而シテ藁草、土石、木竹片、硝子ノ破片、襤褸、紙屑ノ如キ異物ヲ嚥下シ、甚シキハ自己ノ糞尿ヲ啖フモノ尠カラズ。又自己他犬ノ陰部ヲ嗅ギ若シクハ之ヲ舐ム。又嘔吐及秘結スルモノアリ。三四日ノ後狂躁、痙攣ノ發作一張一弛シ、不穩ノ狀倍〻加リ、漫リニ奔逸ヲ企テ欄ヲ毀チ鏈ヲ斷チ窓戸ヲ破ラント欲ス。戸外ニ在ルモノハ目的無クシテ處々ヲ徘徊シ、一日間ニ非常ナル遠距離ヲ往復シ、其ノ間沿道ノ人畜、物品ヲ問ハズ、苟モ遭遇スルモノ殆ド咬着セザルハ無ク、甚シキハ鐵杆烈火ヲ咬ミ自ラ齒牙ヲ折リ、口内出血スルニ至ルモ更ニ意ニ介セザルモノアリ。而シテ音聲一變粗厲ノ嗄聲ヲ放チテ哮吠シ又屢〻發作的ニ咆哮シ、爲ニ漸次音聲鈍弱トナリ終ニ復吠ユルコト能ハザルニ至ルモノ甚ダ多シ。又往々痴鈍ノ狀ヲ爲シ凝眸虛視、漫リニ空中ニ向ヒ捕蠅ノ狀ヲ爲スモノアリ。

以上ノ如キ狀態ヲ呈シツヽ漸次羸瘦シ、相貌一變、絨毛粗剛、眼球陷沒、眼光獰惡ヲ呈シ、咽頭ハ麻痺シテ嚥下スルコト能ハズ。下顎モ亦麻痺ノ爲口ヲ哆開シ舌ヲ挺出シ涎ヲ流下シ、後體麻痺ヲ發シ遂ニ衰弱シテ斃ル。稀ニハ鬱狂性ニ來ルモノアリ。卽チ下顎麻痺ノ爲、口ヲ哆開シ、舌ヲ挺出シ人畜物體ニ觸ルヽモ更ニ之ヲ咬噛スルノ意思ナク、流涎、痴鈍、凝眸、虛視、後體麻痺、步行踉蹌、又吠鳴セズ。食慾絶止漸次衰弱シテ概ネ二三日ノ後斃死ス。

第三項　剖　檢

凡ソ人ヲ咬ミタル犬及狂犬病ニ疑ハシキ犬ハ、成ルベク生キナガラ之ヲ捕獲セシメ繫留シテ視察ノ用ニ供スルモ、狂暴ニシテ捕獲シ難キモノ、又ハ附近ノ人民ニ於テ危險ナリトシ撲殺シテ屆ケ出デタル如キ場合ニハ、常ニ剖檢ヲ行ヒ且動物試驗ヲ施スヲ以テ例トス。

病體解剖ニ於テ認ムル所ノ病的變狀モ亦常ニ特異的ナルコト能ハズ。殊ニ初期ニ於テ撲殺若シクハ斃死シタルモノニ於

方法ヲ試ミタルニ、約四十分ニシテ强直痙攣ヲ發シ横倒遂ニ斃死スルニ至レリ。

（例ノ二）

大正十三年八月五日、埼玉縣入間郡方面ヨリ青梅署管内箱根崎村ニ野犬一頭現ハレ人二名馬二頭ヲ咬傷シ、更ニ福生村熊川村方面ニ逸走人二名馬二頭畜犬三頭ヲ咬傷シ、尚狂奔シテ北多摩郡宮澤村三三番地先道路徘徊中撲殺セラレタル事實アリシガ、其ノ被害馬四中箱根崎村五三七荒井吉五郎所有馬一頭ハ、被害翌日豫防注射ヲ實施シタルニ不拘同月二十八日發病シタル旨青梅署ヨリ衞生部宛報告アリ。檢診スルニ馬房兩側柱ニ繋絆ヲ以テ結付ケラレ、眼光爛々旺ンニ發汗膚ヲ濕ホシ、口角泡沫ヲ吐キ興奮シテ振頭前肢ヲ以テ擂板ヲ叩キ家人ト雖近ケズ。試ミニ繋絆ヲ解キ歩行ヲ强ユルニ後肢ノ運歩蹌踉、後軀ノ麻痺症狀極メテ顯著ナリ。再ビ馬房ニ收メ「ストリキニーネ」ノ脉管内注射ヲ試ミシモ騒狂危險云フベカラズ。依ツテ前胸部ニ一筒（ストリキニーネ約一・五grヲ溫湯ニテ溶解シ五cc入ノ注射器ヲ使用ス）ヲ皮下注射ヲナシタルニ、約三分間ニシテ强直ヲ發シ斃死ス。屍體ハ福生村火葬場ニ於テ燒却ス。

第三項　猫ノ狂犬病

潜伏期ハ平均二乃至四週間トス。發病當初猫ハ靜寂且ツ暗所ニ逃避シ、斃死ニ至ル迄出デ來ラザルコトアリ。刺戟スレバ盛ンニ叫鳴シ攻撃的態度ヲ現ハシ、又突如トシテ猛烈ナル狂暴性ヲ發揮シテ周圍ノ人畜ニ突進襲撃ヲ試ミ、彼ノ鋭尖ナル齒牙ト爪ヲ以テ深キ搔爬傷ヲ負ハシムルコトアリ。彼等ハ人畜ノ顔面ヲ目掛ケテ跳掛リ、或ハ狡猾ニモ後方ヲ襲フコトアルヲ以テ危險ナリ。猫ハ犬ニ次グ狂犬病毒傳播者ニシテ、パストール研究所ノ統計ニ據レば被咬傷者一二、〇〇〇人ノ内一一、〇〇〇人ハ犬ニ、七〇〇人(五・八%)以上ハ猫ニ依ル被害者ナリト。又維納狂犬病研究所ノ取扱ヒタル被害者ノ

內四%ハ猫ニ據ルモノナリト。我東京府下ニ於ケル實例ハ極メテ少數ニシテ

大正二年	狂犬病發生總頭數	三六〇	內猫三	（〇・八%）
同六年	同	四三一	同二	（〇・五%）
同十三年	同	七二六	同二	（〇・三%）

ニ過ギズ。隨ツテ猫ニ據ル被害者ハ總體ノ一%以下ナルベキハ推定ニ難カラズ。斯ク被害者ノ少數ナル所以ハ恐ラク、我國ト歐米各國ニ於ケル犬猫ノ飼養管理方法並ニ人畜ノ接觸程度ノ差異ヨリ來ルモノナランカ。

狂犬病ニ罹レル猫ノ症狀大約左ノ如シ。

病猫ハ憂鬱、不安、騷擾、時ニ假睡ヲ裝ヒ、突如トシテ瞬眼ヲ開キ脾睨シ脅迫ノ氣ヲ示ス。食慾不振或ハ全ク之ヲ缺如シ、或ハ之ニ反シテ異嗜流涎アリ。液體ノ嚥下困難、頻リニ鳴泣シ、爲メニ弱ク嗄レタル音聲ヲ發スルニ至ル。檻中ニ在リテハ憔悴逃走ヲ企テ彷徨ヲ試ム。其ノ際彼等ハ溫順ノ態度忽チニシテ一變シ、奮然攻擊的態度ニ出デ、又犬ニ對スル先天性恐怖心モ消失シ之ニ向ツテ襲擊ヲ敢テスルコトアリ。檻中ニ突入ルル棒片ニ跳掛リ咬付キ唾液ヲ流出ス。發病末期ニ於テハ嚥下不能、麻痺、後肢麻痺ノ徵候顯著ナリ。就中顯著ナル症候トシテハ恐水現象ニシテ水ヲ灌注スレバ、激烈ナル痙攣發作アリ。發病後三乃至六日ニシテ死ノ轉歸ヲ取ル。

鬱狂ハ猫ニ在リテハ稀ニシテ、主要ナル徵候ハ下顎ノ麻痺、咽喉痙攣及ビ早期ニ於ケル一般的麻痺ナリ。發病後二乃至四日ニシテ斃ル。

第四章　野獸ノ狂犬病

研究成果の刊行に関する一覧表

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原　著

0，1，6カ月皮内接種方式による狂犬病抗体価の検討

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目　　的

2006年11月に国内で相次いで輸入狂犬病患者が発生したのち[1,2)]，狂犬病ワクチンの需要が急増した．しかし，需要の増加が供給を上回っていたため，緊急避難的に狂犬病曝露前免疫が制限された．世界保健機関(WHO)は，狂犬病流行地において動物による咬傷を受けた場合，狂犬病免疫グロブリン(RIG)の投与と組織培養不活化ワクチン接種による曝露後発病予防を勧告しているが，曝露前免疫を受けていればRIGの投与が不要になる[3)]．RIGは入手が容易でないため，曝露後発病予防を確実なものにするためには，狂犬病曝露前免疫を普及させることが重要である．わが国における狂犬病ワクチンの生産量は少なく，急な増産ができないことに鑑みれば，ワクチンが不足する事態に備えて，少量のワクチンでも高い効果を上げることができる接種法の検討が必要である．

狂犬病常在地であるタイでは，1人当たりの狂犬病ワクチン接種量を減量した皮内接種法(タイ赤十字方式)が広く用いられ，曝露前免疫にも採用されている[4)]．われわれは，国産の狂犬病ワクチンを4週間隔で2回皮内接種する方式で，発症防御レベルまで狂犬病抗体価の上昇が認められたことを報告した[5)]．今回，日本の標準的な狂犬病ワクチンの接種時期である0，1，6カ月目にワクチンを皮内接種し，狂犬病曝露前免疫の効果と安全性を調査した．

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対象と方法

1．対　　象

本調査の目的，調査項目，接種ワクチンと予測される副反応について文書，および口頭で説明をして，同意が得られた医療関係者9例(男性7例，女性2例；平均年齢30.1±4.3歳)を対象とした．

2．接種ワクチン

化学及血清療法研究所製の組織培養不活化狂犬病ワクチンロットRB02およびRB03を，第1回目および第2回目接種時に用いた．第3回目接種時には，ロットRB04およびRB05を用いた．狂犬病ワクチンは溶解液1 mLに溶解した後，その0.1 mLずつを左右前腕に皮内注射した．

3．局所および全身反応

全例について，皮内接種15分後の接種局所における膨疹，発赤を視診で確認し，瘙痒感の有無を質問した．さらに，次回接種時および採血時に前回注射による局所の腫脹，発赤，疼痛，瘙痒感の自覚症状の有無について問診した．

4．抗体検査

狂犬病ワクチンは0，1，6カ月目に接種し，採血は2回目接種2～3週間後と3カ月後，また3回目接種直前および接種2～3週間後の計4回行った．酵素抗体法(ELISA)による抗体価はPLATELIA™ RABIES KIT Ⅱ(Bio-Rad Laboratories, France)を，中和抗体価は迅速蛍光フォーカス抑制試験法(RFFIT)を用いて測定した．

表1　ELISA法を用いた抗体価の推移

症　例	2回目接種 2～3週間後 (EU/mL)	2回目接種 3カ月後 (EU/mL)	3回目接種 直　前 (EU/mL)	3回目接種 2～3週間後 (EU/mL)
1	1.1	0.5	＜0.5	1.9
2	5.5	1.5	0.5	2.1
3	1.6	0.7	＜0.5	3.1
4	1.0	＜0.5	＜0.5	2.0
5	3.7	0.5	＜0.5	0.9
6	3.6	0.8	＜0.5	3.8
7	5.5	0.9	0.5	13.6
8	3.3	0.9	＜0.5	13.4
9	2.5	＜0.5	＜0.5	6.4
幾何平均	2.6			3.6
範　囲	1.0～5.5			0.9～13.6

表2　RFFIT法を用いた中和抗体価の推移

症　例	2回目接種 2～3週間後 (IU/mL)	2回目接種 3カ月後 (IU/mL)	3回目接種 直　前 (IU/mL)	3回目接種 2～3週間後 (IU/mL)
1	3.5	1.5	＜0.5	1.5
2	6.0	1.5	0.7	2.0
3	2.6	1.5	0.7	2.6
4	4.6	0.7	＜0.5	2.0
5	4.6	＜0.5	0.7	0.7
6	7.9	1.2	0.7	2.6
7	13.7	1.5	0.7	6.0
8	4.6	2.0	0.7	18.0
9	4.6	0.7	＜0.5	6.0
幾何平均	5.2			3.0
範　囲	2.6～13.7			0.7～18.0

結　　果

1．血中狂犬病抗体価

ELISA法を用いた抗体価の測定結果を表1に示す．ワクチンを2回接種した2～3週間後の抗体価は全例で上昇しており，幾何平均は2.6 EU/mL(1.0～5.5 EU/mL)であった．2回目接種3カ月後の抗体価は全例で低下しており，3回目接種直前には7例で0.5 EU/mL未満であった．3回目接種2～3週間後の抗体価は全例で上昇しており，幾何平均は3.6 EU/mL (0.9～13.6 EU/mL)であった．

RFFIT法を用いた中和抗体価の測定結果を表2に示す．ワクチンを2回接種した2～3週間後の抗体価は全例で上昇しており，幾何平均は5.2 IU/mL(2.6～13.7 IU/mL)であった．2回目接種3カ月後の抗体価は全例で低下しており，3回目接種直前には3例で0.5 IU/mL未満であった．3回目接種2～3週間後の抗体価の幾何平均は3.0 IU/mL(0.7～18.0 IU/mL)と上昇を認め，全例発症防御レベルの0.5 IU/mLを超えていた．症例5は3回目接種前後で抗体価の変化が認められなかった．

2．接種後の局所反応および全身症状

ワクチン接種15分後，局所の発赤を呈した者は1例，腫脹を認めた者は4例，疼痛を認めた者は0例，瘙痒感を認めた者は1例であった．局所の発赤は，数日間残ったと報告した者があったが，発熱，頭痛，倦怠感などの全身症状を報告した例はなかった．

考　　察

本邦での狂犬病曝露前免疫は，組織培養不活化ワクチン1回量1.0 mLを4週間隔で2回，その後6～12カ月後に1回皮下注射する方式が標準である[6]．しかし，ワクチン供給不足の状況において，1人に1回量1.0 mLを投与すれば，短期間にワクチンが枯渇して，実際，狂犬病ワクチン接種が不可能となるであろう．そのため，われわれは以前，接種量を減量しても効果が得られる方法として皮内接種法を考案し，4週間隔で2回皮内接種することで十分な抗体価の上昇が認められたことを報告した[5]．今回の小規模接種試験では，狂犬病ワクチン2回接種後，時間経過とともに抗体価の低下が認められた．この傾向は，国産の狂犬病ワクチンを用いて，高山らが考案した皮内・皮下併用法(狂犬病ワクチン0.1 mLを左右前腕に1カ所ずつ皮内注射し，残りを上腕1カ所に皮下注射する接種法)でも同様に認められている[7]．しかし，狂犬病抗体価が発症防御レベルを満たさなくなった場合でも，3回目接種後に十分な抗体価の上昇が認められ，追加免疫効果が確認された．また，全身症状を呈する重篤な副反応は認められなかった．

今回の検討では，狂犬病ワクチン3回目接種後，ELISA法で測定した抗体価の上昇は全例で認められた．しかし，3回目接種後，RFFIT法を用いた中和抗体価の上昇は1例(症例5)で認められなかった．また，中和抗体価の幾何平均が2回目接種後よりも3回目接種後が低値であった．狂犬病抗体価は，中和抗体価を用いるのが標準的で，WHOは0.5 IU/mL以上を発症防御レベルと定めている．本例において，中和抗体価の上昇が認められなかった原因は明らかでないが，中和抗体価と強い相関をもつELISA法[8]での抗体価は上昇していたため，実際には中和抗体価は上昇していたのではないかと推測される．加えて，2回目接種3カ月後で中和抗体価が0.5 IU/mL未満であったにもかかわらず，3回目接種直前には0.7 IU/mLと上昇していた点に鑑みると，測定時における手技的な問題も否定できない．

わが国における狂犬病ワクチンの生産量は，定期接種のワクチンに比べれば非常に少なく，急激な需要の増大が起これば，狂犬病ワクチンが不足する事態は避けられない．しかし，狂犬病はいったん発病すると有効な治療法はなく，現時点では狂犬病ワクチンの投与が唯一の発病予防策である．加えて，狂犬病ワクチンによる曝露後発病予防の効果を確実にするためには，RIGが入手困難である以上，曝露前免疫を提供することが極めて重要である．今回検討した皮内接種方式は，接種量が通常の5分の1と少ないにもかかわらず，効果が認められ，かつ副作用も軽微であった．小規模接種試験であるため，今後はより多くの被接種者を対象として，さらなる検討を行う価値がある．

追　　記

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Pre-Exposure Intradermal Rabies Vaccination Using Japanese Rabies Vaccine Following the Japanese Recommended Schedule

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In November 2006, two consecutive imported rabies cases were reported in Japan. The demand for rabies vaccine has grown rapidly, resulting in a shortage of the vaccine. In order to prepare for the vaccine shortage, it is necessary to consider a method that is effective yet uses less amount of the vaccine. We examined the efficacy and safety of the Japanese rabies vaccine when administered intradermally following the Japanese recommended schedule. The intradermal method we tested uses only 20% of the vaccine dose required under the standard method, but every subject tested had a sufficient rise in their anti-rabies antibody titer. Overall, the vaccine was well tolerated by all subjects. This was a small inoculation trial, but intradermal vaccination is an effective method, and may be used on a regular basis not only when vaccine is short.

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ORIGINAL ARTICLE

Pre-exposure immunization against rabies using Japanese rabies vaccine following the WHO recommended schedule

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Abstract We examined the efficacy and safety of the Japanese purified chick embryo cell rabies vaccine (PCEC-K) when administered on days 0, 7, and 28, as recommended by the WHO. Post-vaccination serum samples were obtained from 53 human subjects, and rabies antibody titers were determined by a combination of enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody (NA) assay. By day 42 of the experiment, which was 2 weeks after the third dose, all subjects had developed NA titers of 0.5 IU/ml or higher. The geometric mean titers of ELISA antibody and NA were 3.8 EU/ml and 5.7 IU/ml, respectively. Overall, the vaccine was well tolerated by all subjects. These results suggest that PCEC-K used for pre-exposure immunization according to the WHO schedule is as immunogenic and effective as the current pre-exposure immunization regimen in Japan, which consists of vaccines administered on days 0, 28, and 180. An accelerated schedule would be of great advantage to Japanese travelers, who could complete the required three doses for primary immunization in 1 month.

Keywords Rabies vaccine · Pre-exposure immunization · Post-exposure prophylaxis

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Introduction

Infection with the rabies virus, a Lyssavirus, causes acute, progressive, and fatal viral encephalitis. Mortality is almost 100% once clinical symptoms of rabies appear, because we lack effective therapy [1]. Rabies is a serious cause of human mortality in many developing countries. Post-exposure prophylaxis (PEP) using the rabies vaccine is the only means of preventing clinical rabies. In cases of severe exposure, WHO recommends the administration of rabies immunoglobulin (RIG) in addition to vaccination. However, the supply of RIG is scarce worldwide. Moreover, RIG is not marketed in Japan, which makes it impossible to follow the WHO recommendation regarding PEP for severe cases [2].

The problems associated with the unavailability of RIG could be mitigated by routine pre-exposure immunization (PEI). With regard to PEI, the WHO recommends rabies vaccines to be administered intramuscularly on days 0, 7, and 21 or 28 [2]. In contrast, the approved PEI regimen in Japan is performed by administering Japanese purified chick embryo cell rabies vaccine (PCEC-K) subcutaneously on days 0, 28, and 180. Therefore, completing PEI by administering the recommended three doses is inconvenient because of the long intervals between doses [3]. Following the WHO recommended schedule would present great advantages for potential travelers, who could complete the three-dose rabies vaccine needed for primary immunization in 1 month.

The aim of this study was to determine the efficacy and safety of PCEC-K when following the WHO recommended schedule. We have previously reported that this accelerated schedule could result in protective titers, as measured by enzyme-linked immunosorbent assay (ELISA), but neutralizing antibody (NA) assay was not performed in that

study [4]. NA assays are considered by the WHO to be the gold standard for measuring successful seroconversion against rabies [2]. However, ELISAs present key advantages over other serological techniques [5], including speed, ease, and lower biosafety requirements, and we were interested in measuring how results obtained with an ELISA correlated with those obtained with the NA assay.

Subjects, materials, and methods

Ethics approval and informed consent

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was submitted to and approved by the institutional review board. All participants were informed of the study procedures, and written consent was obtained from all subjects.

Subjects

A total of 53 subjects (40 men and 13 women; ages ranging from 24 to 59 years, with a mean of 37.0 ± 10.5 years) were enrolled. Of these, 26 and 27 subjects were enrolled in 2007 and 2008, respectively. The subjects were interviewed and examined by a physician and confirmed to have had no previous animal bites; no previous rabies vaccines; no acute or chronic infectious diseases; and no concomitant use of corticosteroid therapy or antimalarial or immunosuppressive drugs. The subjects were all volunteers who considered receiving the rabies vaccine for their travel abroad in the near future.

Administration of vaccines

The PCEC-K vaccine used in this study was manufactured by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). The 26 subjects enrolled in 2007 were vaccinated with lot RB02 for the first and second doses. For the third dose, 18 subjects were vaccinated with lot RB02 and 8 subjects with lot RB03. The 27 subjects enrolled in 2008 were vaccinated with lot RB06 for the first and second doses, and lot RB07 was used for the third dose. Vaccination was performed by subcutaneous injection in the anterolateral aspect of the upper arm, using 1.0 ml of PCEC-K, on days 0, 7, and 28.

Serological testing

Blood samples were collected from all vaccinees on days 7, 28, and 42. ELISA antibody titers were measured with the PLATELIA™ RABIES KIT II (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's protocol. NA titers were measured by using the rapid fluorescent focus inhibition test (RFFIT), following the international standard procedure [6]. ELISA antibody titers of 0.5 EU/ml or higher, or NA titers of 0.5 IU/ml or higher were considered positive.

Adverse effects

The subjects were told to notify the investigators of any adverse effects after the vaccine injection on their next visit.

Statistical analysis

The correlation of $\log_{10}$ ELISA antibody titers and $\log_{10}$ NA titers was calculated using Pearson's correlation test. Statistical analysis was conducted using StatView 5.0 (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

Results

ELISA antibody titers measured on days 7, 28, and 42 are shown in Table 1. On day 7, 1 week after the first dose, none of the subjects had a detectable ELISA antibody titer. On day 28, 3 weeks after the second dose, 84.9% (45/53) were seroconverted, as defined as 0.5 EU/ml or higher. Among the seroconverted subjects, the geometric mean titer (GMT) was 1.7 EU/ml (range <0.5–3.5 EU/ml). On day 42, 2 weeks after the third dose, all subjects (53/53) were seroconverted, with a GMT of 3.8 EU/ml (range 0.8–17.3 EU/ml).

The NA titers measured on days 7, 28, and 42 are shown in Table 2. Again, on day 7, no subject had a detectable NA titer. On day 28, 94.3% (50/53) were seroconverted, defined as 0.5 IU/ml or higher. Among the seroconverted subjects, GMT was 1.2 IU/ml (range <0.5–6.0 IU/ml). On

Table 1 ELISA antibody titers after administration of Japanese rabies vaccine on days 0, 7, and 28

Day	7	28	42
GMT (EU/ml)	–	1.7	3.8
Range	All <0.5	<0.5–3.5	0.8–17.3
n = ≥0.5 EU/ml	0/53	45/53	53/53
Seroconversion (%)	0	84.9	100

Blood samples were collected on days 7, 28, and 42. Subjects were considered to have seroconverted when antibody titers were 0.5 EU/ml or higher

ELISA enzyme-linked immunosorbent assay, *GMT* geometric mean titer, *range* lowest–highest value

Table 2 Neutralizing antibody titers after administration of Japanese rabies vaccine on days 0, 7, and 28

Day	7	28	42
GMT (IU/ml)	–	1.2	5.7
Range	All <0.5	<0.5–6.0	0.7–23.7
n = ≥0.5 IU/ml	0/53	50/53	53/53
Seroconversion (%)	0	94.3	100

Blood samples were taken on days 7, 28, and 42. Subjects were considered to have seroconverted when antibody titers were 0.5 IU/ml or higher

GMT geometric mean titer, *range* lowest–highest value

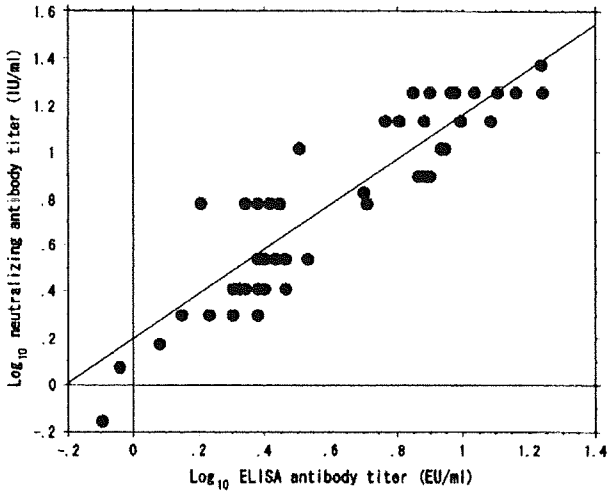

Fig. 1 Correlation of $\log_{10}$ enzyme-linked immunosorbent assay (*ELISA*) antibody titers with $\log_{10}$ neutralizing antibody titers in 53 subjects. The correlation analysis was performed using Pearson's correlation test ($r = 0.891$, $P < 0.0001$, $n = 53$)

day 42, all subjects (53/53) were seroconverted, with a GMT of 5.7 IU/ml (range 0.7–23.7 IU/ml).

Figure 1 shows the comparison between the $\log_{10}$ ELISA antibody titers and $\log_{10}$ NA titers. There was a clear correlation between the two assays ($r = 0.891$, $P < 0.0001$, $n = 53$).

Overall, the vaccines were well tolerated by all subjects. One subject complained of a headache, which resolved on its own the following day. Other systemic reactions such as fever or malaise were not reported. Mild local reactions were observed, erythema being the most frequent, recorded in 18.8% (10/53). This was followed by pruritus in 17.0% (9/53), swelling in 13.2% (7/53), and local pain in 7.5% (4/53).

Discussion

We studied the efficacy of PCEC-K following the schedule recommended by the WHO. After three doses of PCEC-K, all subjects developed protective ELISA and NA titers. There were no significant adverse effects.

Previous studies have reported on the efficacy of the officially approved PEI in Japan, with NA GMT values ranging between 2.7 and 4.0 IU/ml [7, 8]. In our study, all subjects had seroconverted after the third dose of the vaccine, with an NA GMT of 5.7 IU/ml. Our results cannot be directly compared to those in these previous studies, due to differences in the age, gender proportions, sample sizes, and timing of the samples taken. However, the fact that all subjects in the present study seroconverted suggests that administering PCEC-K on days 0, 7, and 28 is as effective as administering it on days 0, 28, and 180.

There was a strong correlation between the ELISA antibody and NA titers in the present study, suggesting that the ELISA test could be as reliable as the neutralizing test in this context. Feyssaguet et al. [5] likewise reported that the ELISA test was as sensitive and specific as the current standardized reference method, with sensitivity of 98.6% and specificity of 99.4%. Moreover, the ELISA kit is simple, safe, and quick to use compared to the neutralizing test, which is time-consuming and expensive, and requires extensively trained technicians and laboratory facilities equipped to handle the live rabies virus.

A difference in the seroconversion rate between the two assays used in the present study was observed on day 28. The seroconversion rate was significantly lower when titers were measured by ELISA as compared to measurement by the neutralizing test (84.9 vs 94.3%, respectively). Moreover, none of the subjects with elevated ELISA antibody titers had negative NA titers. Taken together, these data suggest that the ELISA test could be more stringent in determining seroconversion. All subjects had seroconverted after the third dose, indicating that the completion of the recommend schedule was the most important factor in achieving protection.

Japan is one of the few rabies-free countries in the world. Yet two human imported rabies cases were reported in Japan, in November 2006 [9, 10]. The victims in question did not receive rabies PEP after being bitten by a rabid dog. To prevent further tragedies, travelers must be encouraged to receive PEP immediately after being bitten by an animal suspected of being rabid. Furthermore, it is important to encourage PEI to insure the efficacy of PEP and economize on the short supply of RIG worldwide.

In conclusion, PCEC-K used for PEI and administered according to the schedule recommended by the WHO is as immunogenic and effective as the currently approved PEI in Japan. The use of the schedule recommended by the WHO would also foster traveler compliance with vaccination recommendations by providing protective immunity with a shorter, more manageable time course.

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Equine Herpesvirus Type 9 in Giraffe with Encephalitis

To the Editor: Herpesviruses have been isolated from many mammals. Herpesvirus infection in natural hosts is often mild and is usually followed by a latent infection; however, cross-species herpesvirus infections cause severe and fatal diseases. Equine herpesvirus (EHV)–1 causes abortion, respiratory disease, and, occasionally, neurologic disorders in horses. EHV-1 infection is usually limited to equine species, although it has also been found in other species (*1*), in which it causes fatal encephalitis. Recent sequence analyses suggested that the equine herpesviruses isolated in the United States from onagers (*Equus hemionus*), Grevy's zebras (*E. grevyi*), and Thomson's gazelles (*Gazella thomsoni*) are a subtype or variant of EHV-1 (*2*). With respect to epizootiology, the nonequine animals affected by EHV-1 or EHV-1–related virus were kept in enclosures adjacent to those of zebra species (Grevy's or Burchell's).

Another EHV-related virus was isolated from 2 Thomson's gazelles that had encephalitis and were kept with zebras (*3*). The virus was later found to be a new type of EHV, EHV-9, although it was serologically cross-reactive with EHV-1 (*3*). Recently, neutralizing antibodies against EHV-9 were found among Burchell's zebras in the Serengeti ecosystem (*4*).

A herpesvirus was recently isolated from a reticulated giraffe (*Giraffa camelopardalis reticulate*) with neurologic symptoms; the giraffe was from a zoo in the United States (*5*). Nonsuppurative encephalitis was found by histopathologic examination of the giraffe brain. Several Burchell's zebras that were apparently healthy and later determined to be seropositive for EHV-1 were housed in the same pen as the giraffe. The isolated virus was identified by PCR and a monoclonal antibody assay as EHV-1 (*5*). In the present study, we analyzed 4 gene sequences of the giraffe herpesvirus to show its relatedness to EHV-1 and EHV-9.

We amplified portions of 4 genes from giraffe herpesvirus DNA by PCR. The DNA polymerase catalytic subunit (open reading frame [ORF] 30) gene was amplified by using herpesvirus universal primers (*6*). The genes for glycoprotein B (gB) (ORF33), glycoprotein 2 (gp2) (ORF71), and glycoprotein D (gD) (ORF72) were amplified by using primers specific for EHV-9. The ORF33 primers were gB-F (5′-GGCACAATAGTCCTAGCATG TCTGTTGCTG-3′) and gB-R (5′-AAATATCCTCAGGGCCGGAAC TGGAAAGTG-3′). The ORF71 primers were gp2-F (5′-CCCCGTTGATG AGTTTTGCGTAGAGGTCTA-3′) and gp2-R (5′-GCCACCACTGGTTG TAAAGGCCAAGAGATA-3′). The ORF72 primers were gD-F (5′-TTTACAACCACTGGTGGCGT GTGTGCAGAA-3′) and gD-R (5′-TATCTCCAAACCGCGAAGCTT-TAAGGCCGT-3′). The amplified products were used as templates for direct sequencing (Dragon Genomics, Mie, Japan). The sequences were edited with Phred, Phrap, and Consed (www.phrap.org/phredphrapconsed.html), and the phylogenic trees were constructed with PHYLIP (*2,7*). Accession numbers of the sequences (submitted to the DNA Data Bank of Japan) are given in the Figure.

We used PCR to amplify a part of the gB gene of the giraffe herpesvirus, and we used EHV-1 specific primers for sequencing. However, we could not obtain amplicons (data not shown). Therefore, the more conserved gene, ORF30, was sequenced. The sequence of the 1,066-bp segment of the giraffe herpesvirus ORF30 gene was 99.5% identical to EHV-9 and 94.6% identical to EHV-1, which indicates that the giraffe herpesvirus was most closely related to EHV-9. Therefore, EHV-9 ORF33–specific primers were used to amplify the corresponding region of the giraffe herpesvirus. The sequence of the giraffe herpesvirus ORF33 was 98.8% identical to EHV-9 and 95.9% identical to EHV-1. Also, the sequence of the other envelope glycoproteins (ORF71 and ORF72) of the giraffe herpesvirus were 99.8% and 99.6% identical to EHV-9 and 91.6% and 96.3% identical to EHV-1. A phylogenic tree of maximum likelihood showed that EHV-9 and the giraffe virus formed a genetic group that was apparently distinguished from other genetic groups of EHV (Figure).

Herpesviruses have caused clinical disease in zoo animals, including a case of EHV-9 infection in Thomson's gazelles (*3*) and a recently described endotheliotropic betaherpesvirus in-

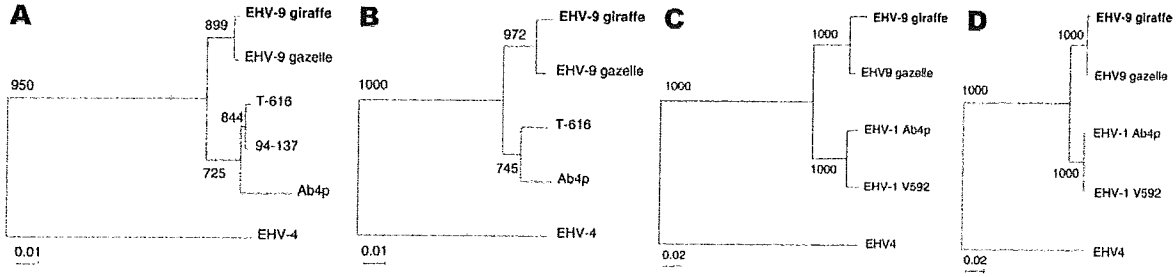

Figure. Phylogenic trees of giraffe herpesvirus and other related viruses. A) Open reading frame (ORF) 30, B) ORF33, C) ORF71, and D) ORF72. EHV-9 giraffe, equine herpesvirus (EHV) type 9 isolated from reticulated giraffe (*5*) (AB453826); EHV-9 gazelle, EHV-9 isolated from a Thomson's gazelle in Japan (*3*) (AP010838); T-616, EHV-1 isolated from a zebra fetus in the United States (EU087295); 94-137, EHV-1 isolated from a Thomson's gazelle in the United States (EU087297); Ab4p, EHV-1 isolated from horses (AY665713); EHV-4, EHV-4 isolated from horses (AF030027). Accession numbers of the sequences are AB439722 for ORF30, AB439723 for ORF33, AB453825 for ORF71, AB453826 for ORF72 of giraffe herpesvirus (DNA Data Bank of Japan, National Institute of Genetics, Japan), and AP010838 for EHV-9 genome sequence (H. Fukushi, unpub.data). Boldface indicates the sequence of EHV-9 derived from the giraffe. Scale bars indicate number of nucleotide substitutions per site.

fection in Asian and African elephants (*8*). The distribution and severity of herpesvirus encephalitis often differ between natural and accidental hosts in terms of enhanced neurovirulence. For example, herpes simplex virus causes a severe and fulminating encephalitis in rabbits but only herpetic stomatitis in humans; herpesvirus B infection is fatal to humans but not to other primates (*9*). These findings may explain why the giraffe had lesions while the zebras in the same enclosure did not.

Alphaherpesviruses can evade the immune system and become latent within lymphoid tissues, peripheral leukocytes, and trigeminal ganglia; they have the potential for reactivation and shedding after immune suppression or stress (*10*). Thus, the fact that the zebras were apparently healthy and seropositive for EHV-1 raises the possibility that the virus was reactivated and shed by one of the zebras, resulting in systemic infection and disease in the giraffe (*5*). This cross-species transmission of equine herpesviruses raises the possibility of latent infection and transmission of the disease from zebras to other animal species kept in zoos; the results could be devastating. Zebras might be EHV-9 carriers in zoos. Cross-species transmission must be considered in terms of screening susceptible animals for subclinical infection in terms of husbandry and housing issues for irreplaceable species.

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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′-CGGGATCCCGGACAACAA ACTCATTGAAC-3′ and 5′-GGAATTCCCATTAGTATGCTCTTCGCTGCC C-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,

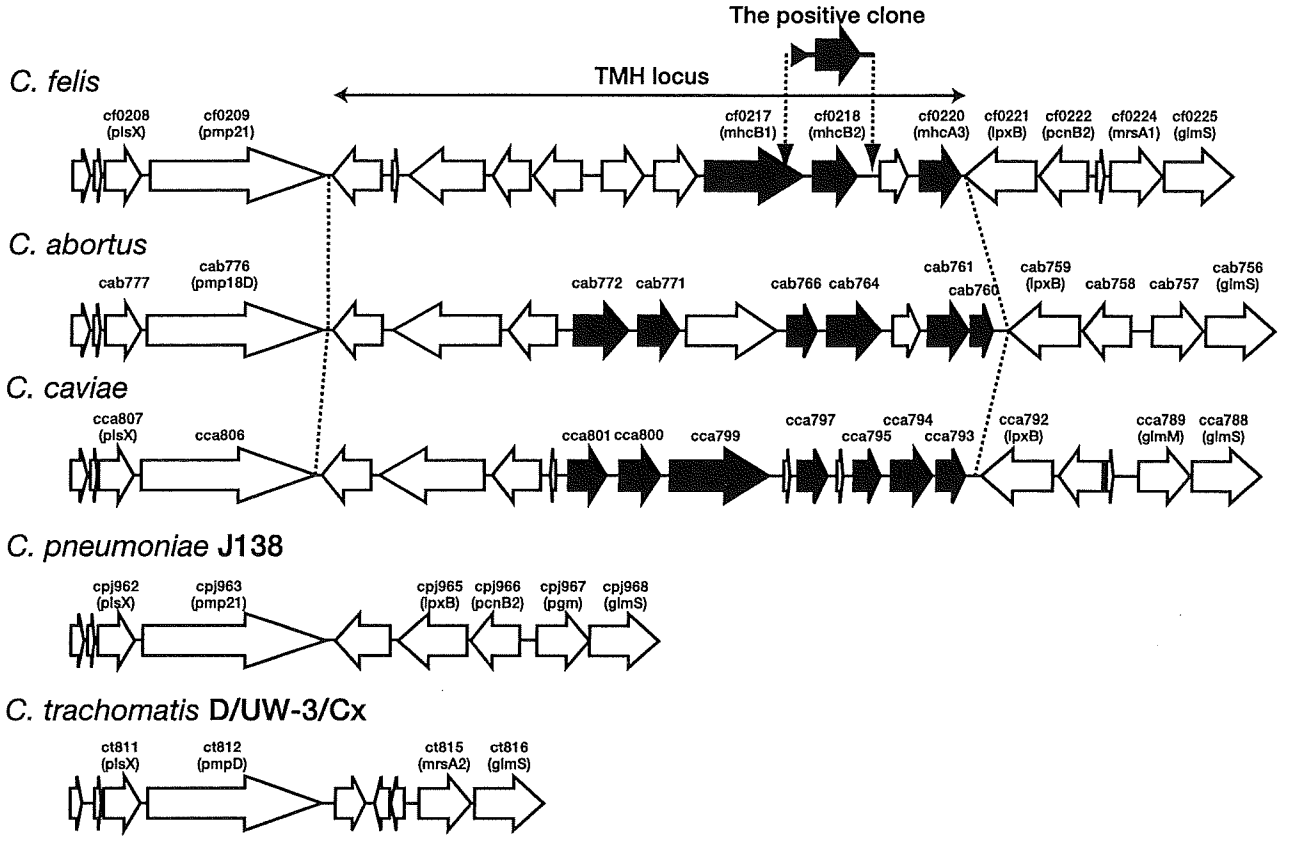

FIG. 1. Gene arrangement of the TMH loci in the *C. felis* (Fe/C-56), *C. abortus* (S26/3), and *C. caviae* (GPIC) genomes and the analogous regions in the *C. pneumoniae* (J138) and *C. trachomatis* (D/UW-3/Cx) genomes. By screening of the *C. felis* genome library with anti-*C. felis* serum, the positive 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 MTTNSLNIGSSSATVVNSVVQKQPLNKVSLNKYQDVAAAIILIAGLVLIGSLVGALVFFA   60
CAB764          1 ---MTTSPVNTNPIATHVSTTQHTLFSTSVSKYQRIATAIALLAGMVLVGTLVGALVFFA   57
CAB766          1 --------MNTTPIATHVPTTQHALSNISSNKYQRLATVIALLAGMVLVGTLIGALVCFA   52
CCA797          1 MTTNPVNTTTTITPPITQKQPLCSVQESKYRRIAAATTLLEGLVLIAPLVGALVFFALPT   60

CF0218         61 LTGPSVLFAAMLMALVSGVVLIAMAGPDLAVGVRKDAKERQLVHEKIQYEVEAALLKDNN  120
CAB764         58 LPTSVTLVALVSVALLASVILLSMAMYNLVSQSRRVSSDPQLGEENTRLEAEMIGLREQL  117
CAB766         53 LPASLTLVALVSTSLLASVILLSMSVYNLVSQFRRASSYAIIGKENIRLEAEMIALQEKL  112
CCA797         61 ATTITVLTVLIGIALGASVILLSMAMYKLLRCRQVPSDPKGIEDSVTLKEEIIEIKAQLT  120

CF0218        121 KLIERKLEELQKHSDALTGQLLGLAGDLECLKDEKIDLEARVMDLQRQNASLSKLMEEGR  180
CAB764        118 IRSENQLAEEQGRCSELSVKLMSVIGDLAQANDEKTSLESKVQALKELVASYPAIAEEAQ  177
CAB766        113 TRSENQLQEEQGRSSELSVKLMSTEIDLDQANDEKASLESKVKALKELVEKYPAIAEESQ  172
CCA797        121 QKAIQLIEEEERSASLLGQLLAITGDLEATQHQKSALEAEVKLLQEQYTSTLKLAEKDHS  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 DEGVSAVVKLQGKVVDLVEETEVKEKNLEFYDGRIKNLINQNSDLVATVRCLESKLDKDQ  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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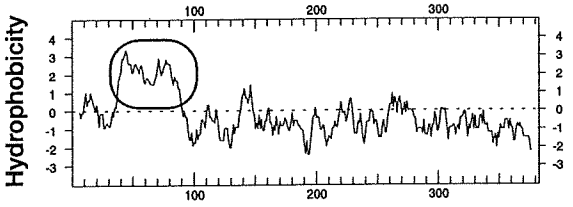
B CF0218



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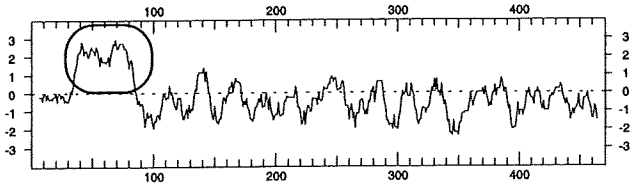

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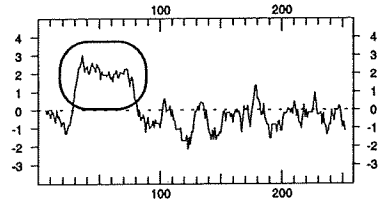

CCA797