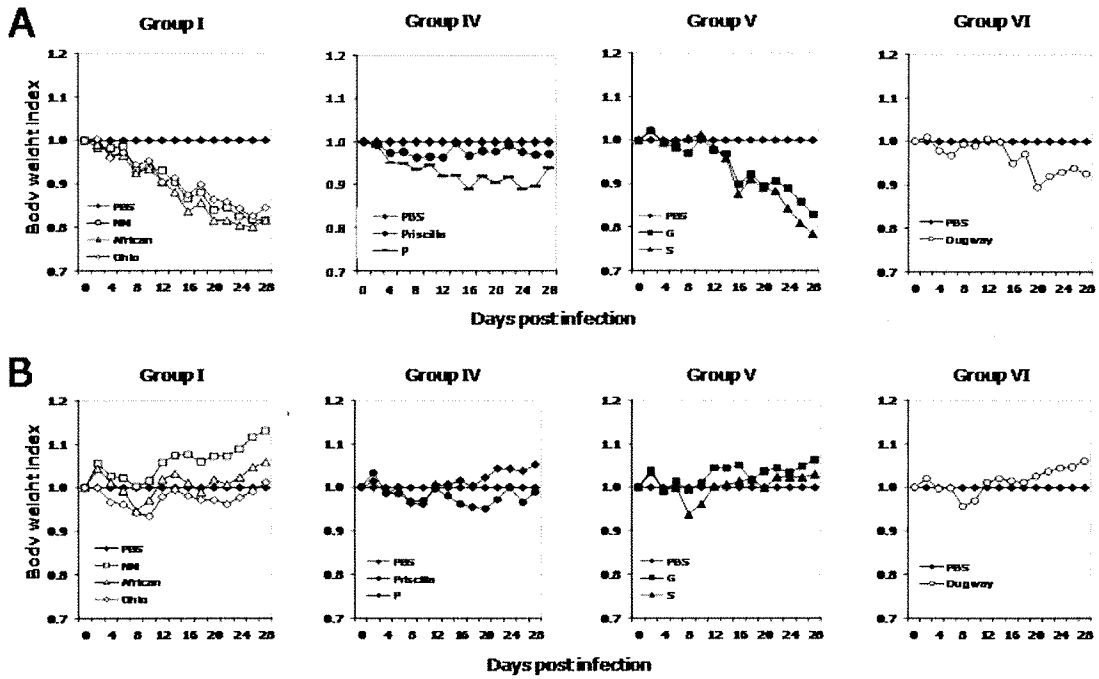

FIG. 1. Average body weight changes in SCID mice (A) and CB-17 mice (B) infected with *C. burnetii* isolates during 28 days of infection. Body weights were significantly lower in SCID mice throughout the infection period and transiently in CB-17 mice infected with all isolates except Priscilla compared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

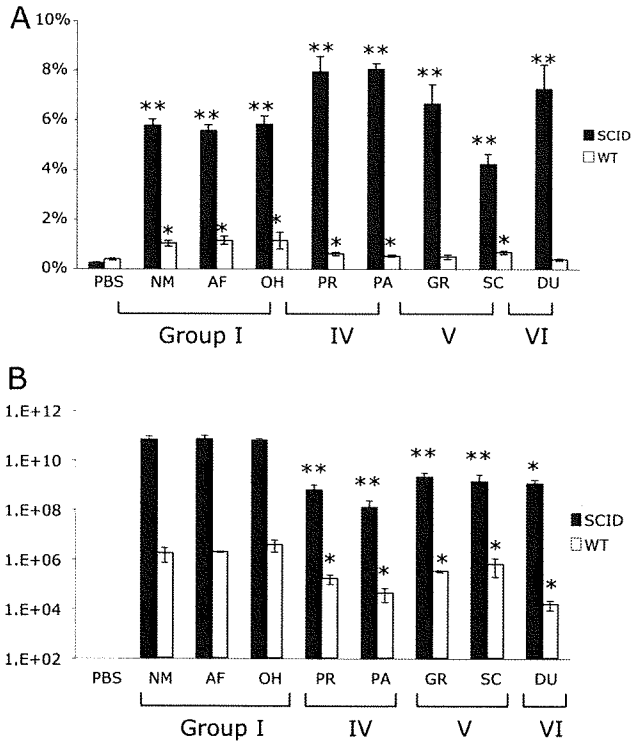

FIG. 2. Splenomegaly (A) and splenic bacterial loads (B) in mice at 28 days p.i. (A) All infected animals developed significant splenomegaly compared to controls, and infected SCID mice had significantly larger spleens than CB-17 mice ($P < 0.05$). (B) Mice infected with group IV, V, and VI isolates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

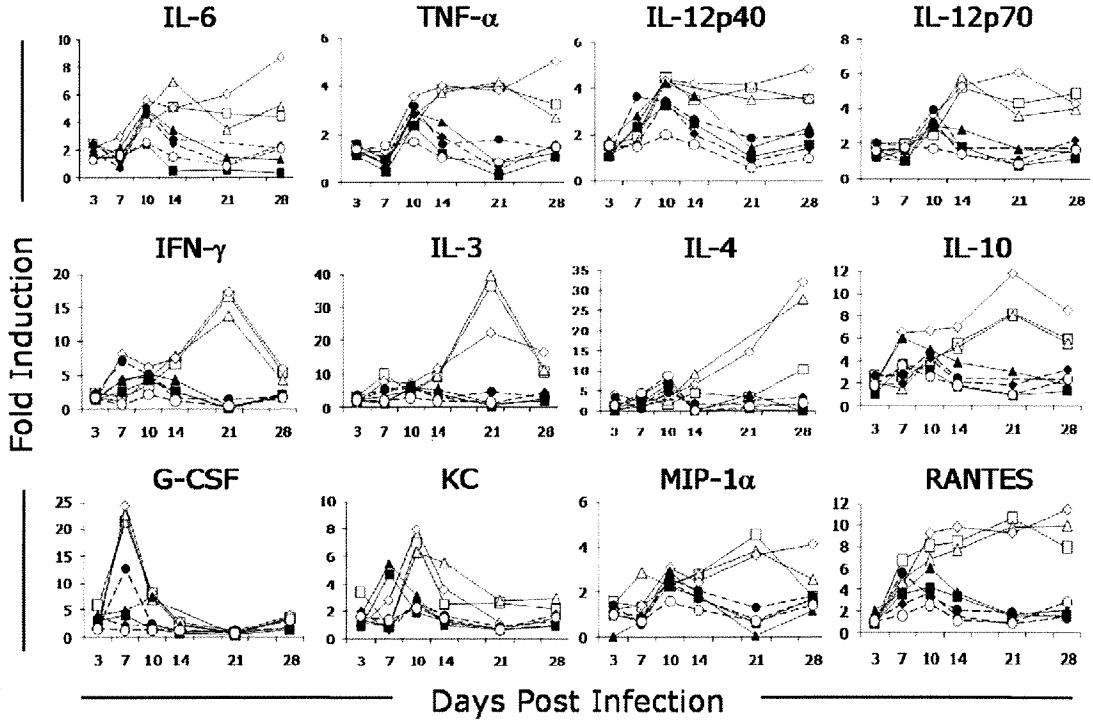

FIG. 3. Mean circulating cytokine levels in response to infection in CB-17 mice with different *C. burnetii* isolates. Isolates from genomic group I induced persistently high cytokine secretion with increased levels of IL-6, TNF-α, IL-12p40, IL-12p70, IFN-γ, IL-3, IL-4, IL-10, MIP-1α, and RANTES compared 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