行った。その結果、4件(マガモ3、オシドリ1)が陽性となった。(他の499件は陰性)。検査の結果、陽性となった鳥については再度隔離・投薬して陰性の確認を行った。

5. 発生原因の推定及び再発防止策

患者発生当時、A施設には検疫室、病鳥隔離室が設置されておらず、1,000羽を超す鳥類は検疫を実施せずに導入され、病鳥の隔離措置が行われることもなかった。また、飼育鳥は一部を除き個体識別が実施されておらず、発病・死亡等の記録がなされていなかった。

A施設には常駐の獣医師が一名配置されていたが、開園準備の為に本来の業務以外の雑務がほとんどを占め、飼育鳥の健康管理を十分に行える状況ではなかった。また、従業員は一部を除き動物由来感染症に関する適正な感染防止の教育を受けておらず、給餌、清掃、死体処理等の作業時に手袋、マスク着用の徹底等の感染防御措置が講じられていなかった。さらに、従業員は目前の開園準備の為に業務量が多くなっており、冬季の環境下で連日深夜まで従事するなど過労気味で、体調維持管理が困難な状況であった。

このような状況の下、オウム病クラミジアを保菌していた一部の鳥の糞便の清掃や死体の処理等、濃厚接触をした飼育担当者の一部が感染・発症したものと推測される。なお、排菌の原因としては、長距離移動や新しい環境でのストレス等が引き金となった可能性がある。

3名の患者の発症日は11月30日から12月3日までの範囲であり、オウム病の潜伏期間が通常7日から14日とされていることから、11月中旬から下旬にかけて曝露された可能性が高いと推測される。感染源となった鳥については肺炎患者から検出された遺伝子遺伝子解析から一部ほぼ特定できている(後述)。本件は上述のようにガイドライン2003に基づく管理運営が遵守されていなかったことが原因であったことは明らかであり、必要な施設設備をもうけ、適正な管理運営体制を確立することが再発防止のための要件であると考えられる。

6. A施設への改善指導

専門家会議での検討内容をふまえ、A施設に対して次のような改善指導が行われた。
①検疫室、病鳥隔離室等の施設設備の整備。
②全飼育鳥の個体識別の実施と個体管理の徹底。③鳥類の展示方法の見直し(飼育鳥数の削減や鳥が隠れる場所の確保などのストレス軽減策、鳥とのふれあいをする区域の限定による管理体制の強化)。④外部委員を含めた感染症対策委員会の組織。⑤飼育管理に関する各種マニュアルの作成とこれに基づく適正な管理運営。⑥全従業員に対する衛生講習会の実施。
⑦その他、ガイドライン2003に基づく適正な管理運営。

7. 施設の開園

上記6.の指導事項に対する改善状況の確認やその他必要な調査のため、中央区保健福祉部及び東部衛生監視事務所は、平成17年12月6日以降3月15日までの間に合計27回の立ち入り調査、14回の面接指導を行った。その結果、上記指導事項に従ってA施設は改善を進め、適正な管理運営を実施できる体制が整備されたことが確認できた。このことから、「患者発生に至った特別な状況は解消されたと考えられ、これに伴い、施設の開園についてこれを止めるべき特別の理由は解消されたと判断する」という専門家会議の意見をふまえ、A施設は2006年3月15日に開園した。

8. 開園後の対応

開園後も、A施設が提出したマニュアルに基づき、適正に管理運営されていることを確認す

るため、東部衛生監視事務所が継続的に監視指導を実施している。

9. 感染源となったトリまたは場所の特定

A 施設由来の菌株を用いた抗体価測定により感染者の範囲を検証するため、神戸市環境保健研究所、国立感染症研究所等にて引き続き検出されたオウム病クラミジアの遺伝子配列の解析や分離培養等、必要な調査研究を行っている。

現在までに肺炎で入院した 1 名の気管支洗浄液より，クラミジア遺伝子が検出でき，MOMP 1,438bp の塩基配列を決定した。PCR で陽性となったトリ由来のクラミジア MOMP の配列と比較したところ、ヒムネオオハシ由来の遺伝子配列が患者のそれと 100%一致した。それ以外のトリ由来のものは、1～5 塩基異なっていた。搬入時期や潜伏時間等を考慮に入れても、ヒムネオオハシが感染の原因となった可能性が高い。ヒムネオオハシは、約 4.3 ㎡の部屋の閉鎖空間に 3 羽放たれており、その排泄物を吸い込んで感染したものと推察された。

10. 本事例から明らかになった課題

今回のオウム病集団発生を経験して，以下のような課題があると考えられた。①対応体制の構築の必要性 ②行政等がどのように感染予防に関与するべきかが不明確（法律上の規制） ③患者の迅速診断法の確立（検体材料の選択） ④オウム病クラミジアの血清診断法の改善（クラミジアの血清型と交差性） ⑤トリ糞便からのクラミジア検出法の改善（PCR 阻害物質の除去）

なお本件に関する内容の主なものについては、厚生労働省健康局結核感染症課長通知として、各都道府県、政令市、特別区衛生主管部(局)宛てに、健感発第 0704002 号平成 18 年 7 月 4 日付けで、「動物展示施設(動物とのふれあい施設を含む。)における動物由来感染症対策について」として通知されている。また同日、各都道府県、指定都市、中核市動物愛護管理主管課(室)長宛に、健感発第 0704001 号、環自総発 060704001 号として、「動物展示施設(動物とのふれあい施設を含む。)における動物由来感染症対策について(協力依頼)」として通知されている。

II. リケッチアに関する研究

1. つつが虫病の血清診断についての課題

2006 年11月にこれまで発生が報告されていない北海道で、つつが虫病疑い例が発生し、発生動向調査に基づく届出が適切かどうかの判断を依頼された。症例は、北海道在住の 11 歳女性で、ここ数年来北海道外には出ていない。経過としては、発熱前 11 月 7～10 日頃に草むらに入ったことがある。11 月 18 日頃、38℃の発熱と頚部痛、腹部の小紅斑（2～3 日で消失）が出現。11 月 24 日、精査のため入院。頚部リンパ節腫大、毎夕 38℃弱の発熱と CRP2～3 台が続き、セフェム系の抗菌薬で改善せず。12 月 1 日、39 度台の発熱と左側腹部痛を訴え、腹部 CT にて多発性脾膿瘍を確認。同日ミノサイクリン 100mg×2/day）点滴を開始、症状、検査数値とも速やかに改善。12 月 5 日に民間検査機関で検査した間接蛍光抗体法（IFA）でのツツガムシ抗体は Karp、Kato がいずれも IgM40、IgG10、Gilliam はともに 10 未満であった。刺し口なし。12 月 13 日には、ミノサイクリン内服中で、CRP は陰性、症状は軽快しているものの、脾膿瘍はやや縮小した程度であった。

以上の情報から、本症例がつつが虫病が確定されれば北海道での初めての発生例となることから、その後の疫学調査等の対応も必要となってくるため、慎重な判断が求められた。そこで国立感染症研究所をはじめ、つつが虫病の

血清診断の経験が豊富な 4 施設にペア血清を送付して確認をしたところ、一部で非特異的な血清反応がみられたが、全施設で特異的な抗体上昇は認められず、最終的に本例については、つつが虫病は否定的であると判定された。

III.コクシエラに関する研究

1.Q 熱病原体 *Coxiella burnetii* の遺伝子検出法の開発と他施設の検出法の検証

近年、わが国で鶏卵や関連食品のマヨネーズに *C. burnetii* 汚染があるとの J 民間研究所からの指摘があり、その方法(J 検出法)の検証と実態の把握が必要になった。これまでに我々は従来の nested-PCR に加え、高感度な検出方法の開発として TaqMan PCR による Real Time PCR 法ならびに LAMP 法による高感度の検出法の開発を行い、実際の市販鶏卵の *C. burnetii* 汚染実態調査を進めてきた。すでに J 検出法の抽出法を再現し、我々の開発したカラム抽出法ならびに DNA 抽出機による抽出法について、卵黄からの DNA 抽出効率について比較してきたが、感度は我々の方法がいずれも高感度であった。またこれまでに 1000 個を超える市販鶏卵の調査を行ったが、すべて陰性であった。

そこで本年度は、J 民間研究所から内閣府食品安全委員会に提出された報告書に示されているLight Cycler とseminested-PCR を組み合わせた方法を再現し特異性について検討した(図 1)。J 検出法による特異性の検討をした各種細菌 39 株は以下のとおりである。

Alcaligenes faecalis, Alcaligenes xylosoxidans, Acinetobacter baumannii, Proteus vulgaris, Chryseobacterium indologenes, Flavobacterium breve, Haemophilus influenzae, Streptococcus pyogenes, Staphylococcus aureus, Pseudomonasu fluorescens , *Pseudomonasu stutzeri, Acinetobacter lwoffii, Flavobacterium odoratum, Pseudomonas aeruginosa, Proteus penneri, Legionella pneumophila, Streptococcus pneumoniae, Listeria monocytogenes, Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Choleraesuis, *Salmonella* Arizonae, *Yersinia enterocolitica, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Chlamydophila psittaci, Rickettsia japonica, Rickettsia prowazekii, Ehrlichia chaffeensis, Anaplasma phagocytophilum, Ehrlichia sennetsu, Orientia tsutsugamushi* Gilliam 株, *Orientia tsutsugamushi* Karp 株, *Orientia tsutsugamushi* Kato 株, *Orientia tsutsugamushi* Kawasaki 株, *Orientia tsutsugamushi* Kuroki 株

図 2 に示すように、J 検出法では Light Cycler の時点では 39 株すべてで何らかの DNA が増幅され、seminested-PCR を終了した時点でも多数の株でバンドが認められた。*Anaplasma phagocytophilum* と *Acinetobacter lwoffii* 2 株では陽性コントロール付近にバンドがみられた。したがって、これらについてはシークエンスまで行わないと実際の陽性かどうかの判定が困難であると思われた。

C.考察

I. クラミジアに関する研究

1.神戸市のトリ展示施設におけるオウム病集団発生例の調査

神戸のトリ展示施設にて発生したオウム病の集団発生事例を経験し、我々が開発した *C. psittaci* のみを特異的に検出可能な PCR 法を用いて疫学調査を行い、有用性が示された。今後、さらに臨床材料を用いた検討を行う予定で

ある。また患者およびトリから検出された *C .psittaci* 株の遺伝子を解析し、感染源の推測がほぼ可能であった。但し血清診断については、今後も分離株を抗原として用いた検討を予定している。

一方で今回のトリ展示施設等でのオウム病発生は、ガイドライン 2003 に示されている予防的措置が十分であれば防ぐことができたものと思われる。過去にも同じ経営者が松江市のほぼ同様の施設でオウム病集団発生を起こしており、その教訓がなぜ生かされなかったのかについては、営業者、さらに営業認可者の真摯な反省が求められる。現在は種々の不備な点が改善され開園されているが、今後も十分な衛生管理と危機管理体制が望まれる。

Ⅱ.リケッチアに関する研究

1. つつが虫病の血清診断についての課題

今回の商業検査機関での陽性判定はおそらく非特異反応を陽性と判断したことによるものと思われ、IFA 法における課題が改めて浮き彫りになった。今後は商業検査機関とも情報交換しつつ検査法の改善や精度管理を進め IFA 法の標準化を目指したい。

Ⅲ.コクシエラに関する研究

1. Q 熱病原体 *Coxiella burnetii* の遺伝子検出法の開発と他施設の検出法の検証

J 検出法は特異性が極めて低く、様々な細菌 DNA 等を増幅した。J 検出法には Light Cycler と seminested-PCR の組み合わせの設計自体に問題があると思われ、卵に *C .burnetii* 汚染があるとの J 民間研究所からの指摘は、実際の *C. burnetii* 陽性とはいえない可能性が高いと考えられた。

D. 結論

I. クラミジアに関する研究

1. 神戸市のトリ展示施設におけるオウム病集団発生例の調査

神戸のトリ展示施設にて発生したオウム病の集団発生事例を経験し、我々が開発した *C. psittaci* のみを特異的に検出可能な PCR 法を用いて疫学調査を行い、有用性が示された。

トリ展示施設等でのオウム病発生は、予防的措置や日常の衛生管理が不十分であれば今後も起こりうる。十分な衛生管理と危機管理体制の維持が望まれる。

Ⅱ. リケッチアに関する研究

1. つつが虫病の血清診断についての課題

今回の商業検査機関での陽性判定はおそらく非特異反応を陽性と判断したことによるものと思われ、IFA 法における課題が改めて浮き彫りになった。今後、抗原や判定法についての精度管理の検討が必要と考えられた。

Ⅲ. コクシエラに関する研究

1. Q 熱病原体 *Coxiella burnetii* の遺伝子検出法の開発と他施設の検出法の検証

J 検出法は特異性が極めて低く、J 民間研究所からの指摘は、実際の *C. burnetii* 陽性とはいえない可能性が高い。したがって現時点で卵の *C .burneti* 汚染は確認できず、もしあったとしてもリスクは低いと考えられた。

E. 健康危機情報

特になし

F. 研究発表等

誌上発表

1. 岸本寿男、安藤秀二、小川基彦：オウム病

および Q 熱ー診断における現状と課題 カレントテラピー24：98-100, 2006
2. 岸本寿男、安藤秀二：リケッチア感染症. 小児の治療指針. 小児科診療 69:169-172, 2006
3. 岸本寿男、安藤秀二、小川基彦：非定型肺炎の治療. クラミジア肺炎. Medical Practice 23:1953-1957, 2006

学会発表
1. 飯島義雄、秋吉京子、大石英明、田中 忍、貫名正文、伊藤正寛、春田恒和、福士秀人、安藤秀二、岸本寿男: トリ展示施設におけるオウム病集団発生例 第 24 回 日本クラミジア研究会・第 13 回 リケッチア研究会 合同研究会 平成 18 年 10 月 22 日 北九州
2. 安藤秀二、坂田明子、佐藤 梢、小川基彦、藤田 修、堀田明豊、宇田晶彦、棚林 清、百田隆祥、小島禎、池戸正成、岸本寿男: 鶏卵からの Q 熱コクシエラ検出に関する研究(第 3 報) 第 24 回 日本クラミジア研究会・第 13 回 リケッチア研究会 合同研究会 平成 18 年 10 月 22 日 北九州

G. 知的財産権の出願・登録状況
なし。

H. 特許取得
なし。

I. 実用新案登録
なし。

J. その他
なし。

Ⅲ. 研究成果の刊行に関する一覧表

別紙5

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fujita O., Tatsumi M., Tanabayashi K., Yamada A.,:	Development of a real-time PCR for detection and quantification of *Francisella tularensis*.	Jpn J Infect Dis	59	46-51	2006
Hotta A., Uda A., Fujita O., Tanabayashi K., and Yamada A	Preparation of Monoclonal Antibodies for Detection and Identification of *Francisella tularensis*.	Clin Vacccine Immunol	14	81-84	2007
Takayama-Ito M., Inoue KI., Shoji Y., Inoue S., Iijima T., Sakai T., Kurane I. and Morimoto K	A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein	Virus Res.	119	208-215	2006
Park C.-H., Kondo M., Inoue S., Noguchi A., Oyamada T., Yoshikawa H. and Yamada A	The Histopathogenesis of Paralytic Rabies in Six-Week-Old C57BL/6J Mice Following Inoculation of the CVS-11 Strain into the Right Triceps Surae Muscle.	J.Vet.Med.Sci	68	589-595	2006
Setiyono A, Ogawa M, Cai Y, Shiga S, Kishimota T, Kurane I	New criteria for immunofluorescence assay for Q fever diagnosis in Japan.	J. Clin. Microbiol.	43, ,	5555-5559	2005

Jpn. J. Infect. Dis., 59, 46-51, 2006

Original Article

Development of a Real-Time PCR Assay for Detection and Quantification of *Francisella tularensis*

Osamu Fujita*, Masashi Tatsumi, Kiyoshi Tanabayashi and Akio Yamada

Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

(Received October 12, 2005. Accepted January 5, 2006)

SUMMARY: The facultative intracellular bacterium, *Francisella tularensis*, is an etiological agent of tularemia and is also considered to be a potential biological threat agent due to its extreme infectivity. We established a real-time PCR assay using the LightCycler (LC) system to detect a *Francisella*-specific sequence of the outer membrane protein (*fop*A) gene. Twenty-five *F. tularensis* strains including 16 Japanese isolates were subjected to this LC-PCR assay, and were tested positive, whereas *Francisella philomiragia* and other bacteria species did not show any specific fluorescent signal. A linear response was observed using *F. tularensis* genomic DNAs of between 20 fg and 2 ng, corresponding to 1.2 to 1.2×10^5 bacteria. The newly established real-time PCR allows the detection of the *F. tularensis* genome specifically, sensitively, and rapidly. This assay may contribute to the standardization of the laboratory diagnosis of tularemia.

INTRODUCTION

Francisella tularensis, the causative agent of tularemia in humans and animals, is a small Gram-negative intracellular bacterium. Although *F. tularensis*, *F. novicida* and *F. philomiragia* are currently considered to be independent species of the genus *Francisella*, Ellis et al. (1) recently proposed that *F. novicida* should be classified as a subspecies of *F. tularensis* based on the nucleotide sequence of the 16S ribosomal DNA. In this study we regarded *F. novicida* as one of the subspecies of *F. tularensis* according to this proposition. Species and subspecies of *Francisella* differ with regard to their biochemical properties, geographic distribution and virulence in humans (1). Of the four *F. tularensis* subspecies, *F. tularensis* subsp. *holarctica* is widely distributed in a wide range of animal reservoir hosts throughout most of the Northern Hemisphere, including Japan. It is transmitted to humans by various routes, including: the direct handling of infectious carcasses; the ingestion of contaminated food, vegetation or water; being bitten by infected arthropod vectors; and the inhalation of infectious dust, soil or aerosols (1). The *F. tularensis* subsp. *tularensis* (also known as Type A) is mainly distributed in North America. This subspecies bacterium is extremely infectious, since as few as 10 organisms are capable of inducing disease in humans following intradermal inoculation or inhalation (2,3). It is also highly virulent in that case fatality rates as high as 30 to 60% were reported in untreated pneumonic and severe systemic forms of the disease (4). *F. tularensis* has been, therefore, considered to be a class A bioterrorism agent by the Centers for Disease Control and Prevention (CDC), Atlanta, Ga., USA (5).

The diagnosis of human cases of tularemia is usually accomplished by the demonstration of an antibody response to *F. tularensis* by tube- or micro-agglutination assay and enzyme-linked immunosorbent assay (ELISA) (6-8). Specific antibody response in patient serum is detectable from 4 to 7 days after the onset of the disease by micro-agglutination assay (8). Furthermore, those immunological assays can be confounded by serum cross-reactivity with antigens of other genera of bacteria (genus *Brucella*, *Haemophilus* and *Yersinia*) (9). For the identification of *F. tularensis*, a conventional culture assay is usually conducted. It not only requires at least 2-4 days for adequate growth of the organism (10), but also is prone to showing false-negative results. Furthermore, the cultivation of *F. tularensis* poses a considerable risk of laboratory-acquired infection. Laboratory work should therefore be performed under biosafety level 3 (BSL-3) conditions. An immunochromatographic hand-held assay has been developed based on polyclonal and monoclonal antibodies against lipopolysaccharide of *F. tularensis* live vaccine strain (LVS), but the sensitivity was relatively low: 10^6 colony-forming units (CFU)/ml in phosphate-buffered saline (PBS) and 10^6 to 10^7 CFU/ml in spiked human sera (11). To overcome these problems, PCR assays targeting the 16S ribosomal RNA (12) or targeting genes encoding the outer membrane proteins such as *fop*A (13) and the 17-kDa major membrane lipoprotein (14-16) have been successfully used to detect *F. tularensis* DNA, but most of them are inferior to real-time PCR assay, both in sensitivity and rapidity (17-19).

The LightCycler (LC) technology enables real-time and high-speed detection of PCR products. It employs two hybridization probes labeled with fluorophore that allow the sequence-specific emission of fluorescence caused by the fluorescence resonance energy transfer (FRET) that occurs when the two probes anneal to the target DNA in close proximity (20). In this study, we describe a real-time PCR assay using the LC system for the specific detection of *F. tularensis* DNA using specific primers and hybridization probes targeting the *fop*A gene.

MATERIALS AND METHODS

Bacterial species and strains: All *Francisella* strains were isolated or collected by Ohara Research Laboratory (Fukushima, Japan) and kindly provided by Dr. Hiromi Fujita. Those strains were originated from various sources such as

*Corresponding author: Mailing address: Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2623, Fax: +81-3-5285-1179, E-mail: esperanz@nih.go.jp

humans, animals, ticks and water (Table 1) (21). The bacteria were grown on Eugon agar (Becton, Dickinson and Company, Sparks, Md., USA) plates supplemented with 8% rabbit defibrinated blood (Nippon Biotest Laboratories, Inc., Tokyo, Japan) (22). All the *F. tularensis* strains, except LVS, were handled in the BSL-3 laboratory in the National Institute of Infectious Diseases (NIID), Tokyo.

To evaluate whether DNA amplification is specific for *F. tularensis*, 24 non-*Francisella* bacterial species (43 strains) were used (Table 2). Some bacteria were chosen because they either represent possible threat agents or were genetically related to these species. Others were selected because they were intracellular parasites with their life cycles similar to that of *Francisella*-bacteria. Eleven were obtained from the American Type Culture Collection (ATCC) (Rockville, Md., USA), and 6 were from the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC) (Chiba, Japan). The others were stocked and/or maintained at NIID. The inactivated *Coxiella burnetii* cultures (23) and *Wolbachia pestis* (24) genomic DNA were kindly provided by Drs. Hideto Fukushi (Department of Veterinary Medicine, Faculty of Applied Biological Science, Gifu University, Gifu, Japan) and Tetsuhiko Sasaki (Department of Biological Science, Graduate School of Science, University of Tokyo, Tokyo, Japan), respectively.

Template DNA preparation: Cultivated bacterial cells were suspended in 200 μl of PBS and inactivated at 95°C for 15 min. DNA was extracted with phenol-chloroform-isoamyl alcohol or with the SepaGene DNA Extraction Kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Purified DNA was dissolved in TE buffer (10 mM Tris-HCl [pH8.0], 1 mM EDTA [pH8.0], Nippon Gene, Tokyo, Japan) at a concentration of 10 ng/ml. All samples were aliquoted and stored at −20°C until use.

Construction of control plasmid DNA: The *fopA* amplicon (708-bp) was generated by PCR from *F. tularensis* LVS genomic DNA using the primer set reported by Higgins et al. (17) and subsequently cloned into pCR2.1 vector (Invitrogen, Darstadt, Germany). The recombinant plasmid, pCR-*fop*A, was amplified in the *Escherichia coli* DH5α, and purified by the Qiagen plasmid mini kit (Qiagen, Hilden, Germany). The absorbance of the DNA solution was measured at 260 nm using a spectrophotometer (DU 530, Beckman, Fullerton, Calif., USA), and the concentration was calculated.

Oligonucleotide primers and hybridization probes: The primers and probes were designed based on the nucleotide sequence of *F. tularensis fop*A gene (GeneBank accession no. M93695) (25) as shown in Table 3. The expected size of the DNA fragment amplified using Ft-F and Ft-R primers was 249-bp. The two hybridization probes, Ft-Flu and Ft-LcR labeled with fluorescein (FL) or LightCycler Red640 (Lc-R) dye, respectively, were designed to bind neighbored on the target DNA. The primers and probes were synthesized by Nihon Gene Research Laboratories Inc. (Miyagi, Japan).

LC-PCR assay and product detection: The amplification mixture consisting of 2 μl of 10× reaction mix (LightCycler FastStart Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany), 3 mM $MgCl_2$, 0.5 μM of each

Table 1. List of strains of *F. tularensis* used in this study

	Isolate	Origin	Year isolated	State	Country
Francisella tularensis subsp. *holarctica*					
1	Ebina	Human lymph node	1950	Miyagi	Japan
2	Yama	*Ixodes* sp.	1957	Fukushima	Japan
3	Naomatsu	Human lymph node	1968	Akita	Japan
4	Yato 96	*Lepus brachyurus*	1968	Akita	Japan
5	GIEM Miura	Human ulcer	1975	Miyagi	Japan
6	Yato 107	*Lepus brachyurus*	1979	Fukushima	Japan
7	Kikuchi	Human lymph node	1982	Fukushima	Japan
8	Ootake	*Heamaphysalis flava*	1982	Miyagi	Japan
9	Suzushichi	Human lymph node	1982	Yamagata	Japan
10	Mitsuo	Human ulcer	1983	Miyagi	Japan
11	Nikaido	Human lymph node	1984	Fukushima	Japan
12	Sami	Human lymph node	1980	Akita	Japan
13	Chiba	Human lymph node	1980	Aomori	Japan
14	Azumaya	Human lymph node	1981	Akita	Japan
15	Kokuchi	Human lymph node	1981	Yamagata	Japan
16	Kato	Human lymph node	1989	Yamagata	Japan
17	N9	*Microtus arvalis*	1948		Russia
18	N503	*Dermacentor* pictus	1949		Russia
19	Tungliao (TyH)	*Citellus* sp.	1957		China
20	N1915	*Lepus europaeus*	1962		Ukraina
21	Russian Vaccine (RV)	*F. tularensis* strain 15	unknown		Russia
22	Live Vaccine Strain (LVS)	RV	1961		
Francisella tularensis subsp. *tularensis*					
23	38 (P38)	Human lymph node	1920		USA
24	Schu	Human ulcer	1941	Ohio	USA
Francisella tularensis subsp. *novicida*					
25	U112	Water	1950	Utah	USA
Francisella philomiragia					
26	029 (Y-29)	Water	1960	Utah	USA

oligonucleotide primer (Ft-F and Ft-R), 0.2 μM FL hybridization probe (Ft-Flu), 0.4 μM LC-Red 640 probe (Ft-LcR), and 1 μl of template DNA in a final volume of 20 μl in the LC capillaries was placed in the LC instrument (Quick System 330; Roche Diagnostics), and initially incubated at 95°C for 10 min to denature the template DNA, and to activate the FastStart *taq* DNA polymerase. The amplification cycle was as follows: 40 cycles at 95°C for 10 sec, 60°C for 15 sec, and 72°C for 10 sec. The temperature transition rate was 20°C/sec. The intensity of fluorescence was monitored at the end of each extension step. After the DNA amplification cycles, melting curve analysis was performed to confirm that the obtained signals were caused by the specific amplicons. The LC software (version 3) produced the standard curve by measuring the crossing point of each standard and plotting them against the logarithmic values of construction.

Table 2. Summary of the results of the LC-PCR conducted on various bacteria strains unrelated to *Francisella*

	Species	Strain	Result of LC-PCR
1	*Bacillus anthracis*	PA I	–
2	*Bacillus anthracis*	PA II	–
3	*Bacillus cereus*	NBRC 3466	–
4	*Bacillus cereus*	NBRC 13494	–
5	*Bacillus cereus*	NBRC 15305	–
6	*Bacillus thuringensis*	NBRC 3951	–
7	*Bacillus thuringensis*	NBRC 13865	–
8	*Bacillus thuringensis*	NBRC 13866	–
9	*Bacillus subtilis*	2	–
10	*Bacillus subtilis*	3	–
11	*Bacillus subtilis*	52	–
12	*Bacillus subtilis*	62	–
13	*Bacillus subtilis*	80	–
14	*Borrelia afzelii*	P/Gau	–
15	*Borrelia burgdorferi*	B31 (ATCC 35210)	–
16	*Borrelia garinii*	FujiP2	–
17	*Borrelia garinii*	HP1	–
18	*Borrelia japonica*	612	–
19	*Brucella abortus* biovar 1	125	–
20	*Brucella canis*	QE13	–
21	*Brucella melitensis* biovar 1	16M	–
22	*Brucella suis* biovar 1	1330	–
23	*Coxiella burnetii*	Priscilla	–
24	*Coxiella burnetii*	Nine Mile	–
25	*Coxiella burnetii*	Ohio	–
26	*Escherichia coli*	DH5α	–
27	*Haemophilus influenzae* Type B	ATCC 10211	–
28	*Klebsiella pneumoniae* subsp. *pneumoniae*	ATCC 13883	–
29	*Legionella feeleii*	ATCC35072	–
30	*Legionella longbeachae*	ATCC33462	–
31	*Legionella pneumophila*	80-045	–
32	*Listeria monocytogenes*	ATCC 15315	–
33	*Mycobacterium tuberculosis*	ATCC 27294	–
34	*Ochrobactrum anthropi*	ATCC 49187	–
35	*Pasteurella aerogenes*	ATCC 27883	–
36	*Proteus mirabilis*	KH492	–
37	*Pseudomonas aeruginosa*	KH683	–
38	*Staphylococcus aureus* subsp. *aureus*	ATCC 29247	–
39	*Streptococcus pneumoniae*	ATCC49619	–
40	*Wolbachia persica*	Ref. 24	–
41	*Yersinia pestis*	Yreka	–
42	*Yersinia pseudotuberculosis*	319 (2a+)	–
43	*Yersinia enterocolitica*	Pa 177 (O9:B2)	–

Sensitivity of LC-PCR assay for *F. tularensis fopA* gene: To assess the analytical sensitivity of the LC-PCR assay, 10-fold serial dilutions of purified plasmid DNA (pCR-*fop*A) or genomic DNA of 6 strains (LVS, Miura, N503, TyH, 38 and Schu strains) were tested in triplicate. We next determined the least number of bacteria detectable by the LC-PCR assay. Single colonies of LVS strain were picked from a fresh culture and suspended in PBS (pH 7.0), and 10-fold serial dilutions were made. The genomic DNA was extracted by the SepaGene DNA Extraction Kit from each bacterial suspension and was tested in triplicate with the LC-PCR assay. For the determination of CFU, 100 μl of each dilution was spread evenly on an Eugon agar plate supplemented with 8% rabbit defibrinated blood in duplicate. The plates were incubated for 37°C for 72 h, and the number of colonies was counted.

RESULTS

Amplification of DNAs from all *F. tularensis* strains: Oligonucleotide primers and hybridization probes were designed to specifically identify *F. tularensis* (Table 3). It was shown that the LC-PCR assay successfully detected 25 of the *F. tularensis* strains tested, including 16 Japanese isolates (Fig. 1). The specific signal became detectable at 28 to 30 cycles when 1 pg of genomic DNA was used. The melting-curve analysis showed that *F. tularensis*-specific *Tm* was approximately 68°C (Fig. 2B).

Sensitivity of the real-time PCR assay: A dilution series (1 fg to 2 ng/reaction) of genomic DNA from the *F. tularensis* LVS strain was tested by the LC-PCR assay. The result showed that significant signals were detected between 20 fg and 2 ng per 20 μl of LC reaction but not at 10 fg or less (Fig. 2). When DNAs from 5 other strains (Miura, N503, TyH, 38 and Schu) were used as templates, similar detection limits were observed (data not shown). To determine whether the linear response was observed with regard to the concentration of the template DNA, known amounts of extracted DNAs were subjected to the LC-PCR. By analysis with LC software, a linear regression curve was obtained between 20 fg and 2 ng of the DNAs with an error of 0.0303 and a correlation coefficient at -1.00 (data not shown). To determine the precise copy number detectable by the LC-PCR, purified plasmid DNA

Table 3. Oligonucleotides used in this LC-PCR assay

Oligonucleotide	Sequence*	Target gene	Nucleotide position	Primer/Probe
Ft-F	5′-GGCAAATCTAGCAGGTCA-3′	*fop*A	824-841	primer
Ft-R	5′-GCTGTAGTCGCACCATTATC-3′	*fop*A	1052-1073	primer
Ft-Flu	5′-ATGGCAGAGCGGGTACTAACATGATTG -[FL]-3′	*fop*A	961-987	probe
Ft-LcR	5′-[Red640]-TGCTGGTTTAACATGGTTCTTTGGTGG-[Ph]-3′	*fop*A	989-1015	probe

*[FL], Fluorescein; [Red640], LightCycler(Lc)-Red 640-N-hydroxy-succinimide ester; [Ph], 3′-phosphorylation.

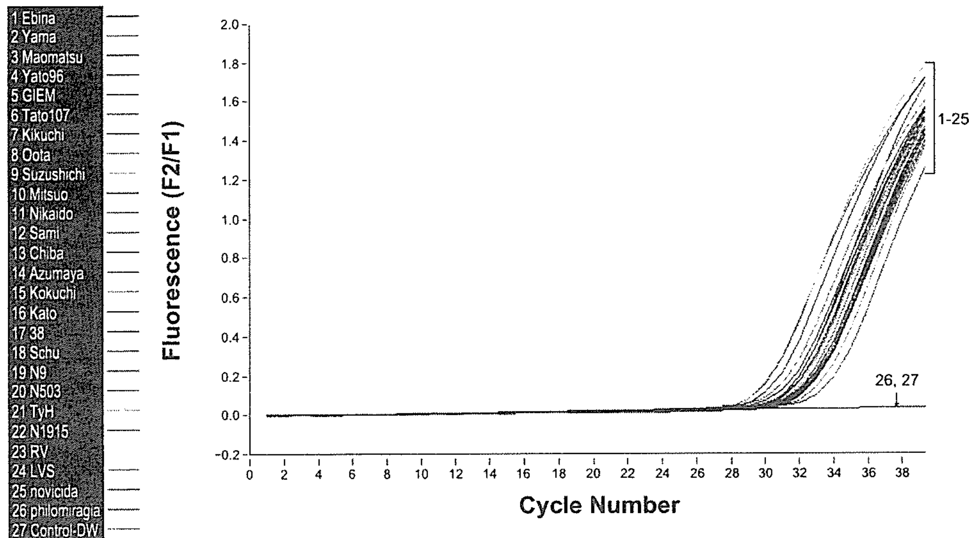

Fig. 1. Detection of the *fop*A gene by the LC-PCR assay. One pg of DNAs from 25 strains of *F. tularensis* and *F. philomiragia* was subjected to the LC-PCR.

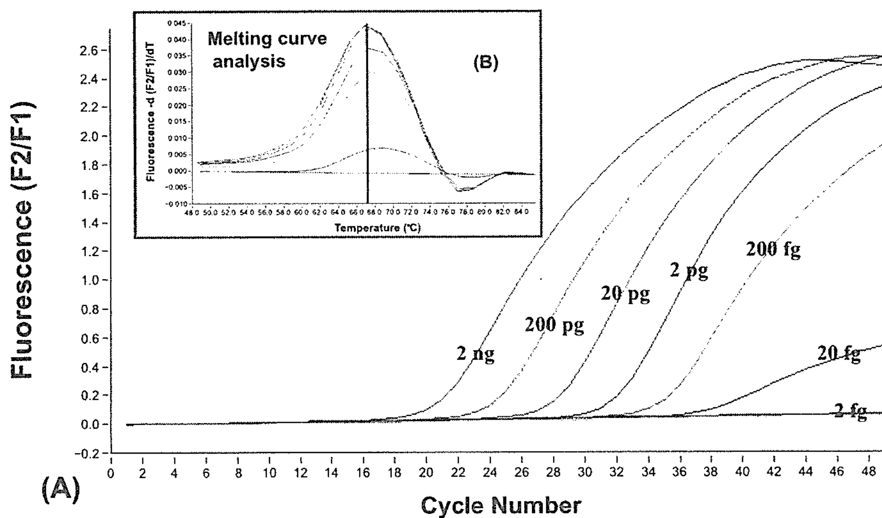

Fig. 2. Quantification analysis of the LC-PCR assay. (A) Twenty fg to 20 ng template DNA were used as template in the LC-PCR assay. (B) Melting-curve analysis of the above reactions.

containing 708-bp of *fop*A gene was subjected to the LC-PCR assay. Twenty femto grams of plasmid DNA corresponding to 10^1 copies of the gene were detected (Fig. 2). The 10-fold serially diluted *F. tularensis* (LVS) cell suspension was aliquoted into two parts. One part was processed for DNA extraction and LC-PCR, while the other was used for counting CFU. A significant fluorescent signal was detected in the LC-PCR assay when the DNAs from 1.2 CFU of the cell suspension were amplified by the LC-PCR (data not shown).

Specificity of the real-time PCR assay: To evaluate the specificity of the LC-PCR assay targeting the *F. tularensis fop*A gene, 1 ng of purified DNA from each of 43 non-*Francisella* organisms was tested. No significantly elevated signal was observed with any of the tested bacterial DNA (Table 2). The absence of amplified DNA was confirmed by agarose gel electrophoresis of LC-PCR products derived from non-*Francisella* organisms (data not shown). It is noteworthy that DNA obtained from *F. philomiragia* did not give rise to any positive response. These results indicated that this LC-PCR assay was highly specific for *F. tularensis*.

DISCUSSION

The development of a highly sensitive and specific PCR assay alleviates the problems associated with microorganisms that are found in low densities in tissue or tissue fluids, and that are difficult to cultivate. In recent years, real-time PCR has emerged as a valuable tool for the rapid identification of various microbes (26). The rapid-cycle real-time PCR method, LC, which is highly sensitive and specific for the detection and quantification of infectious agents, has been applied (27-33) to the diagnosis of a variety of pathogens. This report describes the development of a real-time PCR assay employing two primers for amplification and two hybridization probes for the specific detection of the *F. tularensis* DNA sequence in a rapid, accurate, and quantitative manner.

To establish the LC-PCR assay, we selected a specific primer pair and two independent hybridization probes that were derived from the nucleotide sequences of the *fop*A gene of *F. tularensis*. As shown in Fig. 2A, the limit of detection per reaction was 20 fg of the *F. tularensis* genomic DNA,

which corresponded to 10 copies of the *fop*A gene or 1.2 CFU of bacterial cells. The identification of *F. tularensis* can be achieved within 1 h using our standard protocol. The LC-PCR assay established in this study showed a level of sensitivity that was similar to or higher than the previously reported methods employing TaqMan technology for the detection of the *fop*A gene of *F. tularensis* (17-19). Our method is highly specific, since 37 non-*Francisella* organisms representing diverse genera did not give rise to positive responses (Table 2). Moreover, our LC-PCR assay could detect the *fop*A gene of *F. tularensis* but not that of *F. philomiragia*. Previous reports (17,19) showed that the real-time PCR assays targeting the *fop*A gene from *F. tularensis* could not discriminate it from that of *F. philomiragia*. Because the sequences of TaqMan probes used in the previous reports were highly conserved between *F. tularensis* and *F. philomiragia* (25 of 27 and 27 of 29 nucleotides, respectively), the probes could detect amplified DNAs from both *F. tularensis* and *F. philomiragia*. In contrast, two 27-mer oligonucleotides used as hybridization probes in our LC-PCR assay system contained 4 or 5 nucleotide differences when compared to the *F. philomiragia* sequence. Moreover, most of the differences were located near the 5′-end region of the second probe, Ft-LcR, indicating that hybridization of the probes to the amplified DNA from *F. philomiragia* did not take place efficiently under the conditions employed here. The involvement of two hybridization probes appeared to improve the specificity of the assay. The LC-PCR, therefore, can be used for the discrimination of *F. tularensis* from *F. philomiragia*.

Human cases of tularemia caused by either *F. tularensis* subsp. *tularensis* or *holarctica* have been reported in the United States, Spain, Sweden, Turkey, Bulgaria, and Kosovo in recent decades (34-40). Although more than 40 cases of tularemia were annually reported in Japan for 20 years after World War II, less than 10 cases per year have been reported since 1966 (41). No tularemia case was reported in 2000 or thereafter (Dr. H. Fujita, personal communication). The reason for the decline in the prevalence of tularemia has been discussed. The lifestyles of Japanese farmers and hunters who used to catch and cook wild animals as an important source of nutrition in endemic areas have changed tremendously (42).

Although tularemia is a rare disease in Japan, the possible introduction of the disease from foreign countries in which it is endemic as well as the intentional release of the bacteria should be taken into consideration (43). In recognition of the importance of tularemia, the Ministry of Health, Labour, and Welfare of Japan decided to add "tularemia" to the list of notifiable diseases in Japan beginning in 2003. The LC-PCR assay established in this study could be a powerful tool for conducting passive surveillance of tularemia through laboratory diagnosis.

ACKNOWLEDGMENTS

We thank Drs. Hiromi Fujita, Hideto Fukushi, Tetsuhiko Sasaki, Koichi Imaoka, Hiroki Kawabata, Hirotaka Takagi, Junko Amemura-Maekawa, Kenji Hirose and Toshio Yamazaki for their helpful support, especially with bacterial test strains.

This research was funded by the Health Science Research Grants of the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1. Ellis, J., Oyston, P. C. F., Green, M. and Titball, R. W. (2002): Tularemia. Clin. Microbiol. Rev., 15, 631-646.
2. Saslaw, S., Eigelsbach, H. T., Wilson, H. E., Prior, J. A. and Carhart, S. (1961): Tularemia vaccine study I. Intracutaneous challenge. Arch. Int. Med., 107, 689-701.
3. Saslaw, S., Eigelsbach, H. T., Prior, J. A., Wilson, H. E. and Carhart, S. (1961): Tularemia vaccine study II. Respiratory challenge. Arch. Int. Med., 107, 702-714.
4. Gill, V. and Cunha, B. A. (1997): Tularemia pneumonia. Semin. Respir. Infect., 12, 61-67.
5. Dennis, D. T., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Friedlander, A. M., Hauer, J., Layton, M., Lillibridge, S. R., McDade, J. E., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T. M., Russell, P. K. and Tonat, K. (2001): Tularemia as a biological weapon: medical and public health management. JAMA, 285, 2763-2773.
6. Koskela, P. and Salminen, A. (1985): Humoral immunity against *Francisella tularensis* after natural infection. J. Clin. Microbiol., 22, 973-979.
7. Syrjala, H., Koskela, P., Ripatti, T., Salminen, A. and Herva, E. (1986): Agglutination and ELISA methods in the diagnosis of tularemia in different clinical forms and severities of the disease. J. Infect. Dis., 153, 142-145.
8. Sato, T., Fujita, H., Ohara, Y. and Homma, M. (1990): Microagglutination test for early and specific serodiagnosis of tularemia. J. Clin. Microbiol., 28, 2372-2374.
9. Greiser-Wilke, I., Soiné, C. and Moennig, V. (1989): Monoclonal antibodies reacting specifically with *Francisella* sp. J. Vet. Med. B., 36, 593-600.
10. Doern, G. V. (2000): Detection of selected fastidious bacteria. Clin. Infect. Dis., 30, 166-173.
11. Grunow, R., Splettstoesser, W., McDonald, S., Otterbein, C., O'brien, T., Morgan, C., Aldrich, J., Hofer, E., Finke, E.-J. and Meyer, H. (2000): Detection of *Francisella tularensis* in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. Clin. Diagn. Lab. Immunol., 7, 86-90.
12. Forsman, M., Sandström, G. and Sjöstedt, A. (1994): Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int. J. Syst. Bacteriol., 44, 38-46.
13. Fulop, M., Leslie, D. and Titball, R. (1996): A rapid, highly sensitive method for the detection of *Francisella tularensis* in clinical samples using the polymerase chain reaction. Am. J. Trop. Med. Hyg., 54, 364-366.
14. Long, G. W., Oprandy, J. J., Narayanan, R. B., Fortier, A. H., Porter, K. R. and Nacy, C. A. (1993): Detection of *Francisella tularensis* in blood by polymerase chain reaction. J. Clin. Microbiol., 31, 152-154.
15. Junhui, Z., Ruifu, Y., Jianchun, L., Songle, Z., Meiling, C., Fengxiang, C. and Hong, C. (1996): Detection of *Francisella tularensis* by the polymerase chain reaction. J. Med. Microbiol., 45, 477-482.
16. Sjöstedt, A., Eriksson, U., Berglund, L. and Tarnvik, A. (1997): Detection of *Francisella tularensis* in ulcers of patients with tularemia by PCR. J. Clin. Microbiol., 35, 1045-1048.
17. Higgins, J. A., Hubalek, Z., Halouzka, J., Elkins, K. L., Sjöstedt, A., Shipley, M. and Ibrahim, M. S. (2000):

Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. Am. J. Trop. Med. Hyg., 62, 310-318.

18. Emanuel, P. A., Bell, R., Dang, J. L., McClanahan, R., David, J. C., Burgess, R. J., Thompson, J., Collins, L. and Hadfield, T. (2003): Detection of *Francisella tularensis* infected mouse tissues by using a hand-held PCR thermocycler. J. Clin. Microbiol., 41, 689-693.
19. Versage, J. L., Severin, D. D. M., Chu, M. C. and Petersen, J. M. (2003): Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J. Clin. Microbiol., 41, 5492-5499.
20. Wittwer, C. T., Ririe, K. M., Andrew, R. V., David, D. A., Gundry, R. A. and Balis, U. J. (1997): The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. BioTechniques, 22, 176-181.
21. Fujita, H. (1994): Short historical review of the isolates of tularemia agent in the early years of tularemia research in Japan with list of stock cultures of *Francisella tularensis* and other selected species in Ohara Research Laboratory. Ann. Rep. Ohara Hosp., 37, 5-12 (in Japanese).
22. Sato, T., Fujita, H., Watanabe, Y., Ohara, Y. and Homma, M. (1992): Microbiological and immunological techniques currently used for the diagnosis of tularemia in the Laboratory of Ohara General Hospital. Ann. Rep. Ohara Hosp., 35, 1-10 (in Japanese).
23. Hotta, A., Kawamura, M., To, H., Andoh, M., Yamaguchi, T., Fukushi, H., Amano, K. and Hirai, K. (2003): Use of monoclonal antibodies to lipopolysaccharide for antigenic analysis of *Coxiella burnetii*. J. Clin. Microbiol., 41, 1747-1749.
24. Sasaki, T. and Ishikawa, H. (1999): *Wolbachia* infections and cytoplamic incompatibility in the almond moth and the Mediterranean flour moth. Zoolog. Sci., 16, 739-744.
25. Leslie, D. L., Cox, J., Lee, M. and Titball, R. W. (1993): Analysis of a cloned *Francisella tularensis* outer membrane protein gene and expression in attenuated *Salmonella typhimurium*. FEMS Microbiol. Lett., 111, 331-336.
26. Mackay, I. M. (2004): Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect., 10, 190-212.
27. Pietilä, J., He, Q., Oksi, J. and Viljanen, M. K. (2000): Rapid differentiation of *Borrelia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi* sensu stricto by LightCycler fluorescence melting curve analysis of a PCR product of the *recA* gene. J. Clin. Microbiol., 38, 2756-2759.
28. Lachnik, J., Ackermann, B., Bohrssen, A., Maass, S., Diephaus, C., Puncken, A., Stermann, M. and Bange, F.-C. (2002): Rapid-cycle PCR and fluorimetry for detection of mycobacteria. J. Clin. Microbiol., 40, 3364-3373.
29. Blessmann, J., Buss, H., Nu, P. A., Dinh, B. T., Ngo, Q. T., Van, A. L., Alla, M. D., Jackson, T. F., Ravdin, J. I. and Tannich, E. (2002): Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. J. Clin. Microbiol., 40, 4413-4417.
30. Uhl, J. R., Adamson, S. C., Vetter, E. A., Schleck, C. D., Harmsen, W. S., Iverson, L. K., Santrach, P. J., Henry, N. K. and Cockerill, F. R. (2003): Comparison of LightCycler PCR, rapid antigen immunoassay, and culture for detection of group A streptococci from throat swabs. J. Clin. Microbiol., 41, 242-249.
31. Fournier, P.-E. and Raoult, D. (2003): Comparison of PCR and serology assays for early diagnosis of acute Q fever. J. Clin. Microbiol., 41, 5094-5098.
32. Reischl, U., Bretagne, S., Krüger, D., Ernault, P. and Costa, J.-M. (2003): Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infect. Dis., 3, 7.
33. Mangold, K. A., Manson, R. U., Stephens, E. S., Regner, L., Thomson, R. B., Jr., Peterson, L. R. and Kaul, K. L. (2005): Real-time PCR for detection and identification of *Plasmodium* spp. J. Clin. Microbiol., 43, 2435-2440.
34. Chang, M.-H., Glynn, M. K. and Groseclose, S. L. (2003): Endemic, notifiable bioterrorism-related diseases, United States, 1992-1999. Emerg. Infect. Dis., 9, 556-564.
35. Feldman, K. A., Enscore, R. E., Lathrop, S. L., Matyas, B. T., McGuill, M., Schriefer, M. E., Stiles-Enos, D., Dennis, D. T., Petersen, L. R. and Hayes, E. B. (2001): An outbreak of primary pneumonic tularemia on Martha's Vineyard. N. Engl. J. Med., 345, 1601-1606.
36. Anda, P., del Pozo, J. S., García, J. M. D., Escudero, R., Peña, F. J. G., Velasco, M. C. L., Sellek, R. E., Chillarón, M. R. J., Serrano, L. P. S. and Navarro, J. F. M. (2001): Waterborne outbreak of tularemia associated with crayfish fishing. Emerg. Infect. Dis., 7, 575-582.
37. Eliasson, H., Lindbäck, J., Nuorti, J. P., Arneborn, M., Giesecke, J. and Tegnell, A. (2002): The 2000 tularemia outbreak: a case-control study of risk factors in disease-endemic and emergent areas, Sweden. Emerg. Infect. Dis., 8, 956-960.
38. Gurcan, S., Tatman-Otkun, M., Otkun, M., Arikan, O. K. and Ozer, B. (2004): An outbreak of tularemia in western Black Sea region of Turkey. Yonsei Med. J. 45: 17-22.
39. Christova, I., Velinov, T., Kantardjiev, T. and Galev, A. (2004): Tularemia outbreak in Bulgaria. Scand. J. Infect. Dis., 36, 785-789.
40. Reintjes, R., Dedushaj, I., Gjini, A., Jorgensen, T. R., Cotter, B., Lieftucht, A., D'Ancona, F., Dennis, D. T., Kosoy, M. A., Mulliqi-Osmani, G., Grunow, R., Kalaveshi, A., Gashi, L. and Humolli, I. (2002): Tularemia outbreak investigation in Kosovo: case control and environmental studies. Emerg. Infect. Dis., 8, 69-73.
41. Ohara, Y., Sato, T. and Homma, M. (1996): Epidemiological analysis of tularemia in Japan (yato-byo). FEMS Immunol. Med. Microbiol., 13, 185-189.
42. Ohara, Y., Sato, T., Fujita, H., Ueno, T. and Homma, M. (1991): Clinical manifestations of tularemia in Japan - analysis of 1,355 cases observed between 1924 and 1987. Infection, 19, 14-17.
43. Pertersen, J. M., Schriefer, M. E., Carter, L. G., Zhou, Y., Sealy, T., Bawiec, D., Yockey, B., Urich, S., Zeidner, N. S., Avashia, S., Kool, J. L., Buck, J., Lindley, C., Celeda, L., Monteneiri, J. A., Gage, K. L. and Chu, M. C. (2004): Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. Emerg. Infect. Dis., 10, 419-425.

Clinical and Vaccine Immunology, Jan. 2007, p. 81–84 Vol. 14, No. 1
1556-6811/07/$08.00+0 doi:10.1128/CVI.00057-06
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

NOTES

Preparation of Monoclonal Antibodies for Detection and Identification of *Francisella tularensis*[▽]

Akitoyo Hotta,* Akihiko Uda, Osamu Fujita, Kiyoshi Tanabayashi, and Akio Yamada

Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

Received 13 February 2006/Returned for modification 31 March 2006/Accepted 6 November 2006

Monoclonal antibodies (MAbs) against *Francisella tularensis* were obtained. Three MAbs specifically reacted with *F. tularensis*, while four MAbs reacted with other members of the genus *Francisella* as well. Fluorescent isothiocyanate-conjugated MAbs unequivocally stained bacterial cells in specimens from experimentally infected mice. Two MAbs agglutinated *F. tularensis* antigen in the agglutination tests. These MAbs should improve methods for detection and identification of *F. tularensis*.

Francisella tularensis is a gram-negative coccobacillus that causes tularemia in humans and animals. Tularemia is traditionally diagnosed by the isolation of *F. tularensis* or the detection of specific antibodies. Isolated bacteria were subsequently identified by slide agglutination or immunofluorescence tests using anti-*F. tularensis* immune serum. Specific antibodies are frequently detected by the microagglutination test (18) in most clinical laboratories. However, because such antibodies cross-react with other bacteria (3), there is a need for an improved method for the serodiagnosis of tularemia. Antigenic analysis of *F. tularensis* as well as other members of the genus is important because *Francisella novicida* and *Francisella philomiragia* have biochemical and genetic properties similar to those of *F. tularensis* (9), although they rarely cause tularemia-like diseases (13, 22). Monoclonal antibodies (MAbs) are a useful tool for analyzing the antigenic properties of bacteria (15) because they recognize a single epitope with high specificity. Although some MAbs against *F. tularensis* lipopolysaccharide (LPS) have been produced (5, 10), MAbs against other antigenic components are not available commercially. In this study, we obtained seven MAbs that recognize at least five different epitopes carried by *F. tularensis*. Four MAbs reacted with *F. novicida* and *F. philomiragia* as well. These MAbs can be used for antigenic analyses of *Francisella* organisms as well as for the diagnosis of tularemia and tularemia-like diseases.

Twenty-six *F. tularensis* strains (15 Japanese strains and 11 non-Japanese strains), the *F. novicida* U112 strain, and the *F. philomiragia* 029 strain were kindly provided by H. Fujita, Ohara Research Laboratory, Fukushima, Japan. Two *F. philomiragia* strains (ATCC 25017 and ATCC 25018), and *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae* subsp. *pneumoniae*, *Pasteurella aerogenes*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* were propagated in our laboratory. All *F. tularensis* strains were propagated on Difco Eugon agar (Becton, Dickinson and Company, Sparks, MD) with chocolatized 8% sheep blood in a biosafety level-3 laboratory. The MAb against *F. tularensis* LPS (FB11) (Biodesign International, Saco, ME) was used as a reference, and fluorescent isothiocyanate (FITC)-labeled antirabies virus monoclonal antibody (Fujirebio Diagnostics, Inc. Malvern, PA) was used as an isotype control. All animal experiments were approved by the animal research committee of the National Institute of Infectious Diseases.

Hybridoma clones secreting MAbs (M11D3, M11H7, M13B10, M14B11, M15C6, S11E7, and U22F2) were obtained by the fusion of mouse myeloma cells (P3-X63-Ag8.653) and spleen cells from BALB/c mice, which had been immunized with the formalin-inactivated *F. tularensis* GIEM Miura (Japanese) strain, the Schu (non-Japanese) strain, or the *F. novicida* U112 strain, as described elsewhere (14). Characteristics of the MAbs (Table 1) were based on MAbs obtained from hybridoma supernatant or mice ascitic fluids. Western blotting following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the MAbs recognized at least five different epitopes carried by *F. tularensis* LVS (Fig. 1). The banding patterns obtained with the Schu and GIEM Miura strains were not different from those obtained with the LVS strain (data not shown). MAb M14B11 stained ladder-like bands having molecular masses greater than 15 kDa. Identical ladder-like bands were obtained with MAbs M11H7 and M15C6 (data not shown). These three MAbs also reacted with purified LPS (Fig. 1), a major protective antigen of *F. tularensis* (17). On the other hand, MAb M11D3, M13B10, and S11E7 reactions produced single bands with molecular masses of 40, 17, and 10 kDa, respectively, while MAb U22F2 reactions produced 41- and 43-kDa bands (Fig. 1). These four MAbs did not react with proteinase K-digested antigen (data not shown), suggesting that the MAbs recognized protein components. *F. tularensis* proteins of 10, 17, 40, 41, and 43 kDa were found to be recognized by the sera from tularemia patients (4, 12). In addition, immunoreactive membrane components of *F. tularensis* might play important roles in both the invasion of host cells and escape from phagolysososomes (6, 11). Although it is

* Corresponding author. Mailing address: Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-52851111. Fax: 81-3-52851179. E-mail: ahotta@nih.go.jp.

▽ Published ahead of print on 22 November 2006.

TABLE 1. Summary of the characteristics of monoclonal antibodies

MAb	Immunized antigen[a]	Antigen reacted (kDa)[b]	Reaction against species (no. of strains tested)[c]			Agglutination activity[d]	Ig isotype[e]
			F. tularensis (26)	*F. novicida* (1)	*F. philomiragia* (3)		
M11D3	GIEM Miura	40	+	+	+	−	M
M11H7	GIEM Miura	>15[g]	+	−	−	−	G3
M13B10	GIEM Miura	17	+	+	+	−	G1
M14B11	GIEM Miura	>15[g]	+	−	−	+	G2a
M15C6	GIEM Miura	>15[g]	+	−	−	+	M
S11E7	Schu	10	+	+	+	−	G1
U22F2	U112	41, 43	+	+	−	−	G1
FB11[f]	15	>15[g]	+	−	−	−	G2a

[a] *Francisella* strains used for immunization of mice.
[b] Molecular mass of *F. tularensis* antigen appeared in Western blotting following SDS-PAGE.
[c] Determined by indirect fluorescence assay: +, positive; −, negative.
[d] Determined by microagglutination test: +, positive; −, negative.
[e] Determined with a mouse monoclonal antibody isotyping test kit (Serotec, Oxford, United Kingdom).
[f] Reference MAb purchased commercially.
[g] Ladder-like bands of molecular mass greater than 15 kDa.

unclear whether our MAbs recognize these essential components, they may help to analyze the pathogenicity of *F. tularensis*. We are presently attempting to determine the epitopes recognized by these MAbs.

All MAbs reacted with all Japanese and non-Japanese *F. tularensis* strains but did not react with *B. abortus*, *B. melitensis*, *B. suis*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *E. coli*, *H. influenzae*, *K. pneumoniae* subsp. *pneumoniae* or *P. aerogenes* by indirect fluorescence assay. Since cross-reactions among *F. tularensis*, *Brucella* spp., and *Yersinia* spp. have been discussed by many researchers (3, 19), reactions of the MAbs against *B. abortus*, *Y. enterocolitica*, and *Y. pseudotuberculosis* were further analyzed by Western blotting. The results indicated that our MAbs did not react with the antigens of these three bacteria (data not shown). MAbs M11H7, M14B11, and M15C6 did not react with *F. novicida* or *F. philomiragia* (Fig. 1), indicating that these three MAbs were specific for *F. tularensis*. On the other hand, MAbs M11D3, M13B10, S11E7, and U22F2 appeared to recognize the conserved epitopes among *F. tularensis*, *F. novicida*, and *F. philomiragia* (Fig. 1). Since the antigens of *F. philomiragia* recognized by MAbs M13B10 and U22F2 migrated differently than those from *F. tularensis* and *F. novicida*, *F. philomiragia* seemed to be more distantly related to *F. tularensis* and *F. novicida*. This finding seems to be in good

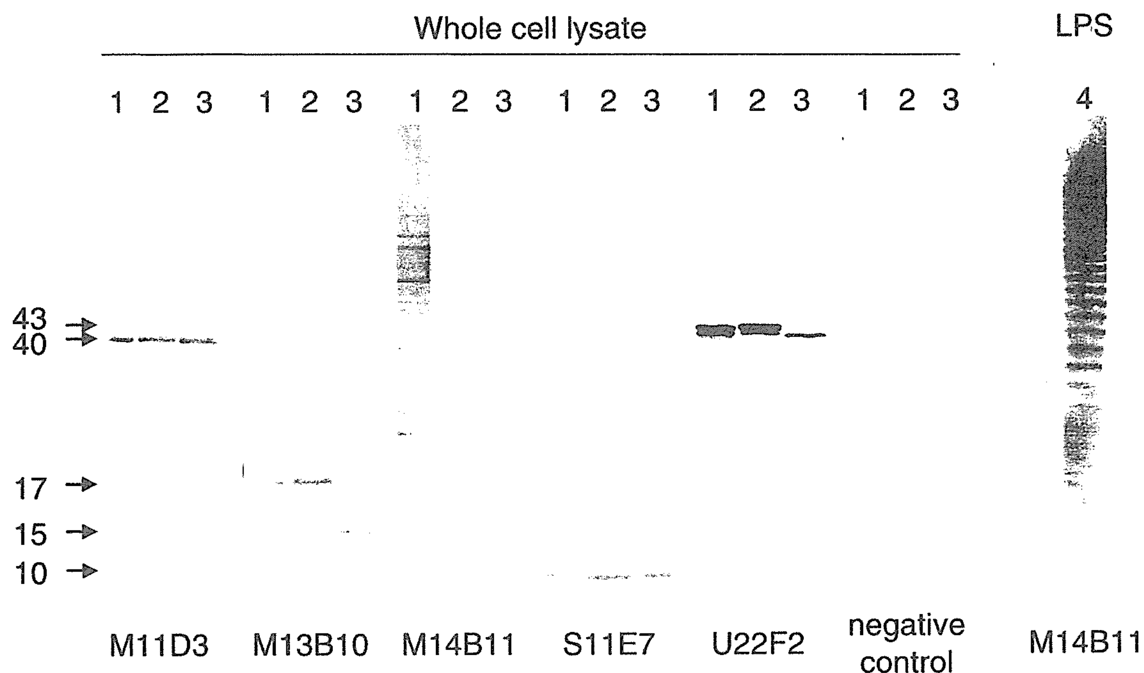

FIG. 1. Reactions of MAbs shown by Western blots following SDS-PAGE. Bacterial lysates from *F. tularensis* LVS, *F. novicida* U112, and *F. philomiragia* 029 (lanes 1 to 3, respectively) were reacted with MAbs M11D3, M13B10, M14B11, S11E7, and U22F2 and normal mouse serum (negative control). The reaction of MAb M14B11 against purified LPS from *F. tularensis* Schu (lane 4) is also shown. The positions of the molecular size markers are indicated (in kilodaltons).

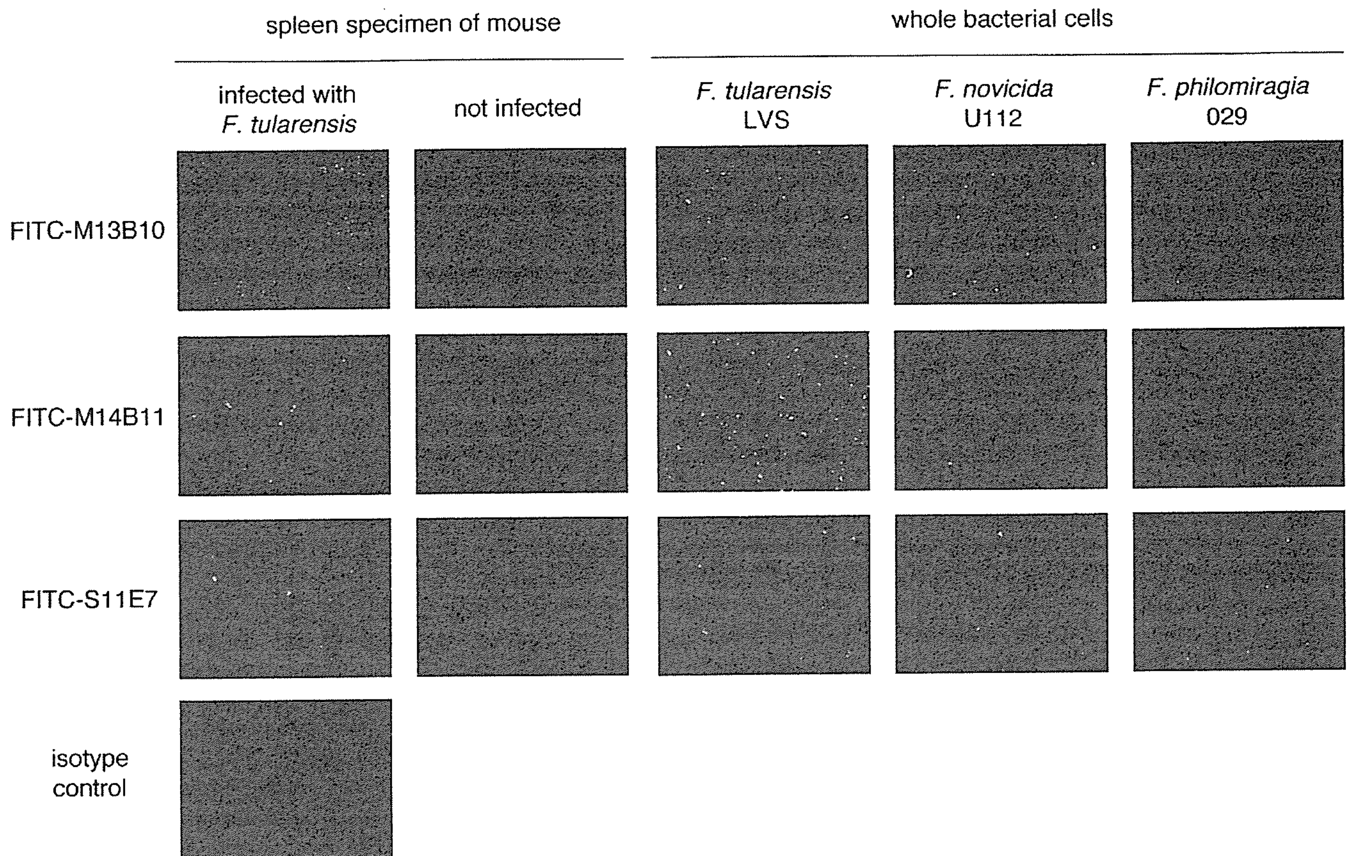

FIG. 2. Reactions of MAbs shown by DFA. FITC-labeled MAbs M13B10, M14B11, and S11E7 were reacted with the impression smears of the spleens from a mouse infected with *F. tularensis* Yama and an uninfected mouse and whole-bacteria cells of *F. tularensis* LVS, *F. novicida* U112, and *F. philomiragia* 029. FITC-labeled antirabies virus monoclonal antibody was used as an isotype control.

agreement with the view that *F. novicida* should be classified as a subspecies of *F. tularensis* (9, 13, 20). Although the numbers of strains tested were limited, it should be possible to use the MAbs to differentiate among *Francisella* species. Unusual *Francisella* organisms, including symbionts of ticks, have been found worldwide (2, 21). Although the antigenic properties of these unusual *Francisella* organisms are mostly unknown, our MAbs might help to characterize the relationships among the different *Francisella* organisms.

F. tularensis antigen was agglutinated by MAbs M14B11 and M15C6 in both the microagglutination and the slide agglutination tests (Table 1). In the slide agglutination test, a solution containing MAb M14B11 (0.2 mg/ml of purified immunoglobulin G [IgG]) agglutinated an equal volume of *F. tularensis* whole-cell suspension (an optical density at 600 nm of 1.8), while solutions containing MAb M11H7 or FB11 (in excess of 0.8 mg/ml of purified IgG) did not show any agglutination at all (data not shown). Thus, *F. tularensis* could be rapidly identified by a simple slide agglutination test using MAb M14B11.

We next determined whether the MAbs could be used to identify *F. tularensis* in the tissue of infected animals by using a direct immunofluorescent assay (DFA). IgG MAbs purified with a protein G Sepharose column (Amersham Biosciences AB, Uppsala, Sweden) were conjugated with FITC with a Fluoro *Taq* FITC conjugation kit column (Sigma-Aldrich Co., St. Louis, MO) according to the manufacturer's protocol. When impression smears of the spleens from mice infected with the Yama strain were reacted with FITC-labeled MAbs M14B11, M13B10, and S11E7, bacterial cells were readily identified by fluorescence microscopy. FITC-labeled MAbs M13B10 and S11E7 also stained bacterial cells of *F. novicida* and *F. philomiragia* (Fig. 2). These results suggest that FITC-labeled MAbs can be used to detect and identify *Francisella* organisms from clinical samples.

Tularemia has been considered to be a disease confined to the northern hemisphere and most frequently in Scandinavia, North America, Japan, and Russia (7). However, it has emerged in other geographic locations recently (16). The prevalence and distribution of *F. tularensis* have received much attention because of fears that the organisms could be used as a bioterrorism agent. Furthermore, *F. tularensis* is associated with protozoa (1) and might reside in the environment in a viable but nonculturable form (8). Therefore, it would be very useful to have a method for detecting *F. tularensis* in environmental samples such as soil and water. The MAbs obtained here appear to be ideal tools for identifying not only *F. tularensis* but also *F. novicida* and *F. philomiragia* for ecological and epidemiological studies as well as for antigenic analyses of *Francisella* organisms, the pathogens of tularemia or tularemia-like diseases.

We thank H. Fujita (Ohara Research Laboratory) for providing the bacterial strains. We also thank K. Imaoka (Department of Veterinary Science, National Institute of Infectious Diseases) for helping with the cultivation of bacteria.

This work was supported by Health and Labor Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare in Japan.

REFERENCES

1. **Abd, H., T. Johansson, I. Golovliov, G. Sandström, and M. Forsman.** 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl. Environ. Microbiol. **69:**600–606.
2. **Barns, S. M., C. C. Grow, R. T. Okinaka, P. Keim, and C. R. Kuske.** 2005. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl. Environ. Microbiol. **71:**5494–5500.
3. **Behan, K. A., and G. C. Klein.** 1982. Reduction of *Brucella* species and *Francisella tularensis* cross-reacting agglutinins by dithiothreitol. J. Clin. Microbiol. **16:**756–757.
4. **Bevanger, L., J. A. Maeland, and A. I. Naess.** 1989. Competitive enzyme immunoassay for antibodies to a 43,000-molecular-weight *Francisella tularensis* outer membrane protein for the diagnosis of tularemia. J. Clin. Microbiol. **27:**922–926.
5. **Bhatti, A. R., J. P. Wong, and D. E. Woods.** 1993. Production and partial characterization of hybridoma clones secreting monoclonal antibodies against *Francisella tularensis*. Hybridoma **12:**197–202.
6. **Clemens, D. L., B.-Y. Lee, and M. A. Horwitz.** 2005. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infect. Immun. **73:**5892–5902.
7. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin. Microbiol. Rev. **15:**631–646.
8. **Forsman, M., E. W. Henningson, E. Larsson, T. Johansson, and G. Sandström.** 2000. *Francisella tularensis* does not manifest virulence in viable but non-culturable state. FEMS Microbiol. Ecol. **31:**217–224.
9. **Forsman, M., G. Sandström, and A. Sjöstedt.** 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int. J. Syst. Bacteriol. **44:**38–46.
10. **Fulop, M. J., T. Webber, R. J. Manchee, and D. C. Kelly.** 1991. Production and characterization of monoclonal antibodies directed against the lipopolysaccharide of *Francisella tularensis*. J. Clin. Microbiol. **29:**1407–1412.
11. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjöstedt.** 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect. Immun. **71:**5940–5950.
12. **Havlasova, J., L. Hernychova, P. Halada, V. Pellantova, J. Krejsek, J. Stulik, A. Macela, P. R. Jungblut, P. Larsson, and M. Forsman.** 2002. Mapping of immunoreactive antigens of *Francisella tularensis* live vaccine strain. Proteomics **2:**857–867.
13. **Hollis, D. G., R. E. Weaver, A. G. Steigerwalt, J. D. Wenger, C. W. Moss, and D. J. Brenner.** 1989. *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease. J. Clin. Microbiol. **27:**1601–1608.
14. **Hotta, A., M. Kawamura, H. To, M. Andoh, T. Yamaguchi, H. Fukushi, and K. Hirai.** 2002. Phase variation analysis of *Coxiella burnetii* during serial passage in cell culture by use of monoclonal antibodies. Infect. Immun. **70:**4747–4749.
15. **Hotta, A., G. Q. Zhang, M. Andoh, T. Yamaguchi, H. Fukushi, and K. Hirai.** 2004. Use of monoclonal antibodies for analyses of *Coxiella burnetii* major antigens. J. Vet. Med. Sci. **66:**1289–1291.
16. **Petersen, J. M., and M. E. Schriefer.** 2005. Tularemia: emergence/re-emergence. Vet. Res. **36:**455–467.
17. **Sandström, G., A. Sjöstedt, T. Johansson, K. Kuoppa, and J. C. Williams.** 1992. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol. Immunol. **5:**201–210.
18. **Sato, T., H. Fujita, Y. Ohara, and M. Homma.** 1990. Microagglutination test for early and specific serodiagnosis of tularemia. J. Clin. Microbiol. **28:**2372–2374.
19. **Schmitt, P., W. Splettstosser, M. Porsch-Ozcurumez, E. J. Finke, and R. Grunow.** 2005. A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. Epidemiol. Infect. **133:**759–766.
20. **Sjöstedt, A. B.** 2005. Family XVII. *FRANCISELLAE*, genus I. Francisella *Dorofe'ev 1947, 176AL*, p. 200–210. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), The proteobacteria, part B. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer-Verlag, New York, NY.
21. **Sun, L. V., G. A. Scoles, D. Fish, and S. L. O'Neill.** 2000. *Francisella*-like endosymbionts of ticks. J. Invertebr. Pathol. **76:**301–303.
22. **Whipp, M. J., J. M. Davis, G. Lum, J. de Boer, Y. Zhou, S. W. Bearden, J. M. Petersen, M. C. Chu, and G. Hogg.** 2003. Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia. J. Med. Microbiol. **52:**839–842.

Available online at www.sciencedirect.com
SCIENCE @ DIRECT•
ELSEVIER
Virus Research 119 (2006) 208–215
Virus Research
www.elsevier.com/locate/virusres

A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein

Mutsuyo Takayama-Ito [a], Ken-ichi Inoue [b], Yoko Shoji [c], Satoshi Inoue [d], Toshio Iijima [b], Takeo Sakai [c], Ichiro Kurane [a], Kinjiro Morimoto [a,*]

[a] *Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Division of Systems Neuroscience, Department of Developmental Biology and Neurosciences, Tohoku University Graduate School of Life Sciences, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan*
[c] *Department of Preventive Veterinary Medicine and Animal Health, Nihon University School of Veterinary Medicine, 1866 Kameido, Fujisawa, Kanagawa 252-8510, Japan*
[d] *Department of Veterinary Sciences, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*

Received 27 September 2005; received in revised form 22 December 2005; accepted 10 January 2006
Available online 13 February 2006

Abstract

An amino acid at position 333 in the glycoprotein of several fixed rabies virus strains is responsible for the pathogenicity in adult mice. Substitution of arginine at this position largely reduces the viral pathogenicity in adult mice. Attenuation by this single amino acid substitution has been established by using escape mutants selected by monoclonal antibodies and point-mutated virus generated by reverse-genetics. A highly attenuated HEP-Flury strain, which was selected by serial passages in cell cultures, has glutamine at this position. In this study, a point-mutated rHEP^{333}R virus, having arginine at position 333, was generated and examined for the responsibility of this substitution in rabies pathogenicity. The rHEP^{333}R acquired an ability to spread and propagate in mouse brain but the parental rHEP did not. The pathogenicity of rHEP^{333}R to adult mice by intracerebral inoculation largely increased. We confirmed that an arginine at position 333 contributed to reversion of the pathogenicity in a highly attenuated HEP-Flury strain.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Rabies virus; Glycoprotein; Pathogenicity

1. Introduction

Rabies virus targets the central nervous system (CNS) and causes fatal encephalitis in almost all species of mammals. Rabies virus belongs to the genus *Lyssavirus* of the family Rhabdoviridae and has an unsegmented negative-sense RNA as the viral genome (Tordo et al., 1986). The genome is about 12 kb in length and encodes five genes for the structural proteins; N, P, M, G, and L. The G protein of rabies virus forms spike projection on the virus particle (Gaudin et al., 1992). Attachment of the G protein with a cellular receptor contributes to retrograde axonal transport and viral distribution in the brain (Etessami et al., 2000; Mazarakis et al., 2001; Yan et al., 2002). Furthermore, the G protein is a main target for virus-neutralizing antibodies (VNAs) (Wiktor et al., 1973; Cox et al., 1977; Perrin et al., 1985). Thus, the G protein has been considered to be a major contributor in pathogenicity of rabies virus.

Various monoclonal antibodies (mAbs) against the G protein have been used to map epitopes and to characterize rabies virus pathogenicity. MAb-escape mutants of fixed rabies virus strains with amino acid substitution of glutamine, isoleucine, glycine, methionine, or serine for arginine or lysine at position 333 of the G protein, have lost their virulence in adult mice after intracerebral inoculation (Dietzschold et al., 1983; Seif et al., 1985; Tuffereau et al., 1989). From these results, it was concluded that a positively charged amino acid at position 333 in the G protein was critical for the pathogenicity of fixed strains. It has been also reported that the amino acid substitution at position 333 affected

* Corresponding author. Tel.: +81 3 5285 1111; fax: +81 3 5285 1188.
E-mail address: kmorimot@nih.go.jp (K. Morimoto).

0168-1702/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2006.01.014

virus invasiveness into the CNS via peripheral routes (Kucera et al., 1985; Coulon et al., 1989) and a rate of cell-to-cell spread of the virus in cultured cells (Dietzschold et al., 1985). However, the mechanism of this attenuation has not been fully elucidated.

There are many fixed rabies virus strains with different levels of pathogenicity. Highly pathogenic strains such as CVS or Nishigahara kill adult mice by either intracerebral or peripheral infection. These strains exhibit high in vivo neuroinvasiveness; an ability of the virus to invade the CNS from a peripheral route of infection, and also high in vitro neurotropism; susceptibility of the virus to neuronal versus non-neuronal cells (Morimoto et al., 1998, 2000; Ito et al., 2001). Other pathogenic strains such as ERA, PV or LEP-Flury cause fatal encephalitis by intracerebral infection but not by peripheral infection, indicating that these viruses exhibit less in vivo neuroinvasiveness and higher in vitro neurotropism. Avirulent strains such as HEP-Flury or RC-HL do not cause fatal infection by either intracerebral or peripheral inoculations, suggesting that these viruses exhibit less in vivo neuroinvasiveness and lower in vitro neurotropism. The avirulent RC-HL strain, which is derived from virulent Nishigahara strain through serial passages in cell cultures, has arginine at position 333 in the G protein (Ito et al., 1994). Recently, it has been reported that regions other than position 333 in the G protein were responsible for the pathogenicity in the RC-HL strain and the arginine at position 333 did not participate in the pathogenic shift during attenuation of the RC-HL strain (Takayama-Ito et al., 2004). These data suggest that the responsibility of amino acid at position 333 for the pathogenicity depends on the strain and structure of the glycoprotein.

A highly attenuated HEP-Flury, high-egg-passage Flury strain which was derived from rabid human isolate by serial passages though brains of chicken, chick embryos, and cultured cells (Koprowski et al., 1954). The HEP-Flury strain causes transient weight loss and mild ruffled fur but does not cause death in adult mice even after intracerebral inoculation. As expected, the HEP-Flury strain has glutamine at position 333 (Morimoto et al., 1989). The LEP-Flury, low-egg-passage Flury strain, has arginine at that position and causes fatal infection after intracerebral inoculation. There are eight amino acid differences including position 333 between G proteins of these two strains (unpublished data). Thus, it is not clear about the extent to which an amino acid substitution at position 333 is responsible for the pathogenic shift during attenuation of the HEP-Flury strain.

In this study, in order to address whether the HEP-Flury strain increases its pathogenicity by a single amino acid change at position 333, we have generated a mutant virus, rHEP^{333}R having a single amino acid substitution of arginine for glutamine at position 333, and characterized its growth properties in vitro and in vivo, neuroinvasiveness, its ability to induce VNA, and pathogenicity.

2. Materials and methods

2.1. Cells and viruses

Mouse neuroblastoma (NA) cells were maintained in Eagle's minimum essential medium (MEM) (SIGMA) containing 10% fetal bovine serum (FBS) (GIBCO). Baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's MEM (SIGMA) supplemented with 10% FBS. A recombinant HEP-Flury (rHEP) virus was rescued from the frill genome plasmid of HEP-Flury strain and propagated in NA or BHK-21 cells (K. Inoue et al., 2003). A highly pathogenic CVS strain was propagated in suckling mouse brains.

2.2. Rescue of mutant rHEP^{333}R virus

For making a mutant rHEP^{333}R, full-length genome plasmid pHEP^{333}R with the genomic backbone of HEP-Flury strain was constructed in which arginine at position 333 in the G protein was encoded instead of glutamine (Fig. 1A). The rHEP^{333}R virus was rescued using the method as described before (K. Inoue et al., 2003). Briefly, BHK-21 cells were grown overnight and transfected with 2.0 μg of full-length genome plasmid (pHEP^{333}R), 0.5 μg of pH-N, 0.25 μg of pH-P, 0.1 μg of pH-L, and 0.15 μg of pH-G using TransIT LT-1 (Panvera) according to the manufacture's protocol. After 16 h, the cells were washed once and maintained for 2 days in Dulbecco's modified Eagle's MEM supplemented with 10% FBS. The culture medium was transferred to NA cells and incubated further 3 days. The rescued viruses were propagated in NA cells and stored at −80 °C until use. To confirm nucleotide sequence of the rescued virus, sequencing of the G gene using ABI PRISM 310 Genetic Analyzer (Applied Biosystems) was performed.

2.3. Virus titration

Viral titers were determined by direct fluorescent test using NA cells. NA cells in 96-well plate were inoculated with serial 10-fold dilution of virus and incubated at 37 °C for 2 days. Cells were fixed with 80% acetone for 20 min and stained with FITC-labeled anti-rabies mAb (Centcor). Antigen-positive foci were counted under a fluorescent microscope (OLIMPUS) and calculated as focus forming unit (ffu) per millilitre.

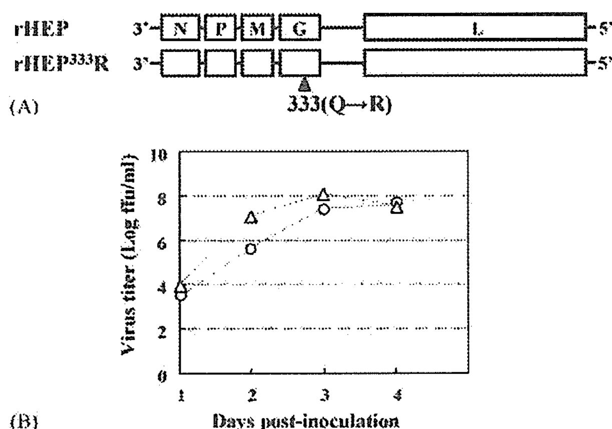

Fig. 1. (A) Schematic representation of genomes of rHEP and rHEP^{333}R. (B) The virus growth curves of rHEP (open circle) and rHEP^{333}R (open triangle) in neuroblastoma NA cells. NA cells were infected with either virus at an m.o.i. of 0.01. Virus titers in the cultured medium of the infected cells were determined by focus forming assay.

2.4. Inoculation of virus to mice and collection of mouse brains

The animal studies have been approved by the Committee for Animal Experimentation of National Institute of Infectious Diseases. Six-week-old ICR mice (Japan SLC Inc.) were inoculated with 10^5 ffu/0.03 ml of rHEP or rHEP^{333}R by intracerebral inoculation under diethyl ether anesthesia, and the mice were observed for 14 days for death or clinical signs of rabies. To determine 50% lethal dose (LD_{50}) of each virus in adult mice, groups of six ICR mice were intracerebrally inoculated with 0.03 ml of serial 10-fold dilution of rHEP or rHEP^{333}R. The LD_{50} of each virus was calculated by the method of Reed and Muench (1938). To determine the virus growth in mouse brains, the virus-infected C57BL/6 mice were anesthetized with diethyl ether, and the brains were removed and weighted. The brains were homogenized in nine-fold volume of phosphate-buffered saline (−) [PBS (−)], and the homogenates were centrifuged at $4000 \times g$ for 10 min at 4 °C. Virus titers of the supernatant were determined by direct fluorescent test as describe above and were expressed as ffu per total of brain.

2.5. Anti-rabies virus-neutralizing antibodies (VNAs) in mouse sera

Sera of the mice inoculated with rHEP or rHEP^{333}R were collected for determination of VNA titers against rabies virus. The VNA titers were determined by a modified rapid fluorescent focus inhibition test (Wiktor et al., 1984). Briefly, mouse sera were diluted into 10-fold with Eagle's MEM containing 0.2% bovine serum albumin, and heated at 56 °C for 30 min to inactivate complement. Serial four-fold dilutions of the sera were prepared and equal volume of virus suspension of rHEP or rHEP^{333}R were added into each well. After incubation at 37 °C for 1 h, 100 μl of sera and virus suspension mixture was transferred into NA cells in 96-well plate. After 2 days incubation at 37 °C, the direct fluorescent test was carried out. VNA titers were defined as the highest serum dilution that neutralized 50% of the challenge virus and normalized to international unit (IU) using the World Health Organization (WHO) anti-rabies antibody standard.

2.6. RNA extraction and RT-PCR

RT-PCR for detecting rabies virus genomic RNA was performed with total RNAs extracted from mock-infected or infected C57BL/6 mouse brains. Total RNAs were extracted with ISOGEN (Nippongene) according to the manufacture's protocol. The cDNAs of rabies genomic RNAs were synthesized using sense primer [N5-a: 5′-ATG GAT GCC GAC AAG ATT GT-3′] with AMV Reverse Transcriptase XL (Takara Bio Inc.), and the N gene of viruses was amplified using N5-a primer and antisense primer [N3-a: 5′-CCC ACT CTG ATT GCC GAA TA-3′]. PCR amplifications were carried out in a volume of 25 μl with Ex-Taq (Takara Bio Inc.). The mixture was denatured at 94 °C for 5 min, and then subjected to 30 cycles of reactions at 94 °C for 30 s, at 50 °C for 30 s, and at 72 °C for 1 min, and the following reaction at 72 °C for 7 min. For an internal control, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was amplified by using G3PDH sense primer [5′-TGC CAA GGC TGT GGG CAA GGT CAT-3′] and antisense primer [5′-AAC AGG GTG GTG GAC CTC ATG GCC-3′]. Sizes of the PCR products of N gene and G3PDH were 684 and 348 bp, respectively.

2.7. Immunohistochemical analysis

Six-week-old C57BL/6 mice were anesthetized by diethyl ether and were inoculated intracerebrally with 10^5 ffu per 0.03 ml of rHEP or rHEP^{333}R. After 4 days post-inoculation (dpi), mice were deeply anesthetized by etherisation, and the brains were perfused with 10% sucrose in PBS and fixed in 4% formaldehyde containing 10% sucrose. After perfusion, the brains were removed and fixed in 4% formaldehyde and 30% sucrose in PBS (−) for 1 day. The brains were stored in 30% sucrose in PBS (−) until use. Samples were frozen and serially sectioned in the coronal plane at a thickness of 50 μm. To identify virus-infected neurons, free-floating tissue sections were processed according to the avidin-biotin-peroxidase method (Vectastain; Vector Laboratories) with anti-rabies N antibody as the first antibody (S. Inoue et al., 2003). The reacted tissue sections were mounted on gelatin-coated glass slides, air-dried, and coverslipped. The slides were observed under a bright field microscope (OLIMPUS).

Adult (12–16-week-old) male Wistar rats weighing 230–260 g were first deeply anesthetized by intraperitoneal injection with pentobarbital (50 mg/kg). They were kept anesthetized by injecting 0.2 ml of (50 mg/ml) ketamine every 2–3 h. The rat was mounted in a stereotaxic frame, and the skull was exposed. Burr holes were made for electrode access according to coordinates by Paxinos and Watson (1986). The animals were inoculated with 10^4 ffu/0.5 μl of rHEP or rHEP^{333}R including pontamine sky blue (as a marker for the injection site) at hippocampus dentate gyrus region. To inject virus into the hippocampal layers, a grass-microelectrode glued to a Hamilton syringe was used. An injection site was identified by recording evoked field potentials with stimulation of the perforant pathway. Thirty-six hours after the inoculation, brains of the infected rats were removed and used for immunohistochemical studies as described above.

3. Results

3.1. Growth property of rHEP and rHEP^{333}R in cultured cells

Single point mutated rHEP^{333}R virus was made by using reverse genetics, in which glutamine was substituted with arginine at position 333 in the G protein (Fig. 1A). The growth kinetics of the parental rHEP and rHEP^{333}R viruses in mouse neuroblastoma NA cells were shown in Fig. 1B. There were no significant differences in growth in NA cell cultures between rHEP^{333}R and rHEP. The single amino acid substitution in the surface G protein did not affect growth property of these two