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A novel genotype of beak and feather disease virus in budgerigars (*Melopsittacus undulatus*)

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Abstract Beak and feather disease virus (BFDV) is a causative agent for psittacine beak and feather disease (PBFD), which shows a characteristic feather disorder in psittacine birds. Nineteen budgerigars, which were clinically suspected to have PBFD, were examined by two polymerase chain reactions (PCR), which target each of open reading frames (ORFs) V1 and C1. All of the 19 samples were detected BFDV by the PCR targeting ORF C1, whereas only two of them were detected by the PCR targeting ORF V1. It was assumed that BFDV derived from budgerigar (budgerigar BFDV) has two genotypes, which are tentatively classified as budgerigar BFDV genotype 1 and genotype 2 by the PCR amplification patterns. Whole genome sequences of six budgerigar BFDVs were determined to reveal the existence of two genotypes. In the phylogenic analysis, six budgerigar BFDV sequences formed a unique group branched from the other 23 published BFDV sequences. The budgerigar BFDV genotype 1 and genotype 2 were also segregated each other, and budgerigar BFDV genotype 2 was particularly distantly related with the other BFDVs. These results suggest budgerigar BFDV is a unique in the known BFDVs and is divided into two genotypes.

Keywords Psittacine beak and feather disease · PBFD · Beak and feather disease virus · Circovirus · Budgerigar

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Psittacine beak and feather disease (PBFD) is a specific disease in psittacine birds caused by beak and feather disease virus (BFDV), a member of genus *Circovirus* [1]. Clinical form of PBFD included chronic, progressive loss of feathers and, in some species, deformities of the beak and claws [2]. Acute-form, which shows signs of septicemia accompanied by pneumonia, enteritis, rapid weight loss, and death, has also been described [2]. BFDV carries a single-stranded circular DNA with a complete genome size of approximately 2.0 kb. The genome contains two major open reading frames (ORFs), encoding the replication-associated protein (V1), and the capsid protein (C1). Another ORF (ORF V2) has been also described, but it is unclear what role the transcriptional product of this ORF may play in the cycle of the virus [1].

PBFD has since been reported to affect more than 60 psittacine species; it is highly probable that all psittacine

bird species are susceptible [3]. So far, phylogenetic analysis of BFDV has revealed an apparent genotypic association with specific psittacine species [4, 5]. The level of genetic diversity has been reported to be similar among several countries such as Australia, New Zealand, and South Africa [5–7].

In the present study, 19 BFDV isolates derived from budgerigars (*Melopsittacus undulatus*) showing PBFD were detected by polymerase chain reactions (PCR), and of which six isolates were determined whole genome sequence. It was shown that 19 BFDV isolates in this study separated into two lineages, in addition, one was unique lineage to be reported previously.

Twenty samples including 13 feathers, 4 livers, and 3 bloods from 19 budgerigars in Japan suspected to be PBFD were examined in this study (Table 1). DNA was extracted from the blood or feathers with a SepaGene nucleic acid extraction kit (Sanko Junyaku Co., Japan) as described previously [8] and stored at −30°C until use. PCR was carried out using two sets of primer, Primer2/Primer4 (Ypelaar's PCR) and PBFDdupF/PBFDdupR (Ogawa's PCR) targeting BFDV genomic DNA previously described [8, 9]. As result of PCR, BFDV genomic DNA was detected in all 19 samples using primer set of PBFDdupF and PBFDdupR, whereas BFDV genomic DNA was amplified in only two samples using Primer2 and Primer4 (Table 1). These isolates were tentatively classified as budgerigar BFDV genotype 1, which was detectable by Ypelaar's PCR, and genotype 2, which was not detectable.

Next, we performed whole genome sequence analysis of these BFDV isolates, 4 and 2, respectively, were from acute-form and chronic-form, to determine their genetic characteristics. Whole genome sequences of six budgerigar BFDVs, of which 2 (MU-JP1P and MU-JP2P) and 4 (MU-JP3P to MU-JP6P) were genotype 1 and 2, were determined. Four fragments covering the whole genome were amplified by PCR using four primers sets for each genotype (Supplementary Table S1). PCR products were cloned and sequenced as described previously [8]. The obtained sequences were edited using Genetyx-Mac version 13 (Genetyx Co., Japan) and Genetyx-Mac/ATSQ version 4.2.4 (Genetyx Co.). The sequences of six

Table 1 Birds used in this study and genotypes of the budgerigar BFDV

Budgerigar	Age[a]	Clinical signs[b]	Clinical forms	Specimen	PCR results		Genotype	BFDVcode	Accession no.[c]
					Ogawa [8]	Ypelaar [9]			
Complete genome sequences determined in the present study									
Budgerigar1	42 d	Acute death	Acute	Liver	+	+	1	MU-JP1P	AB277746
Budgerigar2	2 y 6 m	Feather disorder	Chronic	Feather	+	+	1	MU-JP2P	AB277747
Budgerigar3	3 m	Acute death	Acute	Liver	+	−	2	MU-JP3P	AB277748
Budgerigar4	1 m	Acute death	Acute	Liver	+	−	2	MU-JP4P	AB277749
Budgerigar5	40 d	Acute death	Acute	Liver	+	−	2	MU-JP5P	AB277750
Budgerigar6	3 y	Feather disorder	Chronic	Feather	+	−	2	MU-JP6P	AB277751
Tested by PCR only and not sequenced in the present study									
Budgerigar7	45 d	Feather disorder	Chronic	Feather	+	−	2	MU-JP7P	
Budgerigar8	6 m	Feather disorder	Chronic	Feather	+	−	2	MU-JP8P	
Budgerigar9	4 m	Feather disorder	Chronic	Blood	+	−	2	MU-JP9P	
Budgerigar10	3 y	Feather disorder	Chronic	Feather	+	−	2	MU-JP10P	
Budgerigar11	1 y	Feather disorder	Chronic	Feather	+	−	2	MU-JP11P	
Budgerigar12	2 y	Feather disorder	Chronic	Blood	+	−	2	MU-JP12P	
Budgerigar13	2 m	Feather disorder	Chronic	B&F[d]	+	−	2	MU-JP13P	
Budgerigar14	1 y	Feather disorder	Chronic	Feather	+	−	2	MU-JP14P	
Budgerigar15	9 m	Feather disorder	Chronic	Feather	+	−	2	MU-JP15P	
Budgerigar16	2 y	Feather disorder	Chronic	Feather	+	−	2	MU-JP16P	
Budgerigar17	2 y 6 m	Feather disorder	Chronic	Feather	+	−	2	MU-JP17P	
Budgerigar18	5 y	Feather disorder	Chronic	Feather	+	−	2	MU-JP18P	
Budgerigar19	1 y 5 m	Feather disorder	Chronic	Feather	+	−	2	MU-JP19P	

[a] d, m, and y indicate days, month(s), and year(s), respectively

[b] Budgerigars affected with feather disorder are all alive

[c] Sequence of BFDV isolates derived from budgerigar7 to 19 is not determined

[d] B&F indicates blood and feather

budgerigar BFDV isolates have been submitted to GenBank and have been given accession numbers AB277746–AB277751. The genome sizes ranged from 1996 to 2004 nt (MU-JP1P, 1996 nt; MU-JP2P, 1996 nt; MU-JP3P, 2001 nt; MU-JP4P, 2004 nt; MU-JP5P, 2004 nt, and MU-JP6P, 2002 nt) (Supplementary Table S2). The identities of whole genome sequences of budgerigar BFDV genotype 1 and genotype 2 compared with the 23 published BFDV isolates shown in supplementary Table S3 from 87 to 92% and from 83 to 92%, respectively. Budgerigar BFDV genotype 1 whole genome sequences were from 85 to 88% identical to genotype 2 sequences. The identities of deduced amino acid sequences of budgerigar BFDV genotype 1 and genotype 2 ORF V1 compared to 23 published BFDVs varied from 90 to 99% and from 87 to 93%, respectively. Those of genotype 1 and genotype 2 ORF C1 varied from 75 to 91% and from 74 to 85%, respectively. The deduced amino acid sequences of ORF V1 and C1 of budgerigar BFDV genotype 1 were from 91 to 94% and from 84 to 88% identical to those of genotype 2, respectively.

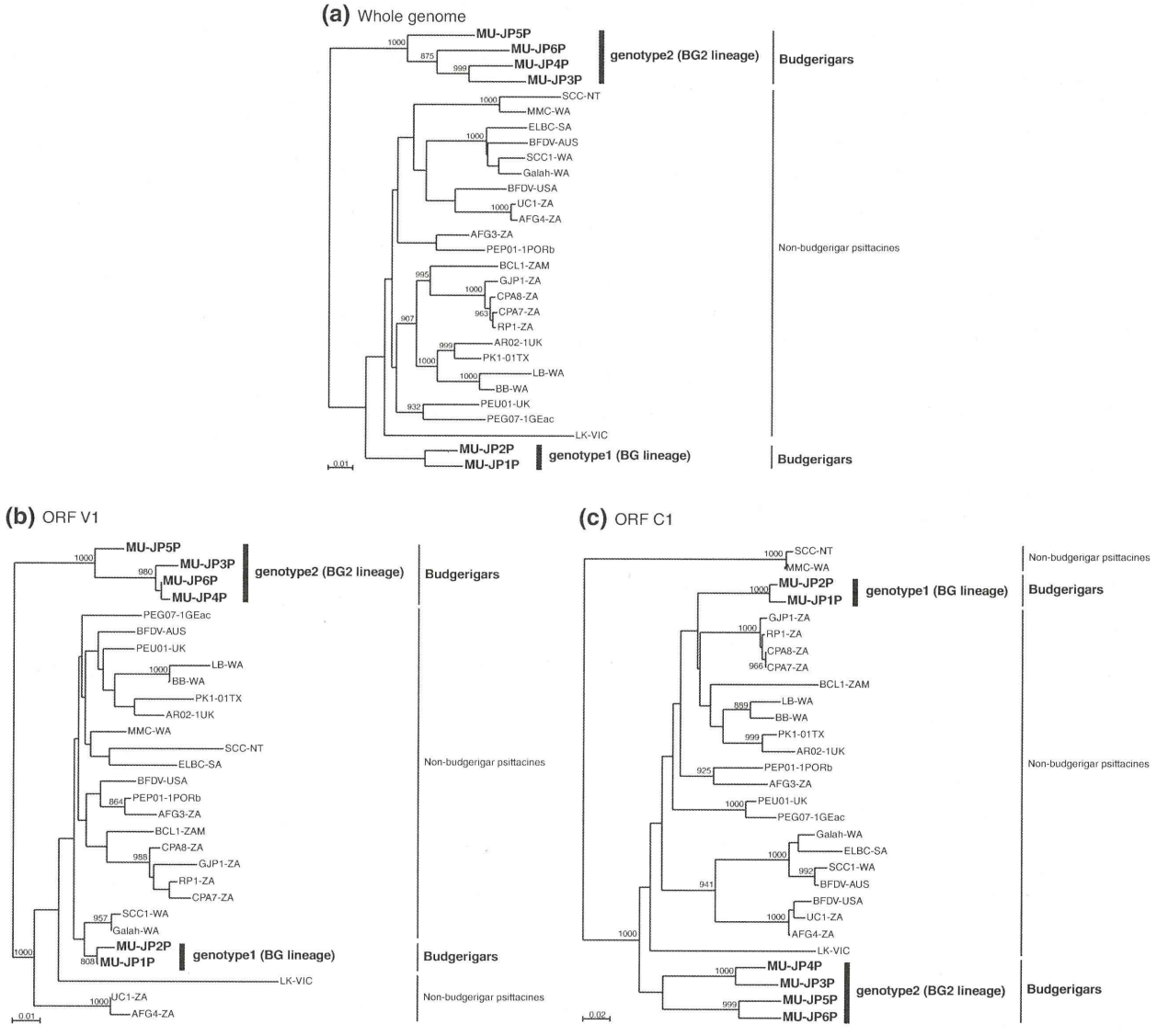

Fig. 1 Neighbor-joining tree of **a** complete genome sequence, amino acid sequences of **b** ORF V1 and **c** C1 of six budgerigar BFDVs and 23 BFDVs [1, 4–6]. The bootstrap values of each node were calculated using 1,000 replications. *Bold fonts* indicate budgerigar BFDV from the present study. Genbank accession numbers we used are AB277746–AB277751 in six budgerigar BFDV isolates, and AF311295–AF311302, AF080560, AF071878, AY521234–AY521238, AY450434, AY450436–AY450439 and AY450441–AY450443 in 23 non-budgerigar BFDV isolates, respectively. **a** Six budgerigar BFDV genome sequences formed different branches from 23 BFDVs, and budgerigar BFDV genotype 1 and genotype 2 were also in independent clusters. **b** and **c** Budgerigar BFDV genotype 1 was closely related to 23 BFDVs in the both trees, whereas genotype 2 was distantly related to them

The deduced amino acid sequences of partial ORF V1 of budgerigar BFDV genotype 1 showed 96.7–99.4% homologies to the sequences of the published sequences derived from budgerigars (BG3-NZ, AY148301; UFS 3 to UFS 5; DQ384623 to DQ384625), whereas genotype 2 showed 91.8–94.6%. A phylogenic tree based on partial ORF VP1 using the UPGMA method revealed that genotype 1 isolates clustered together with BG3-NZ and UFS 3 to UFS 5, which classified into the BG lineage [7, 10], whereas genotype 2 isolates clustered a unique group (data not shown). These results suggested that budgerigar BFDV genotype 2 might be phylogenically distinct from previously described BFDV.

There were two clinical forms of the affected birds with budgerigar BFDV genotype 1, one was acute-form and another was chronic-form. Of the 17 budgerigar BFDV genotype 2 isolates, 3 forms were acute and the other 14 were chronic. No relationship between clinical forms and both genotypes was observed in the present study.

To confirm the further taxonomic position of these six budgerigar BFDV isolates, phylogenic trees were constructed based on the whole genome sequence analysis. The budgerigar BFDV genotype 1 and genotype 2 were located in independently different clusters (Fig. 1a). The genotype 2 isolates were particularly distinct from other BFDV isolates and clustered a unique lineage. This relationship was supported by analysis of the both ORF V1 and C1, respectively (Fig. 1b, c). The phylogenic analysis of whole genome sequence and both ORF V1 and C1 shows identical placement of the previously described BFDVs and the budgerigar BFDV genotype 2. Hereby, it is proposed that budgerigar BFDV genotype 2 belongs to a novel lineage (BG2). During review of this article, three budgerigar BFDV sequences were reported as a unique isolate in South Africa [11]. However, these isolates were also classified into the BG lineage, which was related to genotype 1, and not clustered a novel unique BG2 lineage.

Ypelaar's PCR targeting ORF V1 has been used as a universal method by other researchers [3, 6, 7]. However, it was reported that Ypelaar's PCR might not detect all isolates because of the diversity of BFDV genotypes [3]. In fact, our PCR results showed the existence of budgerigar BFDV, which could not be detected by Ypelaar's PCR. To investigate why the budgerigar BFDV genotype 2 was not detected by Ypelaar's PCR, the sequences targeted by Primer2 and Primer4 were compared on the genomic sequences of both genotype 1 and 2. The sequences of genotype 1 were identical to both primer sequences and those of genotype 2 were identical to Primer4, whereas 6–7 of the 18 nucleotides were different in the sequences of genotype 2, which correspond to the central region of Primer2 (Fig. 2). In the case of Ogawa's PCR, several nucleotide substitutions were scattered in budgerigar BFDV genotype 1 and genotype 2 sequences. However, consecutive nucleotide substitutions such as the genotype 2 in Primer2 were not found. The phylogenic classification of the six budgerigar BFDV genomes was the same as the tentative classification found in both PCR amplification

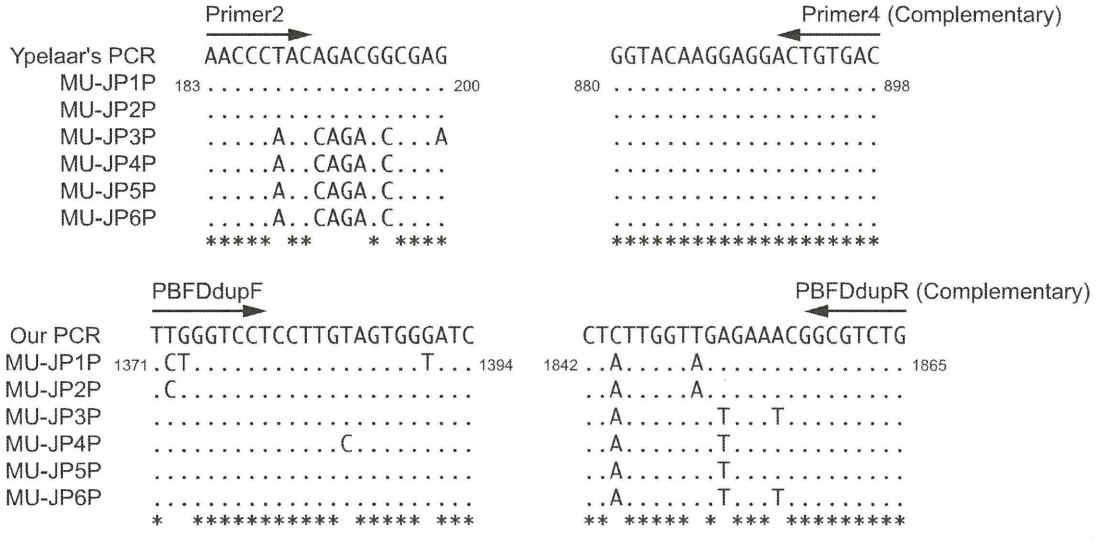

Fig. 2 Sequence comparison of the primer target sites of Ypelaar's PCR [9] and Ogawa's PCR [8] in budgerigar BFDV genotype 1 and genotype 2 sequences. *Dots* indicate positions in agreement with the primer sequences. The numbers to the left and right of the row following MU-JP1P indicate nucleotide positions of the MU-JP1P sequence. *Asterisks* indicate that corresponding positions of alignment are identical. 6–7 of 18 nucleotides were different in budgerigar BFDV genotype 2 sequences which correspond to the above Primer2 sequences. In PBFDdupF and PBFDdupR, several nucleotide substitutions were scattered in budgerigar BFDV genotype 1 and genotype 2 sequences

results. It is conceivable that the combination of the results of both PCRs would be useful to identify the budgerigar BFDV genotypes. Therefore, it is possible that additional uncovered BFDV exist in other psittacine species.

In conclusion, this study revealed the presence of a novel lineage of BFDV in budgerigars, with further investigation involving the identification of any antigenic differences present.

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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 × 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.

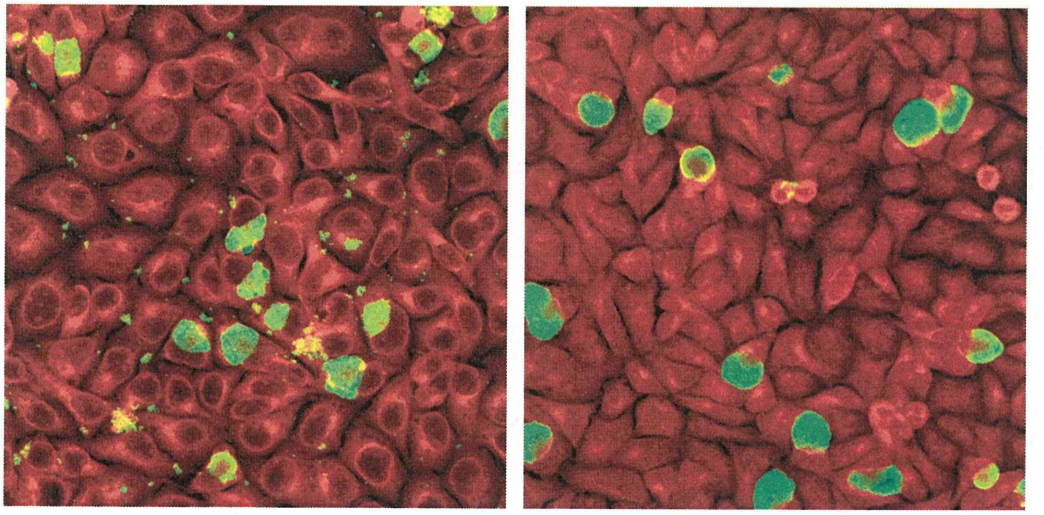
In addition to the chlamydial culture, all swab specimens, from 119 male and 83 female subjects, were examined with the commercially available PCR test kit (AMPLICOR *Chlamydia trachomatis*: Roche Diagnostics K.K., Tokyo, Japan) for *C. trachomatis* and the PCR test kit (AMPLICOR *Neisseria gonorrhoeae*; 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

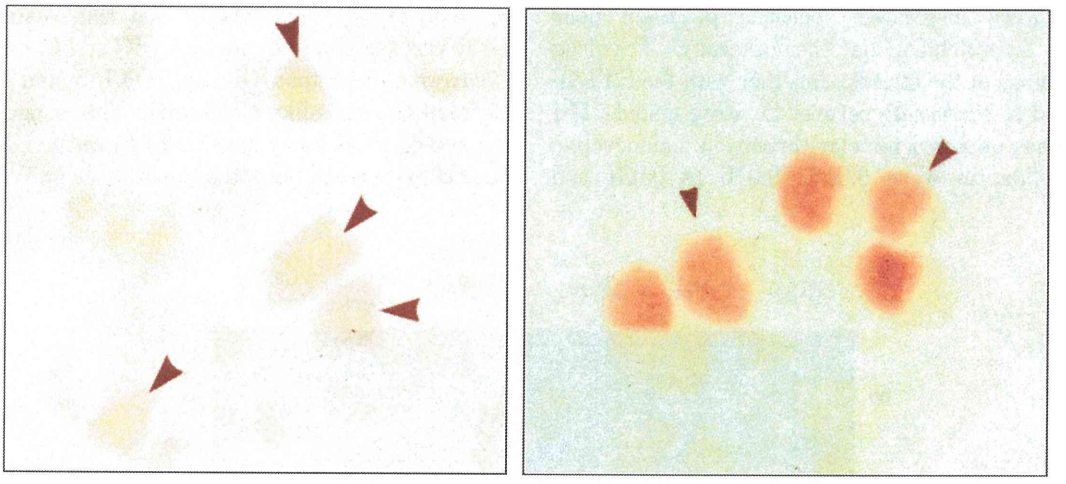
Table 1 PCR primers and conditions used in this study

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



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 0.25 μg/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.

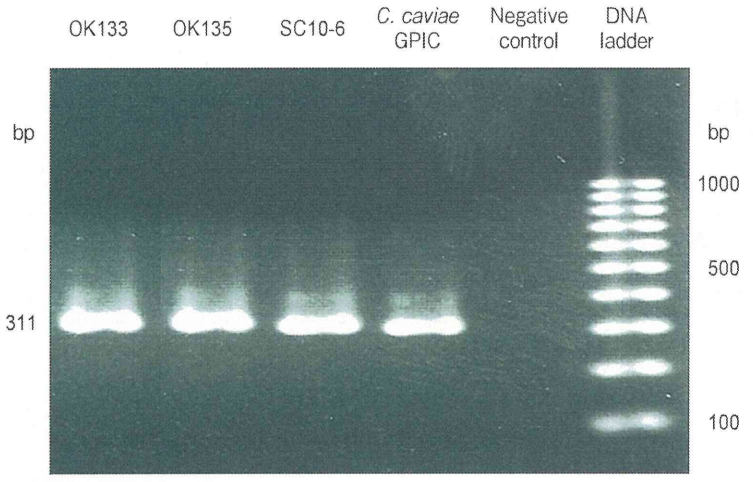


Fig. 3 Electrophoresis of amplification products obtained by nested PCR assay which was performed using two pairs of primers specific to the *ompA* gene of *C. caviae*-like *Chlamydia* OK135 (Table 1). Electrophoresis was conducted using 1.2% agarose gel and a 100-bp DNA ladder as a molecular standard.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

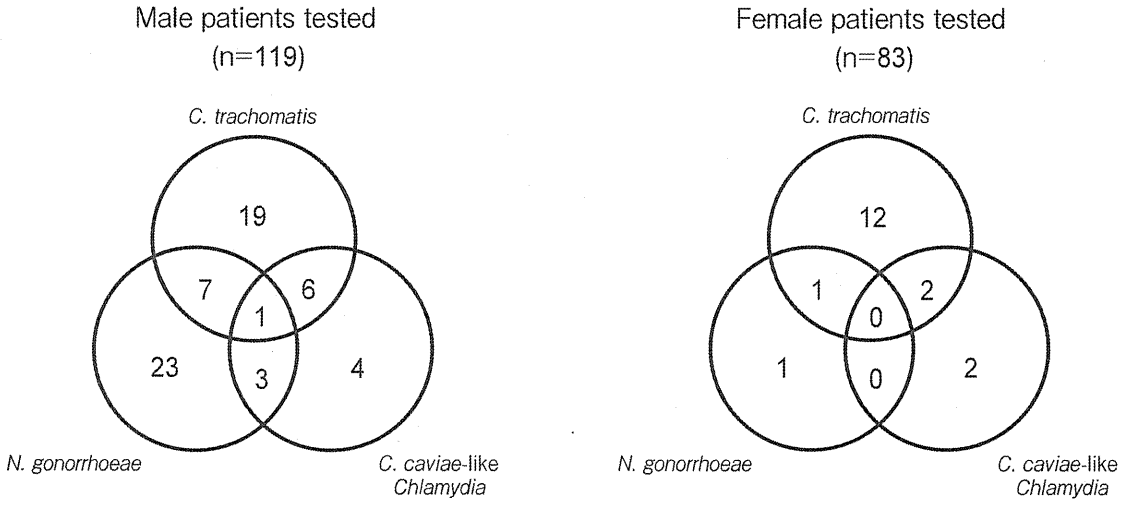


Fig. 4 *C. trachomatis*, *N. gonorrhoeae* and *C. caviae*-like *Chlamydia* infections are summarized for the 119 male and 83 female patients tested. In each patient, the presence or absence of *N. gonorrhoeae* (117 male and 83 female patients tested), *C. trachomatis* (114 male and 83 female patients 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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Part 03

INFECTION

ヒトと鳥が安心して共存していくために

鳥類のクラミジア感染症

ヒトのオウム病として知られる鳥類のクラミジア感染症。
その伝播経路から診断・治療までを解説する。

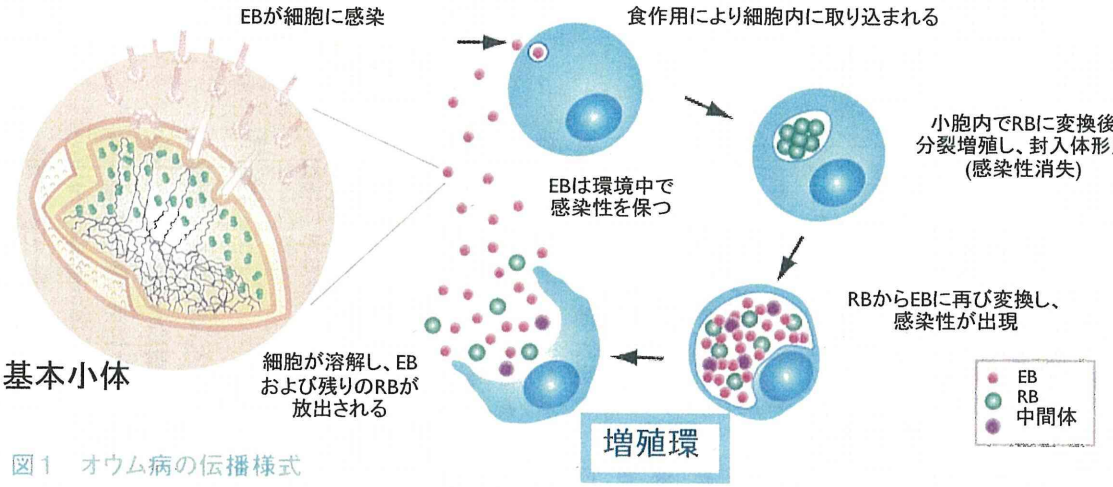

図1　オウム病の伝播様式

鳥類のクラミジア症はオウム病クラミジア *Chlamydophila* (*Chlamydia*) *psittaci* を病原体とする感染症である。ヒトの *C. psittaci* 感染はオウム病として知られている。オウム病は感染症法において全数届出の第四類疾患である。

病原体

本症の原因菌はオウム病クラミジア *Chlamydophila* (*Chlamydia*) *psittaci* である。クラミジアは真核細胞内でのみ増殖可能な細菌の一種である。他の細菌と異なり細胞内で形態学的変化を伴う増殖環を有する（図1）。感染性粒子は基本小体（elementary body：EB）と呼ばれる直径約300nmの小型球型粒子である。このクラミジアの増殖環と薬剤感受性には関連性があり、通常の抗生物質が有効な時期は網様体の時期である。クラミジアに有効な抗生物質は細胞内浸透性が高く、かつ封入体内に入り込む必要がある。

伝播経路

C. psittaci の宿主域は広い。鳥類ではオウム目を含む18目145種から報告されている。鳥類間におけるクラミジアの伝搬様式は接触、吸入、経口による水平伝搬であり介卵伝達はない（図2）。鳥類のクラミジア感染症はほとんどが不顕性感染である。感染鳥が排泄する糞便にはクラミジアの感染性粒子である基本小体が多数含まれる。基本小体は乾燥に強く、環境中で感染性を保っている。ひな鳥の初感染では一部の感染ひな鳥は発症し死亡する。他は保菌鳥となる。

症状

保菌鳥は輸送、密飼いなどのストレス、栄養不良などの要因が引き金となり発症する。通常、元気消失、食欲減退、鼻腔からの漿液性ないし化膿性鼻漏がある。緑灰色下痢便、粘液便が見られることもある。急性例では症状に気づかないまま死亡することもある。鳥類では早期に治療されれば回復するが、時期を逸すると多くの場合、死亡する。

Profile

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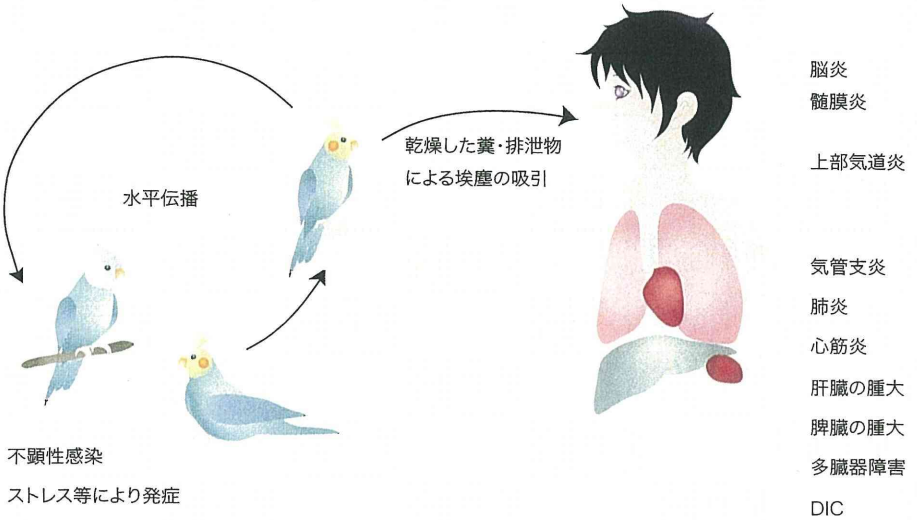

図2　オウム病の伝播様式

不顕性感染鳥は長期間にわたり排泄物中に病原体を連続的ないし間欠的に排泄し、糞便には10^3～10^6／g、鼻分泌液には10^2～10^5／gのクラミジアが存在する。このように持続感染が成立し、クラミジアの生存と伝搬に大きな役割を果たしている。

疫学

日本における感染源の主体である鳥類について見ると環境省が実施し平成15年3月に公表したペット動物流通販売実態調査報告書によれば、平成14年度における鳥類の国内生産数は84500羽、輸入数は1150000羽であった。2003年における我々の調査では健康診断依頼検体491例中25例（5・4%）および何らかの疾病が疑われた検体71例中5例（7・6%）にクラミジアが検出された。斃死鳥では感染症が疑われた59例中13例（28・3%）からクラミジアが検出された。鳥種別にみると、クラミジア保有率はオカメインコ（保有率16%）、セキセイインコ（13%）、ゴシキセイガイインコ（11%）、チャガシラハネナガ（5%）などであった。

オウム病

オウム病についてみると、1999年4月から2009年までに327件の届け出があり、年間の平均届け出数は32件である。感染源はオウムインコ類がほぼ60%である。オウム病の感染源として愛玩鳥に次ぐのは野外のハト（ドバト）である。ドバトは神社仏閣、公園、住宅街など広く生息している。これらドバトからの感染は予防できないため、市民に感染の危険性があることを啓蒙しなければならない。人から人への伝搬は極めてまれである。家族発生の場合でも、同一の感染源からの感染による。感染源となった鳥も発症している場合が多い。鳥との接触や関わりを見出せない症例が報告されているが、原因*C. psittaci*の解析結果から野外のドバトなど自覚のない鳥との接触があったと考えられる。

感染鳥からヒトへの伝播は気道感染である（図2）。感染鳥は排泄物に多量の病原体を排出する。排泄物が乾燥すると塵埃となり、この病原体を大量に含む塵埃の吸入により感染する。7～14日の潜伏期の後に悪寒を伴う高熱で突然発症し、1～2週間持続する。頭痛、羞明、上部ないし下部呼吸器疾患および筋肉痛などのインフルエンザ様症状を主徴とする。

診断

オウム病の診断は遺伝子診断が主となっている。鳥の新鮮糞便からDNAを抽出し、PCRによりクラミジア遺伝子を検出している。Real-time PCRの応用により定量的な検出も可能となっている。

治療

鳥類の治療については確立された処方は必ずしも存在しない。これは鳥種により抗生物質への反応性に相違が見られるためである。一般にはテトラサイクリン系抗生物質の投与が行われているが、マクロライド系の抗生物質も有効である。

おわりに

オウム病の予防は鳥類の健康管理にほかならない。日頃の飼育鳥の健康管理を適正に行うことにより、オウム病の発生を抑えることができる。ヒトと鳥が安心して暮らせるように研究を進めている。

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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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† Supplemental material for this article may be found at http://iai.asm.org/.

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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-