ンテナに入り込んだ動物が生存する可能性はほとんどないが、中国などの近隣アジア地域から、比較的短時間で我が国に到着するコンテナ内で、温度環境などに恵まれれば、潜入した動物が生存できる可能性は十分にある。現に、狂犬病清浄地域である米国ハワイ州の港に狂犬病常在地の米国カリフォルニア州から搬入されたコンテナ内にコウモリが潜入しており、捕獲後にコウモリが狂犬病と判明した事例がある(1)。

上記の症例は、中国から搬入されたコンテナ内に潜んでいたネコを保健所に届けるため、捕獲しようとして受傷した。しかし、港湾部で目撃されたネコ36例（うちコンテナ内14例）のうち当局に通報があったのは15例との調査報告(2)から推定されるように、外国から潜入する動物に関する知識がなければ、コンテナ内に小動物が潜んでいても、捕獲せずに追い出してしまうであろう。このように狂犬病常在地から搬送されたコンテナを介して狂犬病ウイルス感染動物が、日本の港湾部だけでなく、直接内陸部に侵入する可能性があることを知っておく必要がある。

今回の事例では、動物の検疫などを所轄する農林水産省とヒトの健康問題などを所轄する厚生労働省との連携の不備ばかりでなく、侵入動物を検査する上での問題点が明らかになった。加害ネコは、保護目的で保健所から所轄の動物愛護センターに送られ、さらに検査目的で、動物愛護センターから動物検疫所に移送された。この移送は、動物検疫所以外では輸入動物の狂犬病検査が実施できないために必要になったものであり、侵入動物の発見場所が動物検疫所から遠隔地であれば、検査不能となる可能性もある。感染動物の国内侵入を阻止するためには、保健所、動物愛護センター、衛生試験所などが協力して、侵入動物が発見された各都道府県で侵入動物の検査を実施できるよう体制を整えるべきである。

注）保税蔵置場とは、外国貨物の積み卸し、運搬またはこれを保管することができる所として税関長が許可した場所

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2005：135-145

☆

臨床寄生虫学雑誌データベースの構築とその利用

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Key Words：データベース，利用方法

はじめに

1990年に臨床寄生虫学雑誌 Vol. 1 No. 1 が発刊されてから19年の歳月が経過し，この間に臨床寄生虫学雑誌に掲載された論文数は800編以上にのぼっている。掲載された症例報告は国内ではまれな寄生虫疾患もあればいまだ多くの患者の発生を見るものも多い。これらの症例報告の二次利用を図るためには検索可能なデータベースを構築することが有効な手段と考えられる。

今回われわれは，第32回（2007年度）大山健康財団学術研究（代表　名和行文）の助成を受け，研究課題「寄生虫疾患情報の電子化に関する研究」の一部として，臨床寄生虫学会雑誌に掲載された論文のデータベースを構築し運用を開始したので，その概要とアクセス方法，ならびにデータベースを利用した寄生虫感染症の動向について報告する。

結果と考察

データベースの構築

データベースはインターネットを介した検索とPDFファイルのダウンロードを可能にするために，雑誌に掲載されたすべての論文をスキャニングしPDF変換を行った。論文の文字情報は市販のデータベースソフト（ファイルメーカー　Pro Unlimited, FileMaker Inc.）を用いてデータ入力を行い，演題名，著者名，開始ページなどの基本書誌情報を抽出し，データベースの構築を行った。

Creation and Usage of a Database of the Journal of Clinical Parasitology

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論文請求先：赤尾信明　〒113-8519　東京都文京区湯島1-5-45　東京医科歯科大学　国際環境　寄生虫病

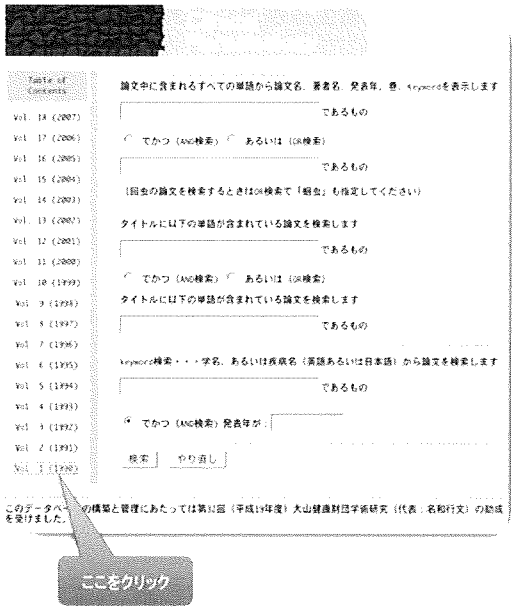

図1　臨床寄生虫学雑誌検索ホームページ.
(http://ascaris.tmd.ac.jp:443/CPtop.html)

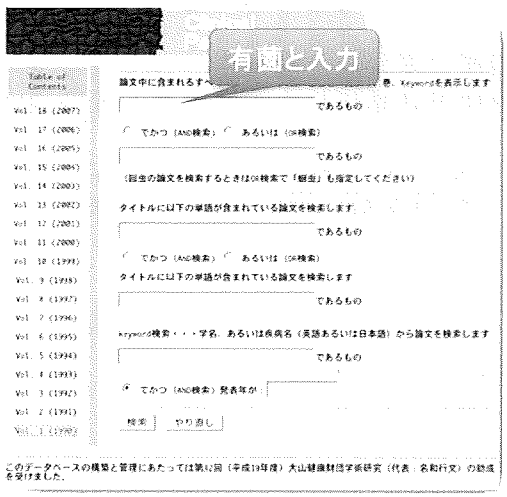

図2a 『有薗』で検索を行う場合の検索方法

図2b 『有薗』が含まれる論文の検索結果

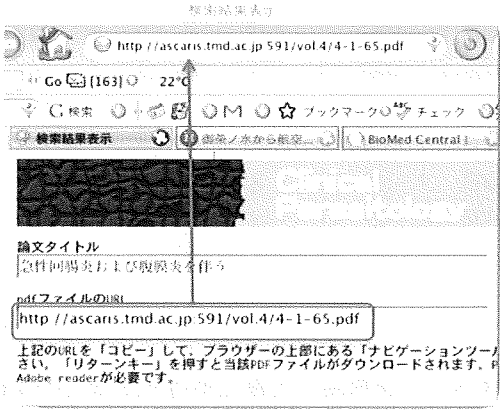

図2c　検索結果の論文のダウンロード方法

また，目次部分のスキャニング情報を基にして，演題名から当該PDFファイルを参照でき，かつダウンロードできるようにした（図1）。

データベースの検索機能は，ファイルメーカーProに付帯するCDMLによる簡易プログラムで実現させた。そして，カスタムWebsiteサイトを作製して公開した。

例えば，第19回臨床寄生虫学会会長の有薗直樹教授の論文を調べたい場合，「論文中に含まれるすべての単語から論文名，著者名，発表年，巻，Keywordを表示します」に『有薗』と入力すると16編の論文がヒットする（図2a，b）。この検索結果一覧の最後のカラムにある『PDFファイル』をクリックすると当該ファイルのURLが明示されるので（図2c），これをブラウザのアドレス欄にコピー&ペーストすると当該論文がダウンロードされる。論文はタイトルやキーワードからも検索が可能で，かつ，and/or検索で論文を絞り込むこともできる。

臨床寄生虫学雑誌データベースは以下のアドレスから利用可能である。ただし，アクセスにはユーザIDとパスワードが必要である。

http：//ascaris.tmd.ac.jp：443/CPtop.html
ユーザID：rinsho　パスワード：kiseichu

表 1　臨床寄生虫学雑誌に発表された論文数

開催年	発表論文数	シンポジウム等	原著論文数	一般演題数
1990	60	1	0	59
1991	45	1	0	44
1992	56	1	0	55
1993	70	1	0	69
1994	65	1	0	64
1995	61	2	0	59
1996	35	2	0	33
1997	42	2	1	39
1998	40	0	2	38
1999	44	1	2	41
2000	39	2	3	34
2001	46	1	3	42
2002	46	12	3	31
2003	32	5	1	26
2004	32	7	1	24
2005	34	7	1	26
2006	40	8	2	30
2007	27	8	0	19
合計	814	62	19	735

表 2　寄生虫疾患別発表演題数

寄生虫	発表演題数	シンポジウム等	一般演題数
1　アニサキス症	84	0	84
2　マラリア	91	11	80
3　アメーバ症	55	2	53
4　裂頭条虫症	51	4	47
5　回虫症	25	0	25
6　糞線虫症	24	1	23
7　肺吸虫症	23	0	23
8　マンソン孤虫症	21	0	21
9　動物由来回虫症	18	1	17
10　包虫症	16	0	16

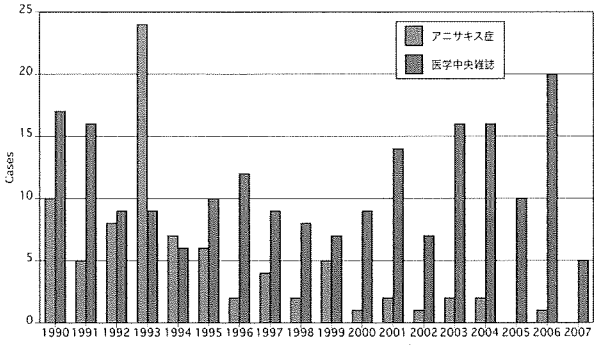

図 3　臨床寄生虫学雑誌と医学中央雑誌に発表されたアニサキス症に関する論文数の年次変化

データベースを利用した寄生虫症発生動向の解析

臨床寄生虫学雑誌データベースを用いて，臨床寄生虫学会で報告された寄生虫症の発生動向を解析した。1990 年～ 2007 年までに発表された総論文数は 814 編であり，その内訳はマラリア関連論文が 91 編，アニサキス関連 83 編，アメーバ関連 52 編と続いた（表 1）。原虫症の演題の増加は感染症法による届け出の義務化により臨床家の関心が増し，シンポジウムで取り上げられる機会が増加したことも一因と考えられる。

814 編の論文のうちシンポジウムや会長講演などを除いた一般講演数は 735 編であり，アニサキス症関連論文が 84 編と最も多く，以下マラリア，アメーバ赤痢と続いた（表 2）。しかし，アニサキス症に関連する報告数は 2000 年を境に減少傾向が見られた。これが本邦におけるアニサキス症の減少を示唆しているのかを検討するために，医学中央雑誌に掲載されたアニサキス症の症例報告数と比較してみたところ，医学中央雑誌に掲載された論文数は 1990 年～ 2007 年の間で 200 編を数えた。そして，その年次変化は臨床寄生虫学雑誌掲載論文数とは異なり，2000 年以降に増加傾向が見られた（図 3）。このことから，アニサキス症自体が減少しているのではなく，臨床寄生虫学雑誌への投稿が減少したためだと推測された。

一方，新興寄生虫感染症であるサイクロスポーラ症は 1996 年に本邦では臨床寄生虫学会で最初の報告があり，以後これまでに 7 編の報告がなされている。一方，医学中央雑誌には 1998 年の第 1 例の報告以後，12 編の症例報告が見られた。6 編は感染症学雑誌，日本臨床微生物学雑誌 2 編，他は熱帯医学会雑誌，臨床病理，医学検査，日本内科学会関東支部会誌にそれぞれ 1 編が掲載されていた。サイクロスポーラ症のような新興感染症では臨床寄生虫学雑誌への投稿数と他の雑誌への投稿数がほぼ拮抗する傾向にあった。

まとめ

臨床寄生虫学雑誌データベースの利用により，臨床寄生虫学会員が関心を持つ寄生虫症の変遷を把握することが可能であった。今後は，本データベースを広く公開し，臨床寄生虫学会の研究成果を社会に還元する方向で検討すべきであると考える。

謝辞：本研究は，第32回（2007年度）大山健康財団学術研究（代表　名和行文）の助成を受けて行われた。

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Case report

A familial case of visceral toxocariasis due to consumption of raw bovine liver

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ABSTRACT

We present 3 adult cases of visceral toxocariasis from the same family, who each consumed thin slices of raw bovine liver weekly, and developed eosinophilia and multiple small lesions in their livers and lungs. Serological examinations using the larval excretory–secretory product of *Toxocara canis* strongly indicated infection with *Toxocara* species larvae. The patients responded well to treatment with albendazole. Ingestion of raw liver from paratenic animals is considered to be a common transmission route of human toxocariasis, especially in adults.

1. Introduction

Human toxocariasis is a common helminthozoonosis caused by infestation with larvae of the nematode worms *Toxocara* (*T.*) *canis* or *T. cati* [1–5]. It has long been considered a parasitic disease that affects pet owners and children, because transmission was thought to only occur via ingestion of infective embryonated eggs after exposure to soil and hair contaminated with the feces of dogs and cats. However, infective stage larvae can also be transferred to other animals and humans through predation, and this type of parasite transfer is now considered to be frequently related to adult cases of toxocariasis in Japan [6]. Therefore, toxocariasis should be recognized as a food-borne parasitic disease, especially in societies where consumption of raw meat is prevalent. Herein, we present 3 adult cases of visceral toxocariasis from the same family who regularly consumed thin slices of bovine liver. Our findings show that consumption of raw liver from paratenic animals is an important source of infestation.

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2. Cases

A 58-year-old man (Patient 1) had never been found with leukocytosis in annual medical check-up examinations until December, 2007, when an increased number of white blood cells (11,800/μl) with marked eosinophilia, absolute count 4250/μl, and elevated IgE (2345 U/ml, normal <100) were found. He was referred to Nara Medical University Hospital. At the initial interview, the patient noted that he and 2 other family members, his 57-year-old wife (Patient 2) and 27-year-old son (Patient 3), consumed raw bovine liver every Friday for the past year, believing that it was good for their health. Their habit was to obtain 100 g of raw bovine liver at a nearby meat shop and serve it as thin slices at dinner. Patient 1 generally consumed the most, followed in order by Patient 2 and Patient 3. In contrast, the mother of Patient 1, who lived in the same house, only ate the raw liver on a few occasions.

We performed blood examinations for all 4 family members. Although none was symptomatic, the 3 regular consumers showed increased eosinophils and IgE (Table 1), while the mother who consumed raw liver only rarely showed no eosinophilia or elevated IgE. Results of a blood examination for Patient 2 obtained by a local physician 1 year previously, prior to beginning the dietary habit, showed a normal number of white blood cells at 6000/μl with a 3%

Table 1
Patient laboratory data

	Patient 1	Patient 2	Patient 3
WBC (/μl)	11,800	8700	8800
Eo (%)	36.0	27.0	19.3
Hb (g/dl)	14.7	14.5	15.6
PLT ($\times 10^4$/μl)	28.4	25.4	29.0
CRP (mg/dl)	0.2	0.5	0.1
AST (IU/l)	23	24	17
ALT (IU/l)	12	28	22
ALP (IU/l)	279	227	201
γGTP (IU/l)	35	53	21
IgE (U/ml)	2345	645	422

Abbreviations and normal ranges:
WBC: white blood cells, 3900–9800/μl.
Eo: eosinophils, 0–5%.
Hb: hemoglobin, 13.2–15.6 g/dl.
PLT: platelet, 13.0–36.0 ($\times 10^4$)/μl.
CRP: C-reactive protein, less than 0.2 mg/dl.
AST: aspartate aminotransferase, 12–32 IU/l.
ALT: alanine aminotransferase, 5–36 IU/l.
ALP: alkaline phosphatase, 120–360 IU/l.
γGTP: gamma-glutamyl transpeptidase, 11–69 IU/l.
IgE: immunoglobulin E, less than 100 U/ml.

eosinophil fraction, though IgE was not examined. Additional tests were performed to determine the etiology of the hypereosinophilia in the patients. Chest computed tomography (CT) demonstrated multiple small pulmonary lesions, nodules with halos and poorly defined margins, and ground-glass opacity with a poorly defined margin in all. Furthermore, contrasted abdominal CT in the portal phase revealed multiple, poorly defined, low-attenuated nodules in the liver of Patient 1, while Patients 2 and 3 each had only a single lesion. Representative CT images from Patients 1, 2, and 3 were shown in Fig. 1. Some nodules in the liver of Patient 1 showed peripheral rim enhancement in the arterial phase (Fig. 2A), while most nodules were undetectable in the equilibrium phase. These CT findings of pulmonary and hepatic lesions were very consistent with those in previous reports of toxocariasis [7–9]. Ultrasonography (US) detected multiple, small, oval, hypoechoic lesions in the liver of Patient 1, and 3 hypoechoic lesions in the liver of Patient 2 including the one lesion detected by CT, whereas none was detected in Patient 3. We also performed contrast US using a newly developed material, Sonazoid® [10], and compared those images with the CT images (Fig. 2B). The lesions were detected as hypoechoic areas in the portal phase and even more clearly in the equilibrium phase, while they were not enhanced in the arterial phase, suggesting that the lesions were poorly supplied with arterial or portal blood. In the post-vascular or so-called Kupffer image phase, the lesions remained un-enhanced, suggesting the absence or scant presence of Kupffer cells (Fig. 2C).

A rapid diagnostic test for toxocariasis, ToxocaraCHECK® [11], which detects IgG antibodies against the larval excretory–secretory (LES) product of *T. canis* on an antigen-sensitized nitrocellulose membrane, showed positive results for all 3 patients. Furthermore, a microplate enzyme-linked immunoabsorbent assay (ELISA) using the LES product and serum from each patient diluted 1:900 revealed the presence of human IgG antibodies at very high titers. The optical density (OD) values at 405 nm for sera from Patients 1, 2, and 3 were 1.58, 1.41, and 1.38, respectively, as compared to the established OD value cutoff level of ≤0.2 for serum from healthy individuals. We also examined immunopositivity against nematode antigens other than the LES product of *T. canis* using a gel diffusion test (Fig. 3), which revealed a strong positivity against the LES products of both *T. canis* and *T. cati*, suggesting a high cross-immunogenicity between them or dual infection, though no formation of precipitate was observed against the LES product of *Ascaris suum* or *Anisakis simplex*. Since no serological examination has been established yet to discriminate between toxocariasis caused by *T. canis* and that by *T. cati*, we made a diagnosis of toxocariasis by *Toxocara species* for all 3 patients.

The patients were instructed regarding prevention of re-infection and treated with a 4-week regimen of daily albendazole at 600 mg (10.8 mg/kg of body weight for Patient 1, 12.8 mg/kg for Patient 2, 10.0 mg/kg for Patient 3). All completed the treatment, though a mild elevation of transaminases up to double the upper limit was observed in Patient 2. During treatment, the eosinophil count decreased in each and became normalized by the end of treatment in Patient 2, while Patients 1 and 3 were further treated with albendazole at the same dose for two more weeks until the eosinophil count became normalized. Hepatic and pulmonary lesions were undetectable by CT and US examinations at the end of treatment in all of the patients. Three months after finishing the treatment with albendazole, we confirmed that a normal eosinophil count was maintained in each patient, along with no recurrent hepatic or pulmonary lesions in CT findings. In addition, the OD values of anti-*T. canis* LES were decreased to 0.95, 0.80, and 0.74 from the initial values of 1.58, 1.41, and 1.38 before treatment in Patients 1, 2, and 3, respectively.

3. Discussion

Visceral toxocariasis is a representative infection of visceral larva migrans (VLM), first reported by Beaver et al. [12], known to be prevalent among preschool children, as they tend to play with dogs in open areas and ingest egg-contaminated soil. However, a recent review of human toxocariasis cases in Japan noted that the disease affects predominantly adults rather than children [6].

There are a number of case reports of adult toxocariasis [13–21], and accumulating evidence [22–27] has revealed that a common route of adult human infection is through ingestion of uncooked or raw liver from a paratenic host. In general, transfer of infective stage larvae through predation is a common mode of helminth transmission among carnivorous vertebrates and this type of parasite transfer can also occur from animals to humans. In experiments with chicken, cattle, and swine, Taira et al. found that the animals were able to function as paratenic hosts for *T. canis* and that the liver was one of the most intensely affected organs [22,23]. Similar observations regarding the importance of predatory cycle have also been reported for cases of infection with *A. suum* [28,29].

Adults with a dietary habit of consuming raw liver have been found to be at high risk for human VLM [24–26]. Morimatsu recently reported an interesting familial case in Japan, in which a father (71 years old) and son (45 years old) developed visceral toxocariasis after consumption of raw chicken livers, and found *T. canis larvae* in the livers of chickens raised in their breeding farm [17]. The present patients began to eat raw bovine liver weekly and continued the habit for about 1 year. Patients 1 and 2 had normal white blood cell counts including eosinophils in routine peripheral blood examinations conducted 1 year and just prior, respectively, to beginning the weekly consumption of raw bovine liver, which suggests that the dietary habit of eating raw liver contributed to toxocariasis in those cases. We strongly suspect that some of the raw liver served at dinner was infected with larvae of *Toxocara species*. Thus, it is important to recognize that toxocariasis can be a food-borne parasitic disease, based on the present findings.

The majority of patients with visceral toxocariasis are asymptomatic and the disease is often discovered during investigation of peripheral eosinophilia [8,9,30], as in the present cases, though those with a high number of worms may complain of vague abdominal discomfort, abdominal pain, cough, dyspnea, fever, or general weakness. Although each of our patients were asymptomatic, the degree of eosinophilia, serum IgE level, and number of hepatic lesions were prominently high in Patient 1, who ingested larger quantities of raw liver as compared to the others, suggesting that the number of worms and disease severity may be proportional to the amount of raw liver intake.

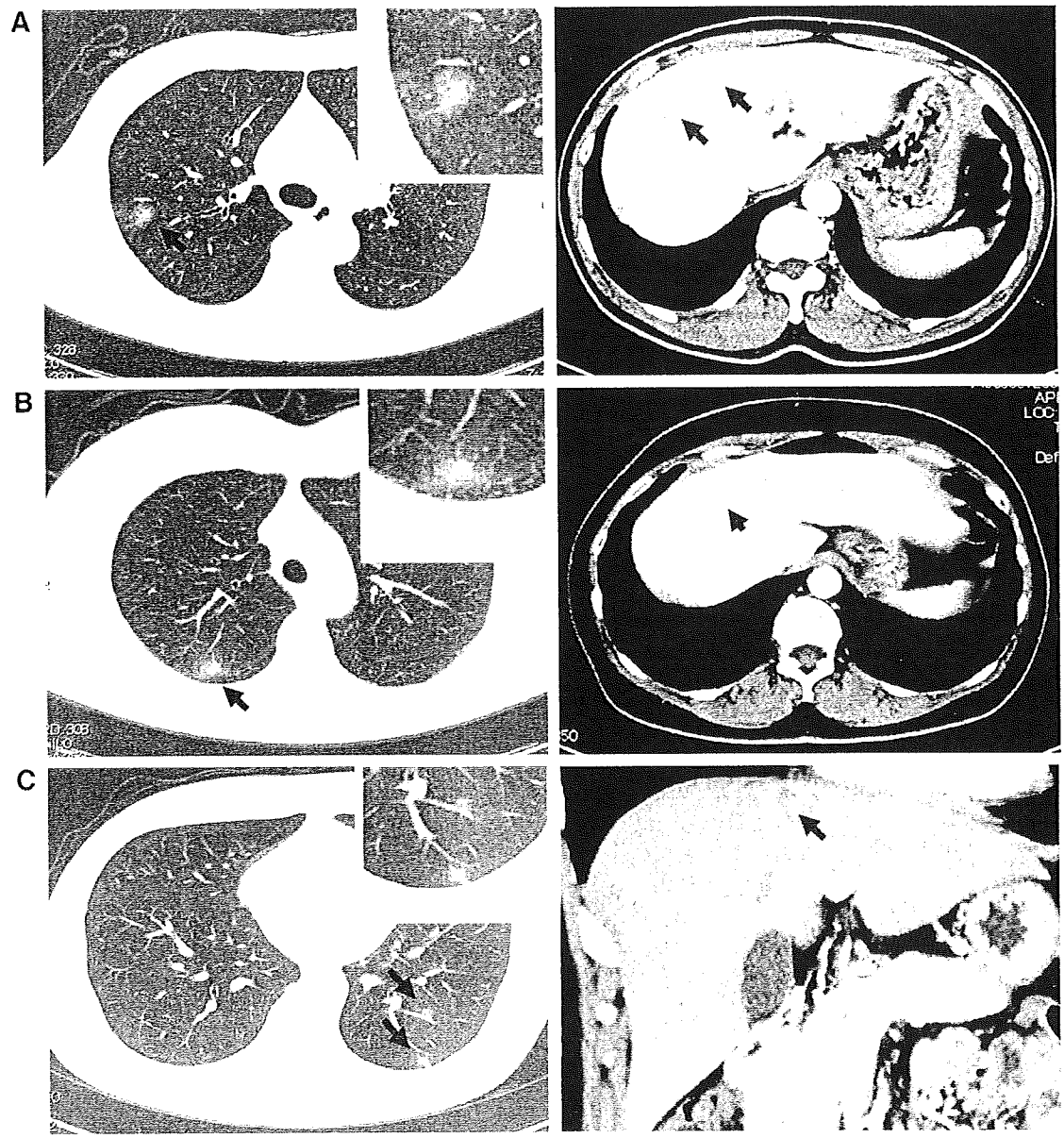

Fig. 1. CT images of pulmonary and hepatic lesions. A, B, and C show representative CT images from Patients 1, 2, and 3, respectively. Pulmonary lesions (arrows, magnified in inset) were shown as nodules with halos and a poorly defined margin or ground-glass opacity with a poorly defined margin. Hepatic lesions (arrows) appeared as small, poorly defined areas of low-attenuation in the portal venous phase of contrast-enhanced CT scanning.

We found that imaging modalities were very useful to reach a diagnosis. Characteristic CT findings of hepatic and pulmonary lesions in visceral toxocariasis reported elsewhere [7–9] are compatible to those found in our patients. Typically, the hepatic lesions are multiple, small (usually less than 2 cm in diameter), poorly defined, oval or elongated, and with low attenuation, and usually best visualized in the portal venous phase of contrast CT. Pulmonary lesions are shown as multiple small nodules (mostly less than 3 cm in diameter), with some associated with halos with poorly defined margins, and also shown as ground-glass opacity with a poorly or well-defined margin. Lesions in the liver and lung tend to be found in the periphery of those organs. In the present cases, we also performed US examinations using Sonazoid, a recently developed microbubble contrast agent, which is phagocytosed by liver-specific macrophages, known as Kupffer cells, following the vascular phase [10]. Sonazoid-contrast US showed that the liver lesions were poorly supplied with arterial and portal blood, and contained no or few Kupffer cells as compared with the surrounding liver parenchyma. These CT and US image findings are compatible to inflammatory granuloma. Although we did not perform a puncture biopsy of the hepatic lesions for histological examinations, eosinophilic granuloma would be expected.

A serological examination is also important for an accurate diagnosis, as it is difficult to obtain worms from patients in most cases. In the present cases, we performed 3 kinds of serological tests, rapid screening ELISA, quantitative ELISA, and an immunodiffusion test, using the LES product of *T. canis*, which is known to be highly immunogenic. Sera from the 3 patients were positive in all of those tests. However, in immunodiffusion tests with LES products of *T. canis*

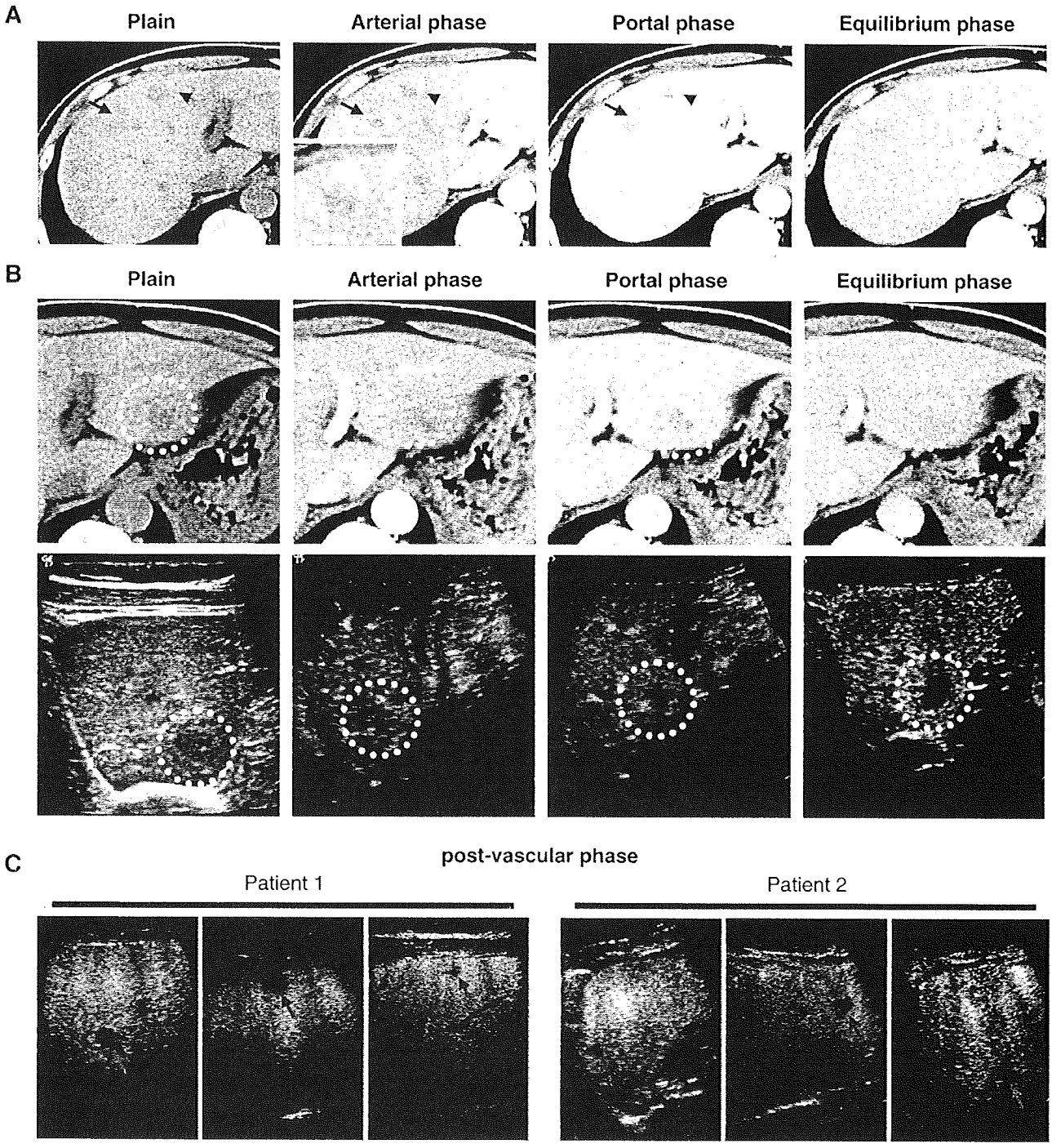

Fig. 2. CT and US images of hepatic lesions. A. Two nodules (arrow and arrowhead) in the liver of Patient 1 are shown. That shown by the arrowhead (magnified in inset) had weak peripheral rim enhancement in the arterial phase, while both were undetectable in the equilibrium phase. B. A lesion (circle with broken line) in the left lobe of Patient 1 was targeted with Sonazoid-contrast US (lower) and the results were compared with contrast CT images (upper). With CT, the lesion was best seen in the portal phase and became undetectable in the equilibrium phase, while it was clearly shown as an un-enhanced area in the equilibrium phase. C. Post-vascular Sonazoid-contrast US images revealed that the lesions (arrows) remained hypoechoic. Three lesions each from Patients 1 and 2 are shown.

and *T. cati*, sera from the patients were reactive to both of the LES products, because of their high cross-immunogeneicity. Finally, we made a diagnosis of toxocariasis by *Toxocara species*.

Covert toxocariasis with eosinophilia alone is often treated conservatively after instruction regarding prevention of re-infection. Stopping the habit of ingesting raw liver alone might have been adequate for the present cases of asymptomatic toxocariasis. However, the existence of living larvae in the lungs and liver for a prolonged period is a potential risk for their migration to other organs, including the spinal cord and brain, leading to serious complications. We decided to prescribe albendazole, which is commonly used for toxocariasis and known to be effective with minimal adverse reactions. A dose of

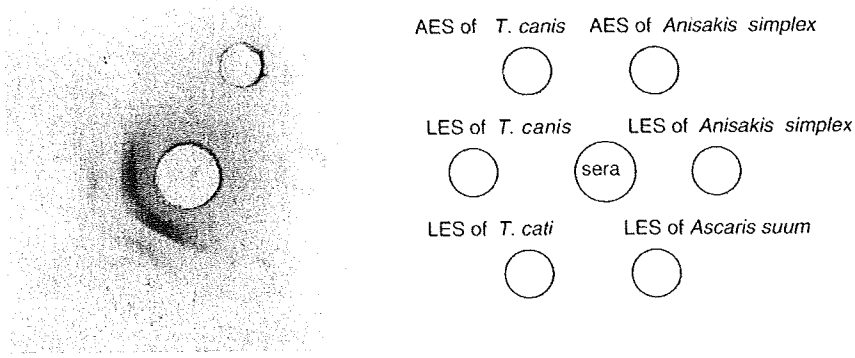

Fig. 3. Results of serological tests. Data from Patient 1 shown with schematic positions of antigens and sera are presented as representative findings. The antigens used were adult worm extract (AES) of *T. canis*, AES of *Anisakis simplex*, larval excretory–secretory (LES) of *Anisakis simplex*, LES of *Ascaris suum*, LES of *T. cati*, and LES of *T. canis*. Strong precipitin bands were observed for the LES products of *T. canis T. cati* in serum samples from all 3 patients.

400 mg of albendazole twice a day or 10 mg/kg of body weight/day in two divided doses for 5 days seems to be the currently recommended therapy [4,31,32], though the optimal duration of therapy is unknown [33]. According to a previous report [31], only 32% of patients with toxocariasis were clinically cured with a 5-day regimen and other reports have noted that additional treatments with other anthelminthic drugs, such as diethylcarbamazine and mebendazole, or the use of albendazole for a longer period was effective [18,34–39]. We adopted a 4-week regimen of daily albendazole at 600 mg, with the disappearance of eosinophilia considered to mark the endpoint of therapy. Clinical improvement appeared soon after the initiation of treatment, demonstrated by a decrease in eosinophil count, with minimum adverse effects related to mild liver dysfunction, followed by the disappearance of hepatic and pulmonary lesions.

Based on our results, we concluded that infestation with *Toxocara species* from paratenic animals is likely a common and important mode of transmission to humans, especially adults, in areas such as eastern Asia where the consumption of raw liver remains a cultural habit.

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

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***Chlamydophila 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 *Chlamydophila pneumoniae* or *Chlamydia trachomatis* genomes. The recombinant CF0218 was not recognized by antiserum against *C. 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 and sexually transmitted disease 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

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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 VR120; isolated in the United States from a cat with pneumonia) (2, 12), Fe/B166 (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 30 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% CO_2 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% CO_2 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 Mn^{2+} 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 *Escherichia coli* XL1-Blue MRF′ 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-1-thio-β-D-galactopyranoside (IPTG) and incubated at 37°C for an additional 4 h. The resulting membranes were reacted with *C. felis*-hyperimmunized feline serum (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% H_2O_2. Several phage clones, which were confirmed as positive by secondary and tertiary screening, were converted to the pBluescript SK(−) phagemid in *E. coli* SOLR according to the manufacturer's instructions (Stratagene). The DNA from the insert in 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 hydropathy 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′-CGGGATCCATGACAACAAACTCATTG-3′ and 5′-GCGTCGACTTAATTAGCGTCATCATT-3′) from *C. felis* FP1 Baker, Fe/B166, and Fe/C-38. Hydropathy 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 region encompassing cf0218 was amplified by PCR with primers (5′-CGGGATCCCGGACAACAAACTCATTGAAC-3′ and 5′-GGAATTCCCATTAGTATGCTCTTCGCTGCCC-3′; the sites of restriction endonucleases are underlined) and was cloned into the glutathione *S*-transferase (GST) fusion protein expression vector pGEX-6P-1 (GE Healthcare). After we checked the correct nucleotide sequence and frame, the resulting plasmid was named pGST-CF0218. GST or GST fused with CF0218 was expressed and purified according to the manufacturer's instructions. Briefly, logarithmic-phase *E. coli* BL21 (GE Healthcare) harboring pGST or pGST-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 glutathione Sepharose 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 antiserum 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 antiserum for 2 h at room temperature. The binding antibody specific for CF0218 was eluted from the membrane 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.

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 recom-

binant CF0218 product was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon-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. Fukushi 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 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 I (Invitrogen), according to the manufacturer's instructions. For reverse transcription-PCR (RT-PCR), cDNA was synthesized from 1.0 μg total RNA by using random primer 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′-CGGGA TCCATGACAACAAACTCATTG-3′ and 5′-GCGTCGACTTAATTAGCGTC ATCATT-3′) were used to detect cf0218-specific message, whereas previously described primers were used to examine levels of *Chlamydophila ompA* (CMGP-2F, 5′-GCCTTAAACATCTGGGATCG-3′, and CMGP-2R, 5′-GCACAACCA CATTCCCATAAAG-3′) (8). *C. felis* genomic DNA was used as a positive control, and total RNA samples without reverse transcription were used as controls for DNA contamination.

Indirect IF microscopy. HeLa cells grown on coverslips were used for the IF study. Seventy-two hours after infection by *C. felis* Fe/C-56, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and subsequently fixed again with cold methanol for 1 min on ice. The coverslips were blocked in 2% (wt/vol) bovine serum albumin in PBS and stained with rabbit anti-CF0218 diluted at 1:50 in PBS, anti-rabbit IgG-fluorescein isothiocyanate (ICN Pharmaceuticals) diluted at 1:200 in PBS, anti-chlamydial LPS monoclonal antibody (13), anti-mouse IgG-Alexa 594 (Invitrogen) diluted at 1:200 in PBS, and 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) (Dojindo Laboratory, Kumamoto, Japan) diluted at 1:5,000 in PBS. The coverslips were mounted in SlowFade antifade reagent (Invitrogen), and examined with an IF microscope (BZ-8000; Keyence, Osaka, Japan) for deconvoluted fluorescence imaging.

Experimental vaccination and infection of cats. Six specific-pathogen-free female cats were used. They were 5 months old and weighed 1.6 kg to 1.8 kg at the start of the experiments. They were divided into two groups. On day 0 (0 weeks) and at 3 weeks, cats in one group were vaccinated twice with the five-antigen-containing vaccine for cats, Fel-O-Vax 5 (Kyoritsu Seiyaku Corp., Tokyo, Japan), which contains inactivated *C. felis* Cello strain EB, via the intramuscular route according to the manufacturer's instructions. Cats in another group were inoculated with saline via the intramuscular route, at 0 weeks and 3 weeks. At 5 weeks (2 weeks after the second vaccination), cats in both groups were inoculated with 10^4 50% embryo infectious doses of live *C. felis* Cello strain via the mucosal (conjunctival, oral, and nasal) route. Sera of all cats were collected from 0 to 8 weeks.

ELISA. The antibody response against *C. felis* EB and CF0218 of infected cats was measured by ELISA. For ELISA, *C. felis* EB (1 mg/ml) was inactivated in 1% Triton X-100 and 5% 2-mercaptoethanol at 37°C for 30 min as described previously (28). To assess levels of antibody against CF0218 in feline sera, purified GST and GST-CF0218 were used as antigens. Each antigen (EB, GST, and GST-CF0218) was diluted at 1 μg/ml with 0.05 M carbonate-bicarbonate buffer (pH 9.6, 15 mM Na_2CO_3, 35 mM $NaHCO_3$) and subsequently applied to the 96-well plates (100 ng/well) (F96 Maxisorp; Nunc, Roskilde, Denmark) at 4°C overnight to coat wells. After blocking of each well with 5% (wt/vol) skim milk in PBS containing 0.05% Tween 20 (PBST), the sera diluted at 1:100 with PBS were added and incubated for 1 h at room temperature. After washing with PBST, the anti-cat light chain-HRP diluted at 1:2,000 with PBS was added as the secondary antibody. The plates were incubated for 1 h at room temperature and washed twice with PBST and then twice with distilled water. Tablets of 2,2′-azino-bis(3-ethlbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, St. Louis, MO) dissolved in 0.05 M citrate buffer (pH 4.0) containing 0.0075% H_2O_2 were added to each well according to the manufacturer's instructions. The plates were incubated at 37°C for 30 min, and optical density (OD) at 405 nm was measured using a Microplate Reader Model 550 (Bio-Rad, Hercules, CA). Net OD values for CF0218 were calculated by subtracting the OD values of the GST wells from those of the GST-CF0218 wells as described elsewhere (19). Each sample was measured in duplicate.

Statistical analysis. Overall antibody responses in cats were analyzed by using repeated-measures analysis of variance, and subsequently differences between control serum (at 0 weeks) and serum at each time point were analyzed by using a Bonferroni correction. For all statistical analyses, P values of <0.01 were considered statistically significant.

Nucleotide sequence accession numbers. The sequences of each cf0218 were deposited in the DDBJ/GenBank/EMBL database under accession numbers AB444855 (FP1 Baker), AB444856 (Fe/B166), and AB444857 (Fe/C-38).

RESULTS

Identification of the cf0218 gene. Seven positive clones were obtained by immunoscreening of the *C. felis* genomic expression library (1.5×10^5 PFU) with the cat serum raised against *C. felis*. Phagemids 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* genomes 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 CF0128 from Fe/B166 (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,

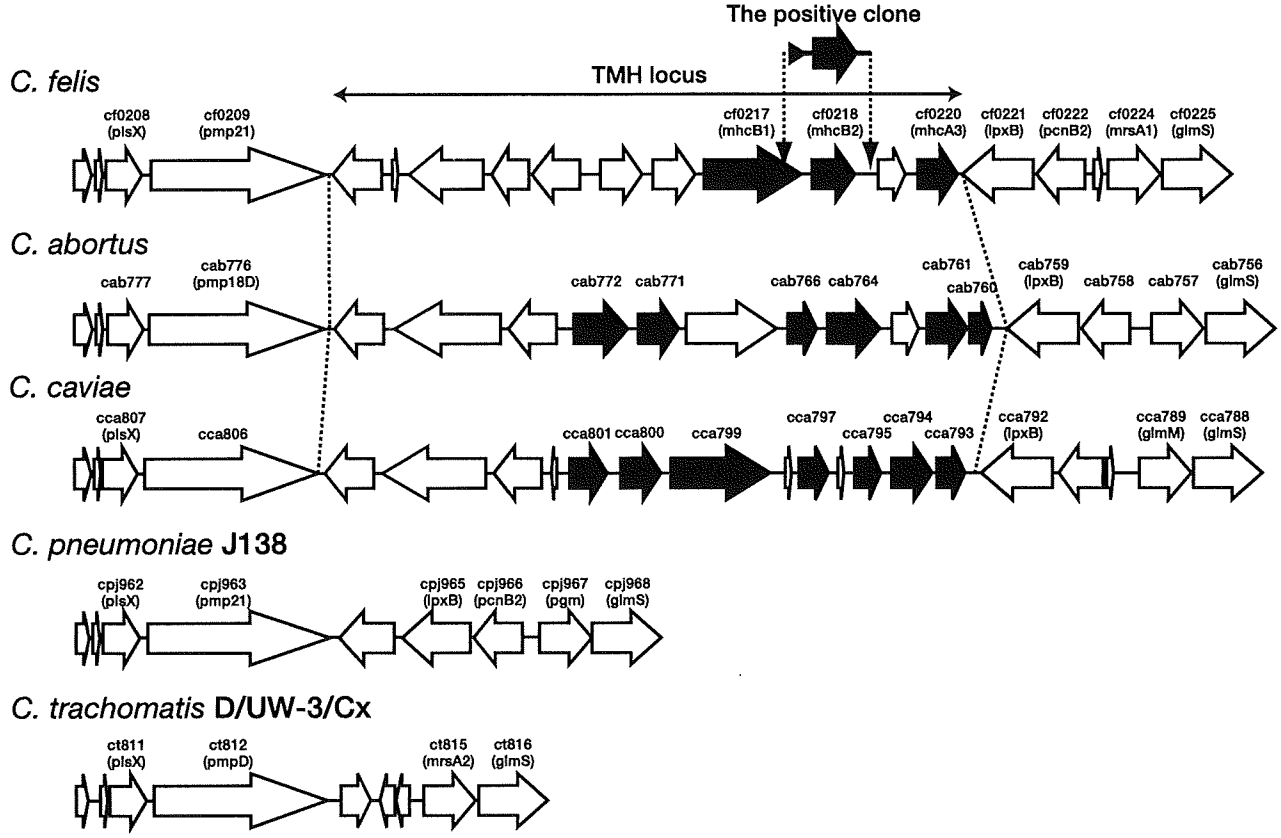

FIG. 1. Gene arrangement of the TMH loci in the *C. felis* (Fe/C-56), *C. abortus* (S26/3), and *C. caviae* (GPIC) genomes and the analogous regions in the *C. pneumoniae* (J138) and *C. trachomatis* (D/UW-3/Cx) genomes. By screening of the *C. felis* genome library with anti-*C. felis* serum, the positive clone obtained was placed at the TMH locus (between the *lpxB* and *pmp* genes; an arrow at both ends) in the *C. felis* genome. The TMH loci are present in the *C. felis*, *C. abortus*, and *C. caviae* genomes but not present in the *C. pneumoniae* and *C. trachomatis* genomes. Representative locus tags (and gene names) are shown on each ORF (arrows). Black arrows show putative TMH family proteins.

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.

A

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CF0218          1 MTTNSLNIGSSSATVVNSVVQKQPLWKVSLNKYQDVAAAILIAGLVLIGSLVGALVFFA   60
CAB764          1 ---MTTSPVNTNPIATHVSTTQHTLFSTSVSKYQRIATNIALLAGMVLVGTLVGALVFFA   57
CAB766          1 --------MNTTPIATHVPTTQHALSNISSNKYQRLATVIALLAGMVLVGTLIGALVFFA   52
CCA797          1 MTTNPVNTTTTITPPITQKQPLCSVQESKYRRIAAATTLLEGLVLIAPLVGALVFFALPT   60

CF0218         61 LTGPSVLFAAMLMALVSGVVLIAMAGYQLAVGVRKDAKERQLVHEKIQYEVEAALLKDNN  120
CAB764         58 LPTSVTLVALVSVALLASVILLSMAMYNLVSQSRRVSSDPQLGEENTRLEAEMIGLREQL  117
CAB766         53 LPASLTLVALVSTSLLASVILLSMSVYNLVSQFRRASSYAQIGKENIRLEAEMIALQEKL  112
CCA797         61 ATTITVLTVLIGIALGASVILLSMAMYKLLRCRQVPSDPKGIEDSVTLKEEIIEIKAQLT  120

CF0218        121 KLIERKLEELQKHSDALTGQLLGLAGDLECLKDEKIDLEARVMDLQRQNASLSKLMEEGR  180
CAB764        118 IRSEAQLAEEQGRCSELSVKLMSVIGDLAQANQEKTSLESKVQALKELVASYPAIAEEAQ  177
CAB766        113 TRSEVQLQEEQGRSSELSVKLMSTEIDLDQANQEKASLESKVKALKELVEKYPAIAEESQ  172
CCA797        121 QKAIQLTEEEERSASLLGQLLAITGDLEATQHQKSALEAEVKLLQEQYTSTLKLAEKDHS  180

CF0218        181 PDLVAEIENQRKQCKNLQELLLQAEEKNKSLESTVKKCQTELMIVEQQNTLLIERNKEL-  239
CAB764        178 KMQAELKKRIADVTSAKQDV-GRLEEQVQDLETQLIQSLKQVDTLSKEKEELELCLKQLQ  236
CAB766        173 LIQS-LDQ-VAVLVKQKEELKSALEKIKEEGAGKLVTVQGEQIDLADLDRQLVEAYVQL-  229
CCA797        181 ELTEVQQELDEKTARLEDFVKTTVLLEKQIAEKEQEIGQLNARVAELEAAAVVKAPESAE  240

CF0218        240 ---KVQKAALEKMLEDSKQEIKILKEVLADFEA--MNIPGNIAELQAKIEEQKLELESLK  294
CAB764        237 DEGVSAVVKLQGKVVDLVEEIEVKEKNLEFYDGRIKNLINQNSDLVATVRCLESKLDKDQ  296
CAB766        230 EACTKRFSDLQKQLLEVSEAIQSRDSSS----------------------------------  257
CCA797        241 ASETVEATEEVEASEEGDSVHAGNLDSESGPDLD                             274

CF0218        295 DQAEKLLLEKDAVMKESQDLKDLLNSSKLEISKNKLDISKLQSELESLQSQPHGDDGADV  354
CAB764        297 RSIEDLQDRVSLLAYMRDELTEKMKNLHCDLDTKVAAMLRLENRVQELEEEKKKVEEDFL  356
CAB766        257 ----------------------------------------------------------  257
CCA797        274                                                             274

CF0218        355 DVQEGAEGDAGDNDVAGSEEHTNDDAN-------------------------------  381
CAB764        357 KAQNQIKALEASVSHKVNVTMQETARLLCQVAELNEQLDESKKEIKVLQSEKSSLISGMD  416
CAB766        257 ----------------------------------------------------------  257
CCA797        274                                                             274

CF0218        381 ----------------------------------------------------------  381
CAB764        417 KLQAQLDELQEQLVASASSVDDSAPSEVDPDGSEVSAADDHVGGSGNSNDLED-------  469
CAB766        257 ----------------------------------------------------------  257
CCA797        274                                                             274

CF0218        381 ---------------------------------------------  381
CAB764        469 ---------------------------------------------  469
CAB766        257 ---------------------------------------------  257
CCA797        274                                                274
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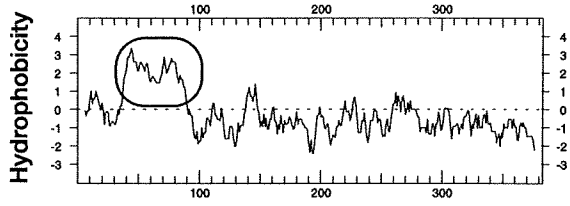
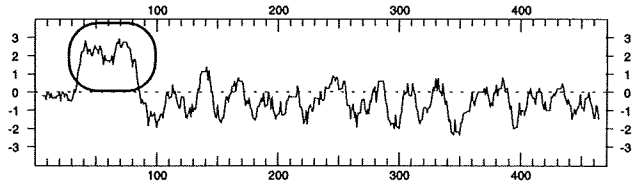
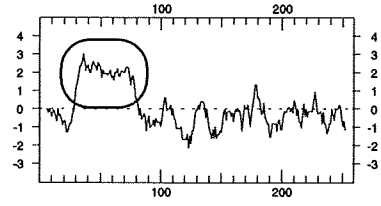
B CF0218



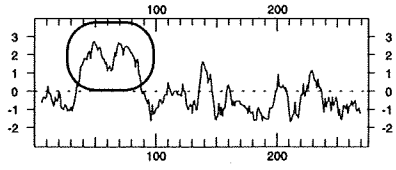
CAB764



CAB766



CCA797



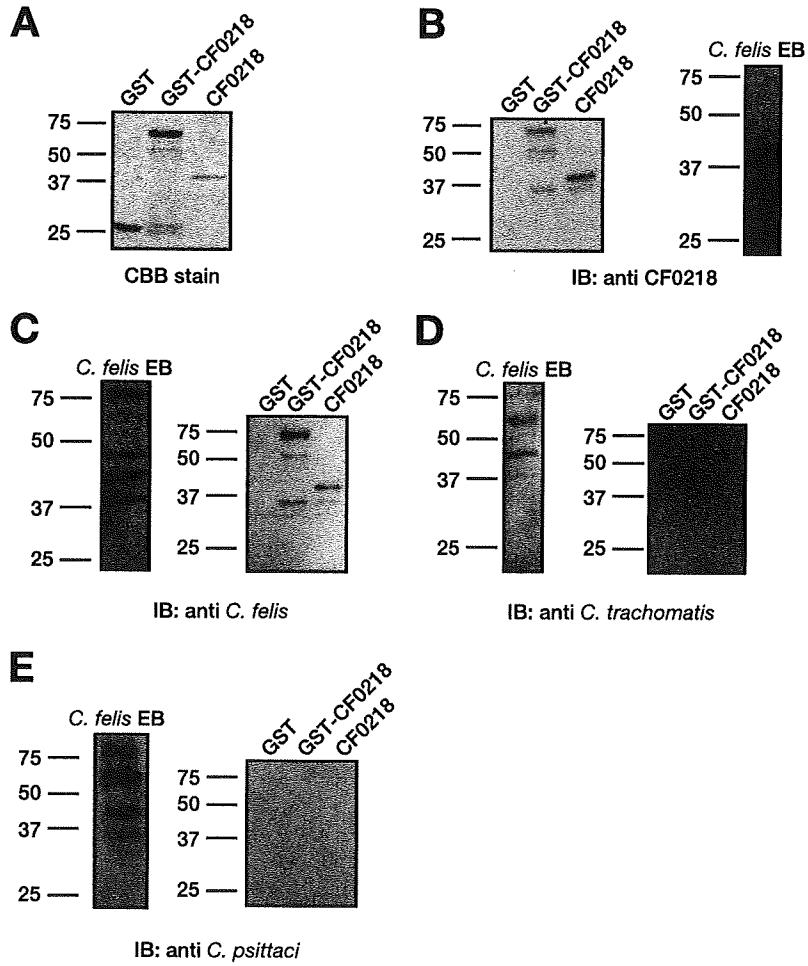


FIG. 3. Production of recombinant CF0218 and its immunogenicity. (A) Purified GST alone, GST-CF0218, and GST-cleaved CF0218 were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB). (B to E) Equal amounts of recombinant CF0218 shown in panel A and purified *C. felis* EB were separated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

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

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

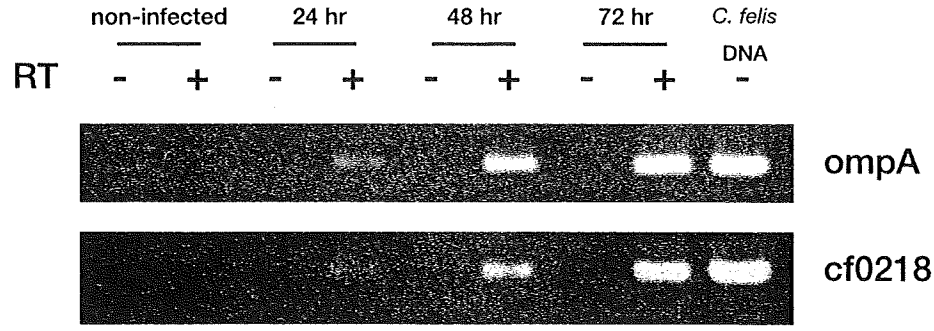


FIG. 4. RT-PCR analysis of cf0218 expression in infected cells. Specific messages for *ompA* and cf0218 were detected from total RNA of HeLa cells infected with *C. felis* at the times indicated (including noninfected cells as negative control). DNase I-treated total RNA was applied to reactions in the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

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

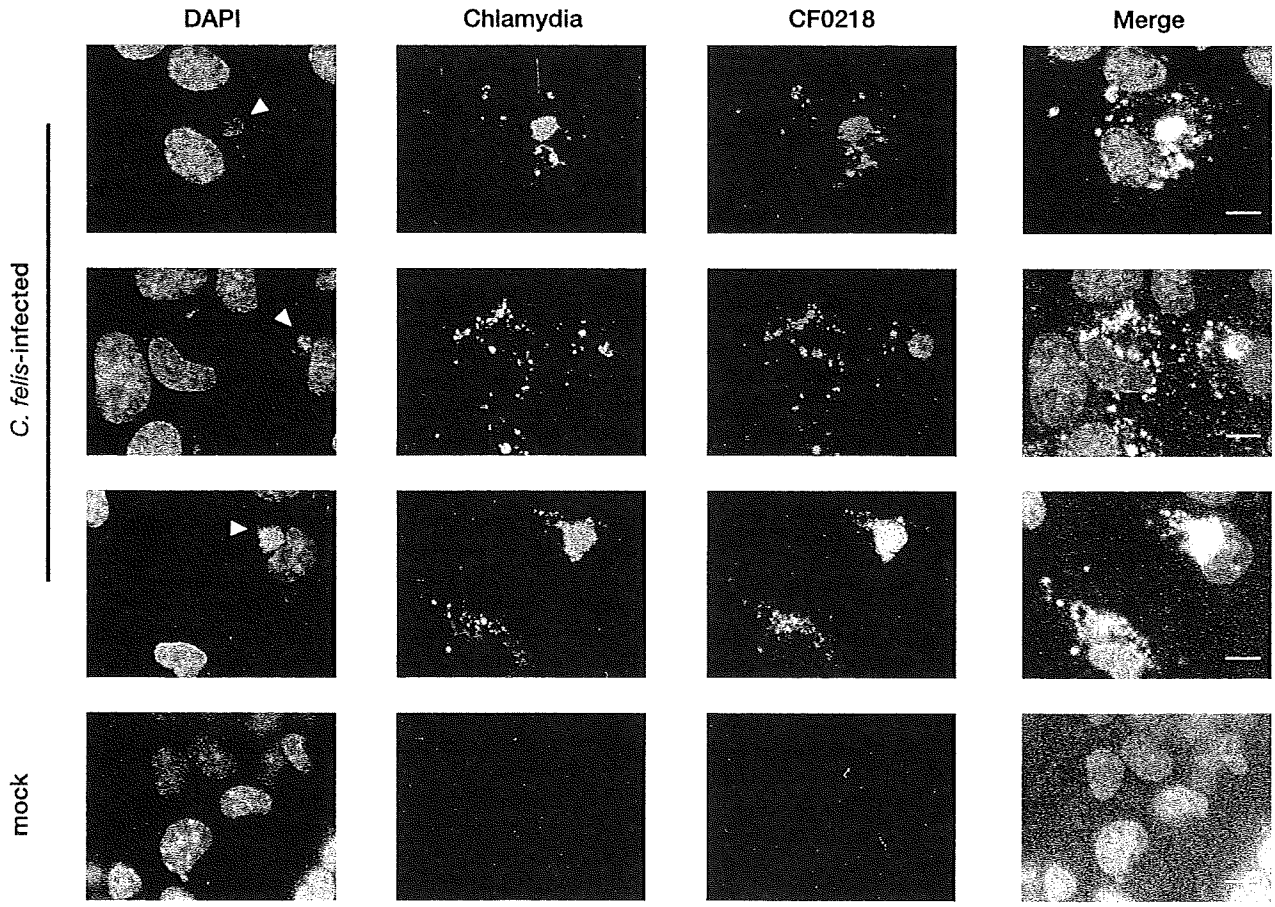


FIG. 5. Localization of CF0218 in *C. felis*-infected cells. HeLa cells infected with *C. felis* for 72 h were fixed and stained with DAPI for host cell nuclei and apparent chlamydial inclusion bodies (blue; white arrowheads show large chlamydial inclusion bodies), anti-chlamydial LPS (red), and anti-CF0218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

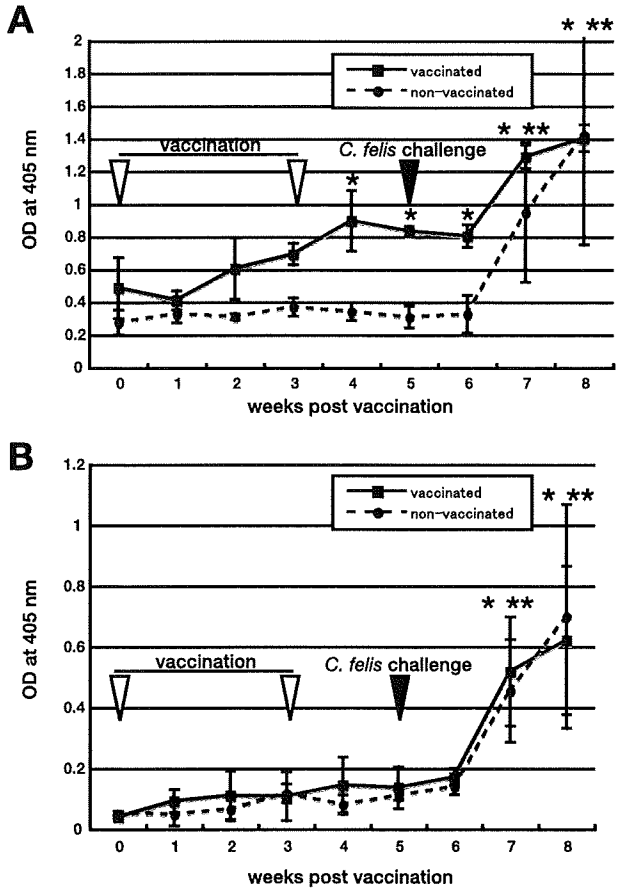


FIG. 6. Antibody response against EB and CF0218 in experimentally vaccinated and *C. felis*-infected cats. Female specific-pathogen-free cats were inoculated with the vaccine containing formalin-inactivated *C. felis* EB (closed squares, solid line) or with saline (closed circles, dashed line) via the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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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