Chlamydia felis CF0218 Is a Novel TMH Family Protein with Potential as a Diagnostic Antigen for Diagnosis of C. felis Infection†

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Chlamydia felis is a causative agent of acute and chronic conjunctivitis and pneumonia in cats (feline chlamydiosis). Also, C. felis is a suspected zoonotic agent of such diseases as non-Chlamydia trachomatis conjunctivitis in humans, although this is controversial. At present, there is no serodiagnostic system that specifically detects C. felis infection conveniently. Current systems use antigens such as lipopolysaccharide that cross-react with all chlamydia species. In addition, it is difficult to distinguish between cats that are vaccinated with the commercial vaccine against C. felis and cats that are infected with C. felis. Here, we describe a new candidate diagnostic antigen for diagnosis of C. felis infection, CF0218, that was obtained by screening a genomic expression library of C. felis Fe/C-56 with C. felis-immunized serum. CF0218 was a putative transmembrane head (TMH) family protein with bilobed hydrophobic motifs at its N terminus, and orthologues of CF0218 were not found in the Chlamydiaceae genomes. The recombinant CF0218 was not recognized by antiserum against Ch. trachomatis, suggesting that CF0218 is C. felis specific. CF0218 transcription during the course of C. felis infection was confirmed by reverse transcription-PCR. By indirect immunofluorescence analysis, CF0218 was colocalized with the C. felis-formed inclusion bodies in the infected cells. The antibody response against CF0218 was elevated following C. felis infection but not by vaccination in experimentally vaccinated and infected cats. These results suggest that CF0218, a novel TMH family protein of C. felis, possesses potential as a C. felis infection-specific diagnostic antigen.

The chlamydiae are obligate intracellular bacterial pathogens, possessing a biphasic developmental cycle, consisting of a metabolically inactive infectious elementary body (EB) and a metabolically active noninfectious reticulate body. The bacteria within host cells occupy vacuoles termed inclusions. Chlamydiae cause a range of diseases in various animals, such as humans, birds, and cats. The family Chlamydiaceae is divided into two genera, Chlamydia and Chlamydophila (9). The genus Chlamydia comprises Chlamydia trachomatis (a human conjunctivitis agent), Chlamydia muridarum (a mouse pneumonia agent), and Chlamydia suis (a pig conjunctivitis agent). The latter genus, Chlamydophila, includes Chlamydophila pneumoniae (an agent for pneumonia and a suspected atherosclerosis agent), Chlamydophila psittaci (an agent for psittacosis), Chlamydophila abortus (a ruminant abortive agent), Chlamydophila caviae (isolated from guinea pigs), Chlamydophila pecorum (infecting ruminants), and Chlamydophila felis (infecting cats) (9).

C. felis is a causative agent of feline chlamydiosis, which is characterized by acute and chronic conjunctivitis and pneumonia in cats (40). The prevalence of C. felis in cats with ocular signs or upper respiratory tract diseases (URTD) has been investigated by PCR or by detection of antichlamydial antibodies. The percentages of cats positive for C. felis infection were 14.7% in Britain (29), 20.0% in Italy (32), 11.5% in Switzerland (42), 15.3% in Sweden (17), and 4.6% in the United States (26). In our previous studies in Japan, the percentages were 26.3% in stray cats, 28.9% in domestic cats, and 59.1% in cats with conjunctivitis and URTD (6, 31, 45). These investigations indicate that C. felis is the most common agent of feline conjunctivitis and URTD in the world.

Since C. felis is susceptible to tetracyclines, doxycycline is the first choice for the treatment of feline chlamydiosis. Systemic administration of doxycycline for 3 weeks can effectively clear the pathogen (40). However, conjunctivitis and URTD in cats are also caused by other pathogens such as feline calicivirus (FCV) and feline herpesvirus 1 (FHV-1), and it is not possible to differentiate feline chlamydiosis from viral conjunctivitis and URTD on the basis of clinical signs (40). Indeed, our previous study showed that in 66 domestic cats with conjunctivitis and URTD, 10.6% of cats had C. felis and FHV-1; 15.2% of cats had C. felis and FCV; and 1.5% of cats had C. felis, FHV-1, and FCV (6). Therefore, to provide adequate treatment and prevent the spread of feline conjunctivitis and URTD, chlamydial infection in cats needs to be differentiated from other viral conjunctivitises and URTD.

Current methods for diagnosing feline chlamydiosis are isolation of the pathogen, immunofluorescence (IF) testing using the infected cells or the purified EB as antigens, or testing by...
conventional PCR and real-time PCR (15, 16, 40). However, these methods require the use of specialized culture techniques (isolation and the IF test) and equipment (PCR and real-time PCR). Therefore, microbiological diagnosis of feline chlamydiosis can be performed only in well-equipped laboratories. Another method to detect *C. felis* infection is enzyme-linked immunosorbent assay (ELISA). There is no ELISA system to specifically detect *C. felis* infection since the ELISA uses whole chlamydial EB and lipopolysaccharide (LPS) as antigens, which are cross-reactive for all chlamydial species (EB and LPS) as well as other bacteria (LPS) (5, 23, 44). Other serodiagnostic antigens include major outer membrane protein (MOMP) and polymorphic membrane proteins (PMPs), which are highly immunogenic and display intraspecies/interspecies diversity (7, 24, 25, 39). For example, Longbottom and colleagues developed the ELISA system by using POMP90 (one of the PMPs in *C. abortus*) as an antigen. The ELISA can specifically detect anti-*C. abortus* antibodies in *C. abortus*-infected sheep (19, 21, 23). ELISA can be performed conveniently in general laboratories and can handle many samples simultaneously. Therefore, ELISA systems which can specifically detect *C. felis* (antibodies or antigens) should be developed for diagnosing feline chlamydiosis (40).

Cases of *C. felis* infection in humans are rarely identified, and whether such cases exist is now controversial (20). However, recently *C. felis* was isolated from a patient with non-*C. trachomatis* conjunctivitis and from one of the patient’s cats (14). In addition, we also reported previously that 5.0% of small-animal clinic veterinarians were seropositive for *C. felis* (45). These results raise the possibility that *C. felis* is a zoonotic agent, as is the case for *C. psittaci* and *C. abortus* (20). Both live and inactivated vaccines for *C. felis* infection have been used in Europe, the United States, and Japan (only the inactivated form is approved in Japan). While the vaccines for *C. felis* do not prevent infection completely, the vaccine can enhance the humoral immune response and reduce the severity of clinical signs in vaccinated cats (22, 37, 40, 43). However, it is difficult to distinguish between vaccinated cats and *C. felis*-infected cats by means of the current serological tests (IF tests and ELISA) because the antibody responses are observed in both cases.

In this study, to discover new diagnostic antigens of *C. felis*, the genomic expression library of *C. felis* was screened with *C. felis*-immunized serum. One of the positive clones was found to encode CF0218 (also named mhcB2). Orthologues of CF0218 were not present in genomes of other chlamydia species such as *C. trachomatis* and *C. pneumoniae*. The recombinant CF0218 was not recognized by *C. trachomatis*-immunized serum. In addition, CF0218 was transcribed in *C. felis*-infected cells and was colocalized with *C. felis*-formed inclusions. Finally, the antibody response against CF0218 was elevated only following *C. felis* infection but not by vaccination in experimentally vaccinated and infected cats. It is likely that CF0218 possesses potential as a diagnostic antigen of *C. felis* which can specifically detect *C. felis* infection.

**MATERIALS AND METHODS**

Chlamydial strains and infection of cultured cells. *C. felis* Fe/C-56 isolated in Japan from a cat with conjunctivitis was used as a standard strain of *C. felis* since this strain had already been subjected to full genomic DNA sequencing (1, 6).

For analyzing the diversity of cf0218 sequences obtained in this study, *C. felis* Fp1 Baker (ATCC VR1210; isolated in the United States from a cat with pneumonia) (2, 12), Fe/B16 (isolated in the United Kingdom from a cat with conjunctivitis) (30, 31), and Fe/C-38 (isolated in Japan from a cat with conjunctivitis) (6) were used. *C. felis* strains were grown in HeLa cells. HeLa cells were treated with minimal essential medium α (Wako Pure Chemical Ltd., Osaka, Japan) containing 50 μg/ml DEAE-dextran at room temperature for 30 min before inoculation. After inoculation of bacteria at a multiplicity of infection of up to 10, flasks (or plates) were centrifuged at 700 × g for 60 min at room temperature and subsequently incubated in the presence of 5% CO₂ at 37°C for 60 min. Afterward, the inocula were exchanged into minimal essential medium α supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1 μg/ml of cycloheximide in the presence of 5% CO₂ at 37°C until formation of the mature inclusion body or until the time indicated. *C. felis* EB was purified from infected HeLa cells by sucrose gradient centrifugation as described previously (12). The purified EB was diluted at 2.0 mg/ml in 0.01 M Tris-HCl (pH 7.2) and stored at –80°C until use.

**Construction and immunoscreening of *C. felis* genomic DNA expression library.** Genomic DNA of *C. felis* Fe/C-56, which was extracted from the purified EB by sodium dodecyl sulfate (SDS), proteinase K, and phenol-chloroform as previously described (11), was partially digested with EcoRI in the presence of 2.5 mM Mn²⁺ and ligated to EcoRI-digested λ-ZAPII phage arms (Stratagene, La Jolla, CA). The ligated DNA was packaged in vitro with Gigapack extracts according to the manufacturer’s instructions (Stratagene). Recombinant phage were plated on *Echerichia coli* XLI-Blue MRF² cells (Stratagene) and incubated at 37°C for 6 h to allow development of the plaques. The plates were sequentially overlaid with nitrocellulose membranes (GE Healthcare, Buckinghamshire, United Kingdom), which were soaked in 20 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 37°C for an additional 48 h. The nitrocellulose membranes were reacted with *C. felis*-hyperimmunized serum culture (28) as the first antibody, subsequently reacted with anti-cat light chain-horseradish peroxidase (HRP) (Bethyl, Montgomery, TX) as the secondary antibody, and visualized by 3,3'-diaminobenzidine in the presence of 0.01% H₂O₂. Several phage clones, which were confirmed as positive by secondary and tertiary screening, were converted to the blueprint SK(+) phagemid in *E. coli* SOLR according to the manufacturer’s instructions (Stratagene). The DNA from the insert of each clone was sequenced in both directions by using M13 forward and reverse primers and analyzed by using the *C. felis* Fe/C-56 genomic DNA sequence data as published previously (1).

**Analysis of diversity and hydrophathy profile of cf0218.** The diversity of cf0218 from different strains of *C. felis* was examined by sequencing the PCR products of cf0218 with primers (5′- CGGGATCCAGTAGACAAACTCATGGT-3′) and 5′-CGGTGCA-TTATATTACGTCAT-3′) from *C. felis* Fp1 Baker, Fe/B16, and Fe/C-38. Hydrophathy profiles were determined using the algorithm of Kyte and Doolittle (18) with a window size of 11 on the web source ProtScale (available at http://www.expasy.ch/tools/protscale.html) and the DNA Strider program (27).

**Recombinant CF0218 and antibody preparation.** The recombinant protein expression was amplified by PCR with primers (5′-CGGGATCCACGGGACTCATGACAAACTCATGGACGTAAGAGGACTCGTACTCATGATT-3′) from *C. felis* Fe/C-56 isolated in Japan from a cat with conjunctivitis and was cloned into the expression vectors pGEX-FP1 Baker, Fe/B16, and Fe/C-38. Hydrophathy profiles were determined using the algorithm of Kyte and Doolittle (18) with a window size of 11 on the web source ProtScale (available at http://www.expasy.ch/tools/protscale.html) and the DNA Strider program (27).

**Immunoblotting.** *C. felis* EB (1 mg/ml) inactivated in PBS supplemented with 0.5% (vol/vol) Triton X-100 and 5% (vol/vol) 2-mercaptoethanol or each recombinant protein was blotted on a nitrocellulose membrane (GE Healthcare). Briefly, logarithmic-phase E. coli BL21 (GE Healthcare) harboring pGEX or pGSTM-CF0218 in Luria-Bertani broth supplemented with 40 μg/ml ampicillin was further incubated at 30°C for 5 h in the presence of 1.0 mM IPTG. The bacteria were suspended and sonicated in phosphate-buffered saline (PBS) containing 1% (vol/vol) Triton X-100. After centrifugation at 4°C, the soluble form of GST or GST-CF0218 in the supernatant was purified by gel filtration on Sephrose 4B (GE Healthcare). Rabbit (Japanese White, female, 12-week-old) polyclonal antibody was raised against recombinant CF0218 emptied of GST by PreScission protease (GE Healthcare). The antisera obtained was purified using CF0218 blotted on a nitrocellulose membrane (GE Healthcare). Briefly, the CF0218 blotted on a nitrocellulose membrane stained with 1% Ponceau S was excised and blocked in 1% polyvinylpyrrolidone for 1 h at 37°C. After washing, the membrane was incubated with the antisera for 2 h at room temperature. The binding antibody specific for CF0218 was eluted from the membranes with 0.1 M Gly-HCl (pH 2.5) and neutralized with 2 M Tris. All animal experiments described in this study were approved by the Commission for Animal Experiments in Gifu University according to current guidelines.

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binant CF0218 product was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane (Immolon-P; Millipore, Billerica, MA). The following antisera were used as the primary antibodies diluted at 1:2,000 in PBS: rabbit anti-CF0218, rabbit C. psittaci Prk/daruma-hyperimmunized serum (H. Fukushima et al., unpublished material), rabbit C. trachomatis L2/434/Bu-hyperimmunized serum (12), and feline C. felis-hyperimmunized serum. The anti-cat light chain-HRP and the anti-rabbit immunoglobulin G (IgG)-HRP (ICN Pharmaceuticals, Aurora, OH) were used as the secondary antibodies diluted at 1:2,000 in PBS. The membranes were incubated in ECL Western blotting detection reagents (GE Healthcare) and subsequently exposed to X-ray film (Fujifilm, Tokyo, Japan).

RT-PCR analysis. Total RNA was extracted from C. felis Fe/C-56-infected HeLa cells at each time point after infection by Trizol reagent (Invitrogen), and residual DNA contamination was removed by treatment with amplification-grade DNase 1 (Fermont International, Laval, Quebec) diluted at 1:50 in PBS, anti-rabbit IgG-fluorescein isothiocyanate (ICN Pharmaceuticals, Aurora, OH) diluted at 1:5,000 in PBS, and Moloney murine leukemia virus reverse transcriptase for 60 min at 42°C according to the manufacturer's instructions (ReverTra Ace kit; Toyobo, Osaka, Japan). The cDNA was amplified by PCR using each primer. Custom primer sets specific for C. felis cf0218 (5'-CCGGGACACCACAATCTGATG-3' and 5'-GCGTCGACTAATTGGCTGATCCATT-3') were used to detect cf0218-specific message, whereas previously described primer sets for C. felis cf0218 (27) were used to examine levels of Chlamydia pneumoniae ompA (9). HeLa cells grown on coverslips were used for the IF microscopy. Fe/C-56-infected HeLa cells at each time point after infection were fixed with formaldehyde, permeabilized with Triton X-100, and mounted in Diamidino-2-phenylindole dihydrochloride (DAPI) (Dojindo Laboratory, Kumamoto, Japan) diluted at 1:50 in PBS, anti-rabbit IgG-fluorescein isothiocyanate (ICN Pharmaceuticals, Aurora, OH) diluted at 1:5,000 in PBS, and Moloney murine leukemia virus reverse transcriptase for 60 min at 42°C according to the manufacturer's instructions (ReverTra Ace kit; Toyobo, Osaka, Japan). The cDNA was amplified by PCR using each primer. Custom primer sets specific for C. felis cf0218 (5'-CCGGGACACCACAATCTGATG-3' and 5'-GCGTCGACTAATTGGCTGATCCATT-3') were used to detect cf0218-specific message, whereas previously described primer sets for C. felis cf0218 (27) were used to examine levels of Chlamydia pneumoniae ompA (9). HeLa cells grown on coverslips were used for the IF microscopy. Fe/C-56-infected HeLa cells at each time point after infection were fixed with formaldehyde, permeabilized with Triton X-100, and mounted in Diamidino-2-phenylindole dihydrochloride (DAPI) (Dojindo Laboratory, Kumamoto, Japan) diluted at 1:50 in PBS, anti-rabbit IgG-fluorescein isothiocyanate (ICN Pharmaceuticals, Aurora, OH) diluted at 1:5,000 in PBS, and Moloney murine leukemia virus reverse transcriptase for 60 min at 42°C according to the manufacturer's instructions (ReverTra Ace kit; Toyobo, Osaka, Japan). The cDNA was amplified by PCR using each primer. Custom primer sets specific for C. felis cf0218 (5'-CCGGGACACCACAATCTGATG-3' and 5'-GCGTCGACTAATTGGCTGATCCATT-3') were used to detect cf0218-specific message, whereas previously described primer sets for C. felis cf0218 (27) were used to examine levels of Chlamydia pneumoniae ompA (9).

RESULTS

Identification of the cf0218 gene. Seven positive clones were obtained by immunoscreening of the C. felis genomic expression library (1.5 × 10^6 PFU) with the cat serum raised against C. felis. Phagegids were excised from these clones and partially sequenced with M13 primers (see Table S1 in the supplemental material). Among them, one clone (clone E in Table S1 in the supplemental material) was chosen for further analysis since this clone (insert size, 2,241 bp) contained one open reading frame (ORF), designated cf0218 (also named mhcB2), for which orthologous genes were not found in the C. pneumoniae and C. trachomatis genomes (1). As shown in Fig. 1, cf0218 in the C. felis genome is located in the region syntenic with the transmembrane head (TMH) locus in the C. abortus (41) and the C. caviae (33) genomes, whereas this locus is not present in the C. pneumoniae J138 genome (38) or the C. trachomatis D/UW-3/Cx genome (39). cf0218 (1,146 bp) encodes 381 amino acid residues with a calculated molecular mass of 42.0 kDa. CF0218 proteins from different isolates of C. felis were highly conserved. For example, CF0218 from C. felis FP1 Baker (isolated in the United States; accession number AB444855) and Fe/C-38 (isolated in Japan; accession number AB444857) exhibited 100% amino acid identity with that from Fe/C-56. Only CF0218 from Fe/C166 (isolated in the United Kingdom; accession number AB444856) showed one amino acid difference at position 110 (G) compared with that of other isolates (E). The TMH locus in the C. abortus and the C. caviae genomes encodes several TMH family proteins with paired N-terminal transmembrane motifs. For example, C. abortus CAB764 and CAB766 and C. caviae CCA797 at the TMH locus, which are TMH family proteins, had 25.0%, 27.4%, and 34.9% amino acid identities with CF0218, respectively (Fig. 2A). Furthermore, CAB764, CAB766, CCA797, and CF0218 possess similar bilobed hydrophobic motifs at their N termini (Fig. 2B), which implies that CF0218 is a TMH family protein of C. felis.

Expression of recombinant CF0218 and its immunogenicity. Recombinant CF0218 was successfully expressed in E. coli as a soluble fusion protein with GST (GST-CF0218) at the expected size (Fig. 3A). To generate polyclonal antibodies against CF0218, recombinant CF0218 was cleaved from GST-CF0218 and then injected into a rabbit. To evaluate the antigenicity of CF0218, recombinant CF0218 and purified C. felis EB were subjected to Western blot analysis. The generated antiserum specifically reacted with GST-CF0218, purified recombinant CF0218, and purified C. felis EB at the expected size but not with GST alone (Fig. 3B). The C. felis-hyperimmunized cat serum, which was used in the immunoscreening of the C. felis library, reacted with GST-CF0218 and purified CF0218 at the expected size, but it did not react with GST alone (Fig. 3C). On the other hand,
recombinant CF0218 was not recognized by the C. trachomatis-hyperimmunized rabbit serum, which showed cross-reactivity with C. felis EB (Fig. 3D). This result agreed with the fact that orthologues of CF0218 do not exist in the C. trachomatis genome (Fig. 1). In addition, we also examined cross-reactivity of CF0218 with non-C. felis chlamydiae. Since we do not have available antisera against C. abortus and C. caviae, we used antiserum against C. psittaci Prk/daruma, which is genetically more closely related to C. abortus than to other C. psittaci strains by Southern hybridization analysis (11). This serum reacted with C. felis EB but not with recombinant CF0218 (Fig. 3E).

Expression of CF0218 in C. felis-infected cells. To determine whether CF0218 is expressed during C. felis infection in host cells, RT-PCR analysis was performed. Total RNA from C. felis-infected or mock-infected HeLa cells was isolated at 24, 48, and 72 h after inoculation of C. felis and subjected to RT-PCR using the primers specific for cf0218 and the gene corresponding to MOMP (ompA) as a control for chlamydia infection. As shown in Fig. 4, specific signals for cf0218 and ompA were detected at 24, 48, and 72 h after inoculation of host cells with C. felis, revealing that cf0218 was transcribed in host cells during C. felis infection. By Western blot analysis, lysates of C. felis-infected HeLa cells reacted weakly with anti-chlamydial LPS, but no specific signal with anti-CF0218 serum was detectable (data not shown). It may be due to low infection efficiency of C. felis and/or the low expression level of CF0218, although we have no quantitative data to describe the infection efficiency of C. felis compared with other chlamydia species. We next performed an indirect-IF assay to visualize CF0218 in C. felis-infected cells. C. felis-infected (72 h postinoculation) or mock-infected HeLa cells were fixed and probed with anti-CF0218, anti-chlamydial LPS, and DAPI for host nuclei and apparent chlamydial inclusions (Fig. 5). The 72-h point was chosen because the inclusions are fully developed (white arrowheads in Fig. 5). Since the inclusions of C. felis are relatively smaller than those of C. psittaci (data not shown), it is hard to detect C. felis by DAPI staining except for the apparent inclusions. However, C. felis in host cells can be visualized by staining with anti-chlamydial LPS (red in Fig. 5). Specific signal for CF0218 (green in Fig. 5) was colocalized with the chlamydial LPS (merged images are yellow in Fig. 5) as well as the apparent chlamydial inclusions (merged images are white in Fig. 5). The IF microscopic analysis clearly showed that the subcellular localization of CF0218 is within the C. felis-formed inclusions.
Antibody response against CF0218 in experimentally vaccinated and *C. felis*-infected cats. As mentioned in the introduction, the modified live and inactivated vaccines against *C. felis* have been used in several countries including Japan (40). It is difficult to differentiate vaccinated and infected cats since both vaccinated cats and infected cats are assessed as positive by means of current serological tests. Therefore, to assess the potential of CF0218 for diagnostic use, antibody responses against CF0218 in vaccinated and *C. felis*-infected cats were examined. Specific-pathogen-free cats were inoculated with the *C. felis* inactivated vaccine twice (at 0 and 3 weeks) and subsequently challenged with *C. felis* at 5 weeks (2 weeks after the second vaccination; for details of the experimental design, see Materials and Methods). Sera were collected at each time point, and antibody responses against *C. felis* EB and the recombinant CF0218 were measured by ELISA (Fig. 6). As shown in Fig. 6A, antibody response against *C. felis* EB was elevated following the *C. felis* vaccination from 4 weeks after the first vaccination in the vaccinated group (closed squares) but not in the nonvaccinated group (closed circles). Thereafter, both groups were challenged with *C. felis* (at 5 weeks), and antibody...
against EB was elevated from 7 weeks (2 weeks after the challenge) in both groups. These results confirmed that antibody response due to the vaccination and the infection occurred correctly in the experiments. Antibody response against CF0218 in the same samples was measured by ELISA using the recombinant CF0218 as an antigen (Fig. 6B). The level of antibody against CF0218 was not increased until 5 weeks later in the vaccinated group (Fig. 6B, closed squares and solid line), although the level of antibody against EB was elevated at this time point in the vaccinated group.

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

FIG. 5. Localization of CF0218 in C. felis-infected cells. HeLa cells infected with C. felis for 72 h were fixed and stained with DAPI for host cell nuclei and apparent chlamydial inclusion bodies (blue; white arrowheads show large chlamydial inclusion bodies), anti-chlamydial LPS (red), and anti-CF0218 (green). The rightmost columns show merged triple fluorescence images. Bars, 10 μm.
CF0218 was increased following C. felis group (Fig. 6A). In contrast, the level of antibody against C. felis vaccination but only by live inoculated C. felis nonvaccinated cats). Indicated as asterisks (single for the vaccinated cats and double for the control serum (at 0 weeks) and at each time point are 0.01) between control serum (at 0 weeks) and at each time point are shown in Fig. 3B, although several Inc proteins of C. caviae (formerly C. psittaci GPC) and C. trachomatis were not reported to be detected in purified EBs (4, 36). On the other hand, C. trachomatis IncA was detected in purified EB to an even lesser extent (36) and CopN (a component of the type III secretion system) was also detected in purified EB (10). Both IncA and CopN play pivotal roles in the chlamydial infection process. It remains to be elucidated whether CF0218 is a structural component of EB or acts as an Inc-like protein in infected cells during the C. felis infection process.

In general, serological diagnosis of chlamydiosis is exclusively performed by using EB or chlamydial cell surface components (LPS, MOMP, and PMPs) as antigens (see the introduction), while other chlamydial products represent immunogenicity in infected animals or human patients. For example, C. caviae IncA and IncC were initially identified by sera from C. caviae-infected HeLa cells and was colocalized in C. felis-formed inclusion bodies; (v) the level of antibody against CF0218 was elevated by C. felis infection but not by vaccination in experimentally vaccinated and infected cats.

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

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

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

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

**DISCUSSION**

In this study, we describe the cloning and molecular characterization of C. felis CF0218 as a new diagnostic antigen. Our results show the following: (i) CF0218 was obtained by immunoscreening of a C. felis genomic library with the C. felis-immunized cat serum; (ii) the location of cf0218 in the C. felis genome is the syntenic region of the TMH locus in the C. abortus genome, which was not present in the genome of C. trachomatis or C. pneumoniae; (iii) recombinant CF0218 was recognized by serum against C. felis but not by serum against C. trachomatis and C. psittaci; (iv) CF0218 was expressed in C. felis-infected HeLa cells and was colocalized in C. felis-formed inclusion bodies; (v) the level of antibody against CF0218 was elevated by C. felis infection but not by vaccination in experimentally vaccinated and infected cats.
putative Inc proteins are recognized by sera from *C. trachomatis* and *C. pneumoniae*-infected patients (3). These reports indicate that chlamydial products other than LPS, MOMP, and PMPs are immunogenic and can be used as diagnostic antigens. CF0218 is highly conserved at the amino acid level among different *C. felis* isolates. The antigenicity of CF0218 was examined by using serum against *C. felis* and was confirmed (Fig. 3C). Furthermore, levels of antibody against CF0218 were increased in experimentally infected cats (Fig. 6B). These results indicate that CF0218 is immunogenic, with potential as a diagnostic antigen of *C. felis*.

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

As described in the introduction, *C. felis* is a suspected zoonotic agent (20). The fact that the recombinant CF0218 was not recognized by the serum against *C. trachomatis* (Fig. 3D) raises the possibility of using CF0218 to clarify whether *C. felis* infection in humans is the cause of non-*C. trachomatis* conjunctivitis.

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

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

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

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