(DOXY) 単独使用時の再発率は10～20%にのぼるとされる。1986年のWHO専門家委員会による成人に対する推奨療法はDOXY＋リファンピシン (RFP) であった[2]。しかし，RFPは血中からのDOXYのクリアランスを早め，脊椎炎などの局所合併症に対する治療効果がDOXY＋ストレプトマイシン (SM) より劣っている。事実，エジプトで骨関節症状をともなう患者にDOXY＋RFPを用いたところ，高率に再発がみられたとの報告もある[24]。ただ，SMには治療の中断や変更をもたらすような副作用もあることから，現在はDOXY＋ゲンタマイシン (GM) が第一選択として推奨されている[1)23]。しかしながらRFPは経口で使用できることから，その利便性も無視できない。いずれにしても，2剤 (DOXY＋GM/RFP) もしくは3剤 (DOXY＋GM＋RFP) 併用 (より望ましい) が原則である[1)23]。小児にはST (トリメトプリムースルファメトキサゾール) 合剤＋GMの併用，妊婦にはRFPまたはST合剤が推奨されている[1]。

ブルセラ属菌の *in vitro* における薬剤抵抗性に関しては，トルコにおける，セフトリアキソン，SM，RFP，クウェートやメキシコでの，RFP，ST合剤などいくつかの報告があるが，感受性の低下したものの割合は問題となるほどには多くない。ただ2012年の報告[25]に，1999～2007年のあいだにエジプトで分離されたブルセラ属菌355株 (主として *B. melitensis*) に関するものがある。報告では，DOXY, GM, SMなどに対する感受性の低下は認められなかったものの，2001年以降，RFPに対して感受性の低下した株が増加し，218/270 (81%) がMIC (最小発育阻止濃度：minimum inhibitory concentration) ≧2を示していた (通常はMIC≦1)。これが世界中に普遍的な事象ではないとも考えられるが，RFPの使用時には注意が必要かもしれない。

Ⅷ おわりに

ブルセラ症は家畜衛生対策が進んでいるわが国では稀少感染症であるが，世界ではいまだに重要な人獣共通感染症のひとつである。特に家畜ブルセラ菌感染症については今後も輸入感染症として注意しておく必要があり，ブルセラ症の流行地域への渡航歴があり不明熱を示す患者については本症も疑いうる。渡航先での喫食歴，動物との接触歴など，感染機会の有無について詳細に把握することが重要である。また，ブルセラ症を疑いうる患者検体の検査依頼時には，その旨，検査室に連絡し，取扱いに注意を促すことが必要だろう。

万一，ブルセラ症と診断された (後日診断が確定する場合が多いが) 患者検体取扱時に曝露事故があった場合は，直接，病原体を取扱っていた者は予防投薬を受けたほうがよい。同室内にいた者については曝露の程度により判断するが，健康状態のフォローアップが必要である。予防投薬では通常，DOXY＋RFPの3週間投与が用いられているが，事故直後ならびに2ないし3週間後の血清抗体検査の結果，抗体価の上昇が認められれば，さらに3週間，投薬を継続する。その後，定期的に最低3カ月間の血清抗体の検査や，必要に応じて血液培養など，細菌学的検査を実施する。感染の疑いが濃厚なときは事故直後から授乳・性交等は避ける必要がある。いずれにしても，検査に安全キャビネットを使用し，基本的な取扱いを守れば，それほど実験室・検査室感染のリスクは高くない。

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Cutting Edge

Cutting Edge: *Brucella abortus* Exploits a Cellular Prion Protein on Intestinal M Cells as an Invasive Receptor

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Brucella abortus is a Gram-negative bacterium causing brucellosis. Although _B. abortus_ is known to infect via the oral route, the entry site in the gastrointestinal tract has been unclear. We found that _B. abortus_ was selectively internalized by microfold cells (M cells), a subset of epithelial cells specialized for mucosal Ag uptake. During this process, colocalization of cellular prion protein (PrP^C) and _B. abortus_ was evident on the apical surface as well as in subapical vacuolar structures in M cells. Internalization of _B. abortus_ by M cells of PrP^C-deficient ($Prnp^{-/-}$) mice was greatly reduced compared with that in wild-type mice. Furthermore, an oral infection study revealed that translocation of _B. abortus_ into the Peyer's patch was significantly lower in $Prnp^{-/-}$ than in wild-type mice. These observations suggest that orally infected _B. abortus_ invades the host through M cells by using PrP^C on the apical surface of M cells as an uptake receptor. *The Journal of Immunology*, **2012, 189: 1540–1544.**

The mucosal surface of the gastrointestinal tract is continuously exposed to vast numbers of commensal microorganisms and sporadically to pathogens. In this context, GALT such as Peyer's patches (PPs) serve as sentinels for the recognition and initiation of the immune responses against those microbes (1). One of the unique features of GALT is the lack of afferent lymphatic ducts, which necessitates the sampling of luminal Ags across the mucosal epithelium. The luminal side of the GALT lymphoid follicles is covered by the dome-shaped follicle-associated epithelium (FAE), within which are microfold cells (M cells). M cells are a unique subset of epithelial cells that actively transport luminal macromolecules through transepithelial membrane traffic, a process referred to as transcytosis (2, 3). Luminal contents transported via M cells are in turn captured by dendritic cells (DCs) residing beneath M cells to initiate mucosal immune responses, which ultimately leads to the production of Ag-specific IgA by B cells (1). Ag delivery through M cells is thus important for host defense. In contrast, the M cell-dependent Ag uptake process can be exploited by diverse pathogenic microbes, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) and *Yersinia enterocolitica* as a portal for host invasion (4). Proteins and/or oligosaccharides on the M-cell apical surface, including GPI-anchored proteins (5), are postulated to serve as receptors for these pathogens (6). In this regard, we have recently shown that the GPI-anchored protein gp2 (GP2) is specifically expressed on the apical plasma membrane of M cells and serves as an endocytic receptor for *S.* Typhimurium and *Escherichia coli* (7). We have also discovered that another GPI-anchored protein, cellular prion protein (PrP^C), is predominantly expressed on the M-cell apical surface among the intestinal epithelial cells (8), suggesting its role as a similar endocytic receptor.

Brucella abortus is a Gram-negative bacterium that causes brucellosis, a major zoonotic infection. Brucellosis manifests as undulant fever, arthritis, endocarditis, and meningitis in humans, as well as abortion and infertility in domestic and wild animals. *B. abortus* is a facultative intracellular pathogen that replicates within both phagocytic and nonphagocytic host cells (9). The organism is taken up by macrophages through a process involving initial movement on the cell surface and generalized membrane ruffling, leading to swimming internalization (10). The internalized *B. abortus* are enclosed by phagosomes with accumulated lipid rafts to form replicative vacuoles that do not fuse with lysosomes (11). The Type IV secretion system (T4SS) encoded by the *VirB* genes appears to

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Abbreviations used in this article: DC, dendritic cell; FAE, follicle-associated epithelium; GP2, gp2; h, human; Hsp60, heat shock protein 60; m, mouse; M cell, microfold cell; PP, Peyer's patch; $Prnp^{-/-}$, PrP^C-deficient; PrP^C, cellular prion protein; T4SS, Type IV secretion system; WT, wild-type.

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be essential for replicative vacuole formation, because phagosomes containing a *virB* mutant strain of *B. abortus* fuse with lysosomes to form conventional phagolysosomes (12). The inhibition of phagolysosome formation by T4SS has thus been implicated as a mechanism for the intracellular survival of *B. abortus*. Of interest, *B. abortus* expresses heat shock protein 60 (Hsp60) on its cell surface, probably via T4SS-mediated secretion. The surface-expressed Hsp60 binds to the PrP^C on macrophages (13). This interaction facilitates macropinosome formation and subsequent intracellular replication of *B. abortus* within macrophages. Although the above-mentioned in vitro studies have revealed the intracellular survival mechanisms of *B. abortus,* the in vivo infectious route of this bacterium is still unclear. *B. abortus* is classified as a food-borne pathogen; however, it remains to be elucidated how this bacterium can translocate across the mucosal epithelial barrier.

In this article, we report that *B. abortus* is efficiently internalized only into M cells among intestinal epithelial cells, suggesting a role for M cells as an entry portal for this bacterium after oral infection. We also observed colocalization of PrP^C and *B. abortus* on the apical surface of M cells. Importantly, the translocation of *B. abortus* into PPs after oral administration was significantly reduced in PrP^C-deficient mice. These observations indicate that PrP^C on M cells serves as a major uptake receptor for *B. abortus* during oral infection.

Materials and Methods

Animals

BALB/c and C57BL/6 mice were purchased from CLEA Japan. $Prnp^{-/-}$ mice (14) were backcrossed onto a C57BL/6 background. Other PrP^C-deficient ($Prnp^{-/-}$) mice (RBRC00437) were provided by RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan (15). These mice were maintained under specific pathogen-free conditions. Animal experiments were approved by the Animal Research Committees of all institutions.

Recombinant mouse prion protein preparation

To obtain constructs for fusion proteins of mouse (m) PrP^C ($mPrP^C$) with the Fc segment of human (h) IgG_1 ($hIgG_1$) (mPrP–Fc), cDNA prepared from FAE was used as a template for PCR amplification. Primers used were as follows: 5′-CGGGATCCACCATGGCGAACCTTGGCTACT-3′ (forward) and 5′-CGCTCGAGGGATCTTCTCCCGTCGTAATAG-3′ (reverse) cDNA fragments were inserted into the BamHI/XhoI cloning sites of a pcDNA3 expression vector (Invitrogen) containing a fragment encoding the Fc segment of $hIgG_1$, to obtain mPrP–Fc. Recombinant protein was prepared as described (7).

In vitro Brucella *spp. binding assay*

A total of 0.25 μg mPrP–Fc or control hIgG–Fc proteins were immobilized in 96-well flat-bottom plates (Nunc) by incubation overnight at 4°C. After washing, the wells were incubated with 1% BSA in PBS for 2 h for blocking, and then incubated for 2 h with 1×10^6 CFUs *B. abortus* 544 at 25°C. For Ab blocking, *B. abortus* were incubated with 0.5 μg/ml anti-Hsp60 Ab (Enzo Life Science) or isotype-matched control Ab (Jackson Immunolaboratory) for 15 min before binding assay. After washing five times with sterile PBS, genomic DNA was extracted from bound bacteria with a DNeasy Blood & Tissue Kit (Qiagen). Quantitative PCR was performed to quantify copy numbers of *bcsp31* (16), using the SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System (TAKARA).

Ligated intestinal loop assay

Mice were anesthetized with Avertin (0.4 mg/g) and placed on a warming pad during the procedure. Next, 50 μg/ml mPrP mAb (SAF-32; Cayman Chemical) or isotype-matched control IgG (BD Biosciences) was injected into the ligated intestinal loop containing PPs. After incubation for 30–60 min, PPs were excised and fixed with Cytofix/Cytoperm (BD Biosciences) for 1 h at 4°C. Intracellular localization of primary Abs was probed with 10 μg/ml Alexa Fluor 488-conjugated anti-mouse IgG Ab (Molecular Probes). The specimens were further treated with 20 μg/ml Rhodamine *Ulex europaeus* agglutinin-1 (UEA-1) (Vector Laboratories).

To assess the blocking effect of anti-Hsp60 Ab on *B. abortus* uptake by M cells, 1 million GFP–*B. abortus* (17), *B. abortus* 544, and *B. abortus* 544 pretreated with anti-Hsp60 Ab were injected into a ligated intestinal loop of C57BL/6 or $Prnp^{-/-}$ mice (14). After incubation, whole-mount specimens of PPs were immunostained with *B. abortus*-specific rabbit antisera (1:100 dilution) (17), together with anti-PrP mAb (44B1; Ref. 18) or GP2 mAb, followed by Alexa Fluor 594-conjugated anti-rat IgG. The specimens were analyzed with a DeltaVision Restoration deconvolution microscope (Applied Precision).

Evaluation of oral infection

C57BL/6 or $Prnp^{-/-}$ mice (four mice per group), 8–10 wk old were anesthetized with isoflurane or 50 mg/kg sodium pentobarbital. Then, mice were inoculated intragastrically by gavage with 0.2 ml 0.1 M sodium bicarbonate containing 1 million *B. abortus* 544. After 4 h, PPs were dissected and incubated at 25°C in sterile PBS containing 20 μg/ml gentamicin for 30 min. The tissues were weighed and homogenized in sterile PBS. The homogenates were plated on Thayer–Martin Selective Agar (BD) to determine CFUs.

Statistics

Statistical analysis was performed with the Mann–Whitney *U* test. Differences were considered significant at $p < 0.01$.

Results and Discussion

PrP^C on M cells serves as an endocytic receptor

Given that PrP^C is a GPI-anchored protein highly expressed on the M-cell apical surface (8) and that diverse infectious agents often use GPI-anchored proteins to gain entry into host cells (5), we hypothesized that it serves as an endocytic receptor for Ag sampling by M cells. This possibility was first examined by means of an in vivo Ab-uptake assay. We injected an anti-PrP mAb into a ligated intestinal loop containing PPs, to explore whether the mAb bound to PrP^C on the M-cell surface is internalized into M cells. The subcellular localization of the mAb was analyzed by deconvolution microscopy to obtain high-resolution images. Serial X–Y images demonstrated that the PrP mAb was efficiently internalized into vesicular structures in the cytoplasm of M cells (Fig. 1A, 1B). Internalization of the PrP mAb was observed only in M cells, and not in the surrounding FAE cells. It is unlikely that the Ab uptake was mediated by a nonspecific pinocytic pathway, because no internalization of an isotype-matched

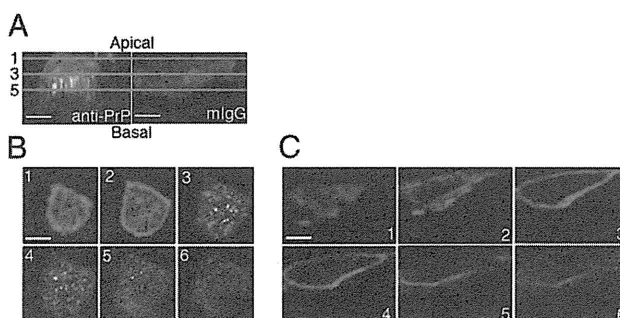

FIGURE 1. Anti-PrP mAb is taken up by murine PP M cells. A PrP mAb or isotype-matched control IgG (mIgG) was injected into the ligated intestinal loop. After incubation, PPs excised from the loop were subjected to whole-mount staining with fluorescent-conjugated secondary Ab to visualize the PrP mAb (green). The specimen was counterstained with UEA-1 (red) and then analyzed using DeltaVision deconvolution microscopy. (**A**) The X-Z image of the M cells. (**B** and **C**) Serial X–Y images from the apical (**1**) to the basal plasma membranes (**6**) of M cells shown in (A). The positions of X–Y images (1), (**3**), and (**5**) are indicated in (A). Scale bars, 5 μm.

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control IgG took place at all (Fig. 1A, 1C). These observations suggest that PrP^C on M cells can serve as an endocytic receptor for the luminal constituents to which it can bind.

B. abortus *are selectively taken up by M cells through interaction with PrP^C*

Because PrP^C plays an important role in the uptake of *B. abortus* by macrophages (13), we examined whether this is also the case in M cells. Our in vitro binding assay using a rmPrP^C–hgG–Fcγ fusion protein (mPrP^C–Fc) confirmed the interaction between PrP^C and *B. abortus* (Fig. 2A). To further examine the interaction between PrP^C and Hsp60 on *B. abortus*, the bacteria were pretreated with anti-Hsp60 Ab before the binding assay. The binding efficiency of *B. abortus* to mPrP^C–Fc was profoundly impaired in anti-Hsp60–treated *B. abortus* compared with that in bacteria not treated or pretreated with isotype-matched control Ab. (Fig. 2B).

We then asked whether PrP^C expressed on M cells binds the bacterium. The ligated intestinal loop assay verified that *B. abortus* bound exclusively to M cells among epithelial cells in FAE and villous regions (Fig. 2C and data not shown). In addition, the X-Z images indicated that *B. abortus* were internalized into the cytoplasm of M cells, where colocalization of the *B. abortus* and PrP^C was evident (Fig. 2D). These results support the idea that *B. abortus* can be taken up by M cells through its interaction with PrP^C. To further confirm this possibility, we tested whether ablation of PrP^C affects the efficiency of *B. abortus* uptake by M cells. In the ligated intestinal loop assay, we found that the number of surface-bound *B. abortus* in PrP^C-deficient (*Prnp*$^{-/-}$) mice was less than half that in wild-type (WT) mice (Fig. 3A, 3B). Moreover, the internalization of *B. abortus* into M cells was markedly reduced in *Prnp*$^{-/-}$ compared with WT mice (Fig. 3B). We also examined the effect of anti-Hsp60 Ab on the interaction between PrP^C on M cell and *B. abortus*. Binding and internalization of *B. abortus* to M cells were decreased in anti-Hsp60–treated *B. abortus* (Fig. 3C). Taken together, these observations suggest an important role for PrP^C, via interaction with Hsp60 on the bacterial surface, in the entry of *B. abortus* into M cells.

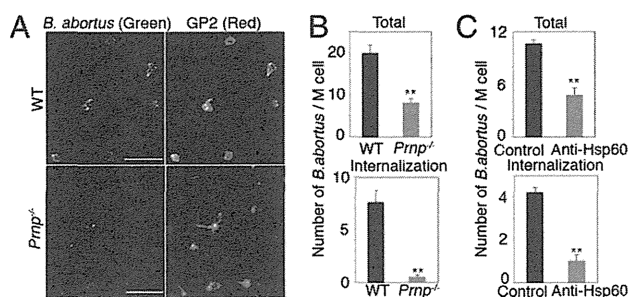

FIGURE 3. Decreased uptake of *B. abortus* by PP M cells in *Prnp*$^{-/-}$ mice. (**A**) GFP–*B. abortus* were injected into the ligated intestinal loop of anesthetized WT or *Prnp*$^{-/-}$ mice. After incubation, PPs excised from the loop were subjected to whole-mount staining with *B. abortus*-specific antisera (green) and GP2 mAb (red), and then analyzed using a DeltaVision deconvolution microscope. The X–Y images of FAE in WT mice (*upper panels*) and *Prnp*$^{-/-}$ (*lower panels*) are shown. Scale bars, 20 μm. (**B**) Quantitative analysis of the number of *B. abortus* associated with the apical surface of M cells and internalized into M cells between WT and *Prnp*$^{-/-}$. (**C**) Quantitative analysis of the number of *B. abortus* associated with the apical surface of M cells and internalized into M cells in the presence or absence of anti-Hsp60 Ab pretreatment. Data are expressed as the mean ± SD of 15 different samples for each group. $^{**}p < 0.01$.

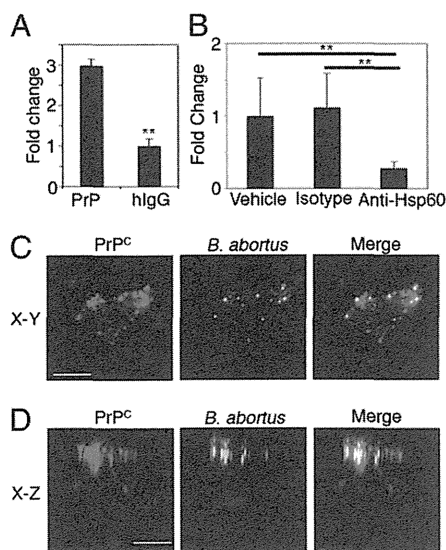

FIGURE 2. *B. abortus* bind to PrP^C in vitro and in vivo. (**A** and **B**) In vitro binding assay with rmPrP^C–Fc or control hIgG–Fc protein. *B. abortus* were pretreated with anti-Hsp60 Ab or isotype-matched control Ab before the interaction with the mPrP–Fc. Data are means and SE ($n = 3$). $^{**}p < 0.01$. (**C** and **D**) GFP–*B. abortus* were injected into the ligated intestinal loop of anesthetized C57BL/6 mice. After incubation, PPs excised from the loop were subjected to whole-mount staining with *B. abortus*-specific antisera (green) and PrP mAb (red), and then analyzed using a DeltaVision deconvolution microscope. The X–Y (C) and X–Z (D) images of M cells are shown. Scale bars, 5 μm.

B. abortus *enters the host via M cells*

To gain further evidence for PrP^C-dependent uptake of *B. abortus*, we performed oral infection with *B. abortus* in *Prnp*$^{-/-}$ and WT mice. After oral administration, a substantial number of viable *B. abortus* organisms were detectable in PPs of WT mice; by contrast, the bacteria were nearly undetectable in *Prnp*$^{-/-}$ mice (Fig. 4). Taken together with the above observations made with the ligated loop assay, this result underscores the biological significance of PrP^C in the uptake of *B. abortus*, as well as supports the idea that *B. abortus* enters the host through M cells, using PrP^C.

Prion protein is the causative agent of the transmissible spongiform encephalopathies. According to the "prion hypothesis," the infectious isoform of prion protein, termed

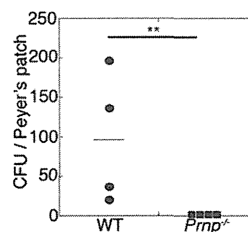

FIGURE 4. *B. abortus* infection through the gastrointestinal tract in WT and *Prnp*$^{-/-}$ mice. C57BL/6 or *Prnp*$^{-/-}$ mice were fed 1 × 10^6 CFU of *B. abortus*. After 4 h, bacterial translocation to Peyer's patches was examined. The horizontal solid line shows the average. Data are expressed as the mean ± SD of four different samples for each group. Representative data of three independent experiments are shown. $^{**}p < 0.01$.

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PrP^{Sc}, replicates by interacting with cellular PrP^C and mediating its conformational change into the disease-causing PrP^{Sc} (19). Compared with its well-defined pathological significance, the physiological function of PrP^C remains unclear. PrP^C is highly expressed not only by cells in the CNS but also by follicular DCs, mature myeloid cells, and activated T cells. This distribution suggests involvement of PrP^C in immune surveillance (20).

Our present study defines a novel role for PrP^C as an M-cell receptor for the uptake of pathogenic bacteria. PrP^C on macrophages has been reported to recognize surface-exposed Hsp60 of *B. abortus* and to facilitate internalization of the bacteria (13); however, Fontes et al. (21) reported a contradictory result. Using $Prnp^{-/-}$ mice, they showed that *B. suis* infection is independent of PrP^C expression. By contrast, WT macrophages had a greater tendency to be infected with *B. abortus* than did $Prnp^{-/-}$ macrophages, although no significant difference between WT and $Prnp^{-/-}$ macrophages was found in intracellular multiplication of *B. abortus*. To shed more light on these observations, we performed oral infection with *Brucella* spp. in WT and $Prnp^{-/-}$ mice. After oral administration, a substantial number of *B. suis* organisms were detectable in both WT and $Prnp^{-/-}$ PPs (data not shown), whereas the translocation of *B. abortus* into PPs was significantly reduced in $Prnp^{-/-}$ mice (Fig. 4). These data are consistent with the observation by Fontes et al. (21). In addition, these authors discussed the spatial proximity of *Brucella* spp. and PrP^C during the early stage of infection. In accordance, our ligated loop assay showed that the internalized *B. abortus* were surrounded by PrP^C in the cytoplasmic vacuolar compartment of M cells (Fig. 2D). Taken together, these results suggest that *B. abortus* is efficiently taken up by M cells in a PrP^C-dependent manner. However, *B. suis* might invade the host independently of PrP^C.

Interaction of PrP^C and Hsp60 family proteins has been demonstrated by several approaches, including a yeast two-hybrid screening as well as a pull-down assay (22). Our in vitro binding assay confirmed the interaction between PrP^C and Hsp60 on *B. abortus* (Fig. 2B). We also showed that PrP^C on M cells interacts with Hsp60 on *B. abortus* (Fig. 3C). Nevertheless, we cannot formally exclude an alternative and mutually not exclusive possibility that PrP^C on M cells acts as a scaffold to coordinate several proteins in a complex, with the complex mediating *B. abortus* internalization (23). The Hsp60 proteins have been recognized as immunodominant Ags of many microbes (24). Hsp60 normally resides in the bacterial cytoplasm, but the protein can be secreted via T4SS and expressed on the outer membrane of the bacteria. In fact, the presence of Hsp60 on the bacterial surface is not restricted to *B. abortus*. A similar phenomenon has also been shown for other bacteria (25–28). The exposure of Hsp60 on the surface appears to increase bacterial adherence to host cells. PrP^C expressed on the apical plasma membrane of M cells in the GALT thus may contribute to immunosurveillance on the mucosal surface by promoting transcytosis of bacteria that express Hsp60 at their surface. This idea is concordant with the observation that *H. pylori* translocation from the intestinal mucosa into PPs, possibly via the M cells, is essential for the induction of humoral and cellular immunity against this pathogen (29).

Accumulating evidence supports the idea that many infectious agents and their toxins use GPI-anchored proteins to gain entry into host cells (5). For example, CD48 on macrophages and mast cells contributes to phagocytosis of *E. coli* via FimH recognition (30). On the basis of our observations that PrP^C and GP2 proteins on the apical plasma membrane of M cells can serve as receptors (this study and Ref. 7), M cells also seem to use GPI-anchored proteins for intestinal immunosurveillance. In conclusion, our findings indicate that the PrP^C-dependent route of bacterial uptake by M cells can be exploited for *B. abortus* invasion into the host. Once *B. abortus* penetrate M cells, the bacteria are capable of surviving inside DCs, which accumulate beneath the M cells, by forming replicative vacuoles with subsequent systemic spread to other organs. This model offers a new insight into the pathogenesis of *B. abortus* infection, a disease that leads to significant economic losses for cattle and other domestic animals and, in turn, transmission to humans. The disruption of the Hsp60–PrP^C interaction on the mucosal surface may provide a useful therapeutic target for protection against *B. abortus* infection.

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Disclosures

The authors have no financial conflicts of interest.

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ORIGINAL ARTICLE

Seroprevalence of Tularemia in Wild Bears and Hares in Japan

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Impacts

- Serological assays for tularemia were performed with 431 Japanese black bears and 293 Japanese hares samples.
- All eight seropositive samples were originated from Japanese black bears from the Tohoku district, northeastern region of the Honshu, Japan.
- Japanese black bears can be used as a sentinel for tularemia.

Keywords:
Francisella tularensis; tularemia; wild animals; seroprevalence

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Summary

Tularemia is a zoonotic disease caused by *Francisella tularensis*. The distribution of the pathogen in Japan has not been studied well. In this study, seroprevalence of tularemia among wild black bears and hares in Japan was determined. Blood samples collected from 431 Japanese black bears (*Ursus thibetanus japonicus*) and 293 Japanese hares (*Lepus brachurus*) between 1998 and 2009 were examined for antibodies against *F. tularensis* by micro-agglutination test (MA) or enzyme-linked immunosorbent assay. By subsequent confirmatory tests using western blot (WB) and indirect immunofluorescence assay (IFA), eight sera from Japanese black bears were definitely shown to be seropositive. All of these eight bears were residents of the northeastern part of main-island of Japan, where human tularemia had been reported. On the other hand, no seropositive Japanese hares were found. These results suggest that Japanese black bears can serve as sentinel for tularemia surveillance and may help understand the distribution of *F. tularensis* throughout the country. This is the first report on detection of antibody to *F. tularensis* in black bears of Japan.

Introduction

Tularemia is a zoonotic disease caused by *Francisella tularensis*, highly infective, intracellular gram-negative coccobacilli. It is primarily a disease of wild animals: mainly lagomorphs and rodents. The disease occurs throughout the northern hemisphere including North America, Russia, Europe and Japan. In North America and Europe, 100–200 human tularemia cases are reported every year (Ellis et al., 2002). Humans are infected through contact with infected animals, arthropod bites, ingestion of contaminated water or food, and inhalation of infective aerosols (Ellis et al., 2002). The clinical type and severity of the disease is dependent on the route of infection. Predominant symptoms are high fever, enlarged lymph nodes, and ulcer at the site of bacterial entry (Ellis et al., 2002). In animals, the severity of the disease varies among species. In susceptible animals such as mice severe collapses are followed by a fatal septicaemia. Other animal species such as cats, dogs and cattle are relatively resistant to the infection (Hopla, 1974).

Understanding of the distribution of the pathogen in animal populations is of particular importance when studying zoonoses. The seroprevalence of *F. tularensis* in wild animals in North America and Europe has been reported for bears (Binninger et al., 1980; Chomel et al., 1998), hares (Mörner et al., 1988; Frölich et al., 2003), rabbits (Shoemaker et al., 1997; Berrada et al., 2006) and wild boars (Al Dahouk et al., 2005). These data are indispensable to assess the risk of future occurrence of tularemia in humans and domestic animals as well as to identify the natural reservoir of *F. tularensis*.

In Japan, tularemia was first reported in 1924, and approximately 1400 human cases have been reported since then (Ohara et al., 1991). The annual incidence of tularemia has decreased from the middle of the 1960s and it became extremely rare thereafter (Ohara et al., 1996).

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Most of human cases occurred in the Tohoku district, the northeastern part of the largest island, Honshu, Japan. The pathogens had been isolated from humans, hares, ticks, and shrew-mole, and a number of wild animals (such as hare, bear, or squirrel) have been suggested to have epidemiological links to human infections (Ohara et al., 1996). However, epidemiological study on wild animals is scarce and the distribution of *F. tularensis* in environment is not well understood.

We developed several tools for diagnosis of tularemia, such as monoclonal antibodies (Hotta et al., 2007), and protocols for DNA amplification and detection (Fujita et al., 2006; Uda et al., 2007). We also reported molecular epidemiological characteristics of Japanese *F. tularensis* isolates (Fujita et al., 2008). In this study, to assess the potential risk of occurrence of tularemia by understanding the distribution of *F. tularensis* in wild animals, we investigated whether Japanese hares and black bears have specific antibodies against *F. tularensis*.

Materials and Methods

Blood samples

Sera or plasma from 431 wild Japanese black bears were collected from 11 prefectures, Iwate, Fukushima, Ibaraki, Yamanashi, Nagano, Gifu, Shiga, Kyoto, Hyogo, Tottori and Tokyo between 1998 and 2007 (Fig. 1).

Samples from 293 wild Japanese hares were collected from nine prefectures, Aomori, Iwate, Akita, Yamagata, Fukushima, Niigata, Kochi, Miyazaki and Kagoshima during the winters (November–April) from 2005 to 2009 (Fig. 1). Apparently healthy wild Japanese hares were captured by licensed hunters. The blood samples were collected onto filter papers (Toyo-Roshi Ltd, Tokyo, Japan) or into plastic tubes. The filter papers were incubated with 1 ml of phosphate-buffered saline (PBS), pH 7.2, containing 0.5% (vol/vol) Tween 20 at 4°C for 4 h on a rotator. After centrifugation at 13 000 ***g*** for 3 min, the supernatant was collected and stored at −80°C until use. Because the filter paper was designed to retain 250 μl of whole blood, resulting extracts were regarded as a 1 : 50 dilution of the sera (De Swart et al., 2001). Rabbit defibrinated blood (800 μl; Nippon Biotest Laboratories Ltd, Tokyo, Japan) mixed with the sera from *F. tularensis* immunized and normal rabbits (200 μl) were used as positive and negative control, respectively. The blood samples collected to tubes were ordinarily processed to obtain sera.

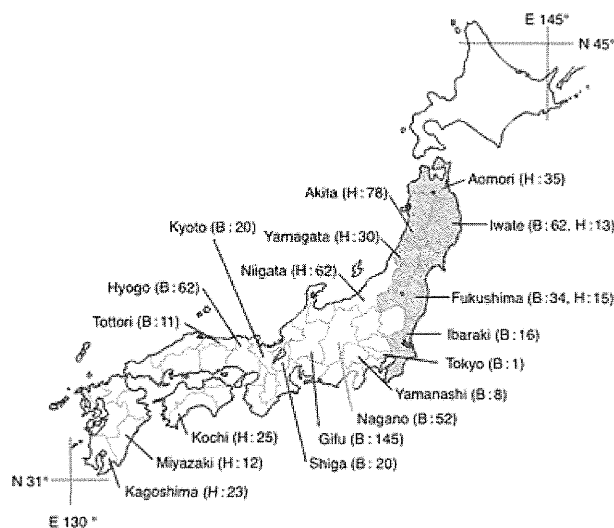

Fig. 1. Map of Japan showing the areas where samples were collected. The numbers of samples collected in each area was shown in parentheses (B, Japanese black bears; H, Japanese hares). The areas coloured grey are the prefectures where more than 50 cases of human tularemia have been reported previously (Ohara et al., 1996).

Bacterial antigens

Francisella tularensis (Yama strain), *Francisella novicida* (U112 strain), and *Francisella philomiragia* (029 strain) were kindly provided by Dr Hiromi Fujita, Ohara Research Laboratory, Fukushima, Japan. *Francisella tularensis* were propagated on Difco™ Eugon agar (Becton, Dickinson and Company, Sparks, MD, USA) with chocolatized 8% (vol/vol) sheep blood under the biosafety level 3 condition. *Francisella tularensis* LPS was purified using a LPS Extraction kit (iNtRON Biotechnology, Kyungki-Do, Korea) according to the protocol provided by the supplier. *Brucella abortus*, *Brucella canis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pasteurella aerogenes* were propagated in our laboratory as described previously (Hotta et al., 2007).

Micro-agglutination test (MA)

Sera or plasma samples from Japanese black bears and Japanese hares were screened by MA according to Sato et al. (1990). Twenty-five microlitres of 2-fold serial dilution of samples were mixed with an equal volume of antigen solution in wells of a round type micro-titre plate. Judgment was made after incubation at 37°C for 18 h. The agglutination titre was expressed as the reciprocal of the highest serum dilution showing a positive response to the antigens.

Enzyme-linked immunosorbent assay (ELISA)

The extracts from the filter paper were screened by ELISA. Six micrograms of purified *F. tularensis* LPS was dispensed into wells of a flat type 96-well microtitre plate and the plate was incubated at 4°C overnight. After washing five times with PBS containing 0.1% (vol/vol) of Tween 20

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(PBST), the wells were incubated with PBST containing 3% (wt/vol) non-fat milk at RT for 1 h. After further washing with PBST, samples were added to the wells at a final dilution of 1 : 100 and the plate was incubated at 37°C for 1 h (Shoemaker et al., 1997). The plate was further incubated with 1 : 8000 horseradish peroxidase (HRP) conjugated anti-rabbit IgG (ICN Products; ICN Pharmaceuticals, Inc., Aurora, OH, USA) at 37°C for 1 h. The bound conjugate was colour developed by addition of 100 μl of substrate solution (0.003% H_2O_2, 0.05 M citric acid and 1 mg/ml of 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid). Absorbance at 405 nm was read by the ELISA reader model 680XR (BioRad, Hercules, CA, USA). Sera from immune and normal rabbits were used as positive and negative control, respectively. All samples were tested in duplicate and the samples that showed OD value over the cut-off value (mean + 2SD) were considered as positive (Al Dahouk et al., 2005).

SDS-PAGE and western blotting (WB)

Whole cell lysate and purified LPS of *F. tularensis* Yama strain were subjected to SDS-PAGE using 12.5% gel and antigens were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corporation, Bedford, MA, USA). After incubating in Immunoblock (Dainippon Sumitomo Pharma, Tokyo, Japan) at RT for 1 h followed by several washings with PBST, the PVDF membrane was incubated with the samples appropriately diluted with a 4-fold dilution of Immunoblock (Dainippon Sumitomo Pharma) at RT for 1 h. Dilution of Japanese black bear and Japanese hare samples were 1 : 1000 or 1 : 200 times, respectively. After further washings with PBST three times, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (ICN Products; ICN Pharmaceuticals, Inc.) or HRP-conjugated recombinant protein A (Pierce, Rockford, IL, USA) at a dilution of 1 : 8000 at RT for 1 h. Finally, antigen reacted with the samples were visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.02% 3,3′-diaminobenzidine (Wako Pure Chemicals, Osaka, Japan) and 0.003% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). Samples were considered to contain specific antibodies when the typical LPS ladder-banding pattern was recognized (Al Dahouk et al., 2005) regardless of whether there were high background reaction. Mouse monoclonal antibody against LPS and serum from mouse experimentally infected with *F. tularensis* were used as positive control.

Indirect immunofluorescence assay

The whole bacterial cells of *F. tularensis* Yama strain suspended in 10 μl saline were placed onto each well of the 24 spots slides (Matsunami Glass Ind., Ltd, Osaka, Japan), air-dried, and fixed with pure methanol at RT for 15 min. Twenty to 160-fold dilution of samples were added to the slides and incubated at 37°C for 30 min. After washings with PBS and distilled water, the slides were incubated with 10 μl of protein A conjugated with fluorescent isothiocyanate (Zymed Laboratories, Invitrogen Immunodetection, Carlsbad, CA, USA) at a dilution of 1 : 200 with PBS at 37°C for 45 min. The specific fluorescence was observed under a Olympus BX51 UV microscope (Olympus, Tokyo, Japan). Because a number of non-specific reactions were observed at dilution 1 : 20, samples were considered positive when they reacted with the antigens at dilutions of greater than 1 : 40.

Criterion of positive reaction

When samples which tested positive in MA or ELISA gave rise to positive reactions both in WB and immunofluorescence assay (IFA), we considered that these samples contained specific antibody directed to *F. tularensis.*

Cross-reactivity with other bacterial antigen

The samples reacted with *F. tularensis* in both WB and IFA were further tested for their reactivity to other bacterial antigens including *F. novicida*, *F. philomiragia*, *B. abortus*, *B. canis*, *E. coli*, *K. pneumoniae* and *P. aerogenes* by ELISA.

Results

Screening assays

At first, 431 sera or plasma from Japanese black bears and 47 sera from Japanese hares were screened for the antibodies to *F. tularensis* using the MA test. Sixteen samples obtained from black bears of Iwate and seven from Fukushima prefectures agglutinated the antigen with titres from 10 to 80 (Table 1). No sample originated from other areas showed agglutination at all. Forty-seven sera from hares did not show any agglutination (data not shown). Because of limited amount of samples, all blood samples of hares extracted from the filter papers were tested by ELISA. Out of 293 samples, only one sample of a hare captured in Akita showed high OD value (1.47).

Confirmatory assays

Twenty-four samples (23 bears and one hare) tested positive in screening assays were subjected to WB and IFA together with several negative samples in screening assays to make sure that these samples did contain specific antibodies directed to *F. tularensis*. Ten samples from

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Table 1. Antibody prevalence to *Francisella tularensis* in Japanese black bears

Area	No. sample	No. positive			No. positive in all assays	Positive rate (%)
		Screening	Confirmatory assay*			
		MA	WB	IFA		
Iwate	62	16	10	8	8	12.9
Fukushima	34	7	0	0	0	
Gifu, Hyogo, Kyoto, Nagano, Shiga, Ibaraki, Tottori, Yamanashi, Tokyo	335	0	NT	NT	0	
Total	431	23	10	8	8	1.9

MA: agglutination at dilutions of 1 : 10 or higher were considered to be positive.
WB: LPS banding pattern observed with 1000 time dilution were considered to be positive.
IFA: immunofluorescence assay titre at dilutions of 1 : 40 or higher were considered to be positive.
NT, not tested.
*Confirmatory assays were performed only for MA positive samples.

Japanese black bears reacted with both whole cell lysate and LPS antigens with similar banding pattern (Fig. 2, sheet nos. 1–10). In IFA, eight of these 10 samples reacted with whole cell antigen at 1 : 40 or 1 : 80 (summarized in Table 1). These eight positive samples did not react with antigens prepared from bacterial species other than *F. tularensis* in ELISA (data not shown). Fifteen remaining samples of screening positive did not give rise to positive reaction against *F. tularensis* in IFA at 1 : 40. According to the criterion described in the *Materials and Methods*, eight samples, which were positive in both WB and IFA, were considered to contain specific antibody to *F. tularensis*. On the other hand, ELISA-positive sample from a Japanese hare did not react with purified LPS in WB (Fig. 2, lane 12). There were, however, several bands when whole cell lysate was used as antigen. Because similar bands were also found when ELISA-negative samples from hares were subjected to WB, the presence of those bands was probably due to non-specific reaction. In addition, this sample did not show any positive reaction in IFA at 1 : 20. We therefore concluded that samples from hares were negative.

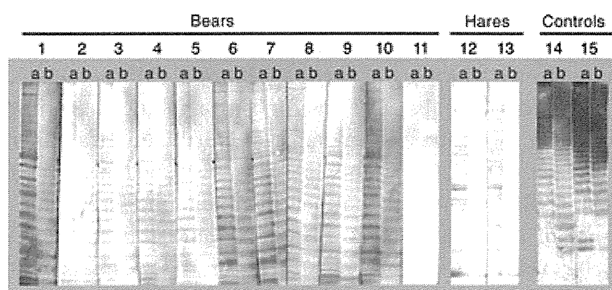

Fig. 2. Detection of antibodies to *Francisella tularensis* antigen by western blot. Sera from 10 black bears (sheets 1–10) reacted specifically with whole cell lysate (a) and purified LPS (b). Thirteen other samples tested positive in the screening MA did not show any positive reaction with either antigens as represented by the sheet 11. Reactions of the screening positive and negative hares samples (sheets 12 and 13, respectively) did not react with LPS. Anti-*F. tularensis* LPS monoclonal antibody (sheet 14) and serum from mouse experimentally infected with *F. tularensis* (sheet 15) were used as positive controls.

Eight samples shown to be positive in all three assays (MA, WB and IFA) were obtained from the bears captured in Iwate between 1999 and 2003. Seven of them were from male bears older than 3 years of age (Table 2). No specific reaction was observed when samples from female bears and bears aged <2 years were examined. The overall prevalence of anti-*F. tularensis* antibody among bears in Japan was 1.9% (8/431) (Table 1); however, nearly one in 10 bears of Iwate had experiences of infections with *F. tularensis* (Table 2).

Discussion

Upon conducting serological survey in wild animals, confirmation of the specificity is extremely important, because false positive reactions are fairly common when samples taken from wild or feral animals are examined. Although MA and ELISA are commonly used for testing a large number of samples, the false-positive reactions may sometimes occur (Grebenchtchikov et al., 2002; CDC, 2008). Recently, WB and IFA have become confirmatory tests for detection of antibody to *F. tularensis* (Porsch-Ozcurumez et al., 2004; Magnarelli et al., 2007). To avoid inclusion of false positives, only samples tested positive in all three assays (MA or ELISA, WB and IFA) were regarded as definitely positive in this study. Because

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Table 2. Distribution of age and gender of positive samples from Japanese black bears captured in Iwate

Age	Male		Female		Unknown		Total	
	Positive samples/*n*	%	Positive samples/*n*	%	Positive samples/*n*	%	Positive samples/*n*	%
<3	0/1		0/1				0/2	
3–5	3/15	20.0	0/10				3/25	12.0
6–9	2/11	18.2	0/5				2/16	12.5
>9	1/6	16.7	0/3				1/9	11.1
Unknown	1/5	20.0	0/1		1/4	25.0	2/10	20.0
Total	7/38	18.4	0/20		1/4	25.0	8/62	12.9

The blanks indicate no sample or 0%.

the criteria adopted in this study is rather strict, it seems possible that the samples regarded as negative here actually contain specific antibody to *F. tularensis*. Nevertheless we believe that the bears identified as seropositive in this assay had definitely been infected with *F. tularensis*. The lack of reaction with antigens prepared from irrelevant bacteria guaranteed the specificity of these tests (data not shown). This is the first report describing the detection of antibodies against *F. tularensis* in bears of Japan. These bears may be infected by direct contact with infected carcasses of hare or mice, or indirectly by bite of infected ticks. It is not known whether infected bears show clinical signs or symptoms, but it is generally thought that bears are relatively resistant (Hopla, 1974). Because Japanese black bears have been suspected as the source of human infection with *F. tularensis* (Ohara et al., 1996), investigating how bears got infected may help understand the ecology of zoonotic *F. tularensis* and the possible roles of bears in the maintenance of *F. tularensis* in nature.

Iwate prefecture is the only place where infected Japanese black bears are recognized. Out of eight samples, two were taken from the bears captured in 2003 (data not shown), indicating that *F. tularensis* still exists in Iwate. We could not definitively conclude that several bears originated from other areas had been infected with the bacteria, because one of the confirmatory tests gave negative results. Although Fukushima is endemic area of tularemia (Table 1), none of animals were proved to be seropositive to *F. tularensis*. This finding may be explained by the fact that the number of Japanese black bears is scarce in the Abukuma mountains where tularemia is most endemic in Fukushima (Francis and Moore, 1926; Yamazaki and Inaba, 2009). The prevalence of tularemia in Japanese black bears were much lower than those estimated for black bears in Alaska (32%) (Chomel et al., 1998) and Idaho (19%) (Binninger et al., 1980). It is not known whether the difference was due to the methods used in those studies.

There was a difference in the prevalence between male and female bears (Table 2). Although the number of samples is small, this finding may help understand the ecology of *F. tularensis* in Japanese black bears. It is known that male bears roam much broader area compared with females, suggesting that the difference in habitat or territory between genders (Tsubota et al., 1998) results in more frequent exposure of male bears to infectious agents including *F. tularensis*.

None of Japanese hares turned out be seropositive even those captured in the areas where human tularemia was reported. Although one sample appeared as positive in the screening assay (ELISA), subsequent confirmatory tests using WB and IFA indicated that the samples did not contain specific antibody to *F. tularensis*. Several bands observed when whole cell lysate was used as antigen in WB were probably due to non-specific reaction caused by unknown components in the sera or cross-reaction with the antibody to other bacteria. Because hares are highly susceptible to *F. tularensis* infection (Mörner and Addison, 2001), it is likely that a majority of infected hares die out before developing an immunological response to *F. tularensis* infection. Their short lifespan in natural environment or habitat may be another reason for the difficulty of finding seropositive hares. Similar findings have been reported on hares in Europe (Mörner et al., 1988; Frölich et al., 2003). Seropositive rabbits were not found in tularemia endemic area in North America either (Berrada et al., 2006). Thus, it is unlikely that hares and rabbits play any role as sentinels in conducting serosurveillance of tularemia; however, there would be an increased chance of isolation of *F. tularensis* if fresh carcasses of hares or rabbits were available (Park et al., 2009). When planning surveillance of tularemia in wild animals, the fact that various factors may affect the results should be borne in mind, in particular, their lifespan, susceptibility to the pathogen as well as assay methods.

It seems likely that Japanese black bears will serve as the sentinel to assess the possible risk of tularemia outbreaks. Because continuous sampling from an individual seems feasible, bears are more useful as the sentinel than other animals like raccoon dogs or skunks (Berrada et al.,

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2006). Japanese black bear is a subspecies of Asiatic black bear, which widely distributed throughout southern Asia, northern China and far eastern Russia (IUCN Red List of Threatened Species, 2009. Version 2009.2; http://www.iucnredlist.org). Therefore, surveillance of other subspecies of bears will help understand the distribution of *F. tularensis* in these areas as well.

This study serologically showed that reservoir animals of *F. tularensis* are rare but definitely thrive in the north-eastern part of Japan, where four human cases of tularemia were reported in 2008 (Infectious Diseases Weekly Report Japan, 19 January 2009). Thus, hunters and veterinarians should be advised to take necessary precautions when treating wild animals because tularemia is maintained in mammalian reservoir animals and outbreaks in humans often parallel those in animal populations (Tärnvik et al., 1996). Further serosurveillance in wild animal species including bears is now in progress.

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Detection of *Francisella tularensis*-Specific Antibodies in Patients with Tularemia by a Novel Competitive Enzyme-Linked Immunosorbent Assay

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A novel competitive enzyme-linked immunosorbent assay (cELISA) was developed and evaluated for detection of antibodies against *Francisella tularensis* in humans. The assay is based on the ability of serum antibodies to inhibit the binding of monoclonal antibodies (MAbs) directed against *F. tularensis* lipopolysaccharide antigens. The assay was evaluated using serum samples of tularemia patients, inactivated *F. tularensis*-immunized rabbits, and *F. tularensis*-infected mice. Antibodies against *F. tularensis* were successfully detected in serum samples of tularemia patients as well as the immunized and infected animals. The cELISA method was compared to indirect ELISA (iELISA) and the commonly used microagglutination test (MA) using serum samples of 19 tularemia patients and 50 healthy individuals. The sensitivity and specificity of cELISA were 93.9 and 96.1%, respectively, in comparison to the iELISA. MA was less sensitive than cELISA with a sensitivity and specificity of only 81.8 and 98.0%, respectively. A high degree of correlation ($R^2 = 0.8226$) was observed between cELISA and iELISA results. The novel cELISA developed in this study appears to be highly sensitive and specific for serodiagnosis of human tularemia. The potential of the MAb-based cELISA to be used in both human and animal samples emphasizes its usefulness for serological survey of tularemia among multiple animal species.

Tularemia is a highly infectious zoonotic disease caused by an intracellular Gram-negative bacterium, *Francisella tularensis*. It was first reported in North America in 1911 during plague studies of rodents (1); subsequently, both human and animal infections were identified in Japan, as well as in European countries and the former Soviet Union (2). Tularemia exists primarily as two clinically relevant strains, the highly virulent type A (*F. tularensis* subsp. *tularensis*) found predominantly in North America and the less virulent type B (*F. tularensis* subsp. *holarctica*) found in the northern hemisphere (3, 4). Transmission to humans is mostly associated with handling of infected animals, arthropod bites, ingestion of contaminated water or food, and inhalation of infective aerosols (2). The clinical manifestation of the disease in humans ranges from skin ulcers to life-threatening pneumonia (3). Clinical signs and the course of the infection vary among species. For example, rodents and hares generally die rapidly after being infected without mounting an antibody response, whereas other animal species, such as cats, dogs, and cattle, are relatively resistant to infection (5).

To date, tularemia outbreaks, both sporadic cases and epidemiological surveillance data, have been reported (6–8); however, little is known about the prevalence rate in Asia. Currently, pathogen isolation, molecular detection, and serology are the most commonly used methods for the diagnosis of tularemia (9). However, a high risk of laboratory infection associated with isolation of the organism and lack of a well-evaluated standardized PCR protocol make these techniques difficult to apply for routine diagnosis of large numbers of samples (10–12). For these reasons, serological assays are the best choice for surveillance of tularemia in humans and animals.

The most commonly utilized serological assays for tularemia are microagglutination (MA), enzyme-linked immunosorbent assay (ELISA), and Western blotting (WB) (13, 14). In patients with tularemia, antibodies appear approximately 2 to 3 weeks after infection and may be detected several years after recovery (15–17). Monitoring of antibody titers in serum during acute and convalescent phases is thus necessary to identify tularemia infection. MA seems to be an appropriate test because it is easy and applicable to various animal species (17, 18); however, it is not applicable to hemolyzed serum, and the sensitivity is relatively low (14), particularly for serum with lower antibody titers. Furthermore, cross-reaction with other bacterial species makes this assay difficult to use for examination of serum suspected to have antibodies against *F. tularensis* (19, 20). Indirect ELISA (iELISA) is appropriate for seroepidemiological studies because the test is relatively sensitive (10, 13, 14); however, iELISA requires enzyme-conjugated secondary antibodies against immunoglobulins of respective animal species. For seroepidemiological surveillance of many wild animals, it is almost impossible to prepare antibodies that are specifically directed against immunoglobulins of each animal species. Although the combination of WB and iELISA is often used for confirmatory serodiagnosis (14), it is difficult for use with a large number of samples. Therefore, there is a need for a high-throughput assay that is specific and sensitive in detecting antibodies against *F. tularensis*. Monoclonal antibody (MAb)-based compet-

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itive ELISA (cELISA) appears to be ideal because it is able to overcome the problems associated with the currently available tests. Therefore, we attempted to develop cELISA for detection of antibodies against *F. tularensis* in serum of humans and animals.

MATERIALS AND METHODS

Serum samples. A total of 84 human serum samples were used in the present study. Twenty serum samples from 15 patients with confirmed tularemia and 5 healthy subjects were obtained by H. Fujita, Ohara Research Laboratories, Fukushima, Japan. Fourteen serum samples were obtained from four patients at several other hospitals in Japan. Patient serum samples were obtained as early as day 1after onset of tularemia symptoms (Table 1). All patients were diagnosed with tularemia by a significant rise of MA or tube agglutination titer. A total of 45 serum samples of healthy donors were also obtained from several hospitals in Japan. The identity of the patients was not disclosed to us and was derived from various contributors. The studies in human subjects were approved by the research and ethical committees of the National Institute of Infectious Diseases (NIID), Tokyo, Japan, and written informed consent was obtained from all participants.

Anti-*F. tularensis* serum samples were obtained by immunizing rabbits or mice with formalin-inactivated *F. tularensis* subsp. *tularensis* or subsp. *holarctica* suspension as follows. To prepare immunized serum samples, specific-pathogen-free 10-week-old female Kbl:JW rabbits (Kitayama Rabesu Co., Nagano, Japan) were inoculated subcutaneously with formalin-inactivated *F. tularensis* subsp. *tularensis* (38 strain) or subsp. *holarctica* (Yama strain, a Japanese isolate) (400 μg of protein/rabbit) suspended in TiterMax Gold (KIEL Lab, Norcross, GA). The protein concentration of *F. tularensis* whole cells was determined by a Bradford protein assay (Bio-Rad). The rabbits were inoculated again with 400 μg of the protein together with the adjuvant 4, 6, and 8 weeks after the first injection. A final booster was injected intravenously 2 weeks after the fourth injection with formalin-inactivated bacteria (50 μg of protein/rabbit) in phosphate-buffered saline (PBS). Similarly, specific-pathogen-free 6-week-old female BALB/c mice (SLC, Shizuoka, Japan) were immunized twice with formalin-inactivated *F. tularensis* subsp. *holarctica* (Yama) or *Francisella novicida* (U112; 100 μg of protein/mouse) suspended in Titer Max Gold 4 weeks apart. At 2 weeks after the second inoculation, mice were boosted by intravenous injection with the formalin-inactivated bacteria (50 μg of protein/mouse) in PBS. Serum samples of mice that had recovered from experimental infection with attenuated *F. tularensis* subsp. *tularensis* (Schu strain) were also included. Eight-week-old female BALB/c mice were infected intraperitoneally with 6.2×10^6 CFU bacterial suspension in saline, and the blood was collected at 6 days postinfection. These animal experiments were approved by the Animal Care and Use Committee of NIID. Serum samples of rabbits immunized with formalin-inactivated *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis* were gifts from Koichi Imaoka of our department.

Bacteria and purification of LPS. *F. tularensis* subsp. *holarctica*, NVF1 strain, which was isolated from a hare in 2009, was grown at 37°C for 72 h on chocolate agar (II) plates (Becton Dickinson, Tokyo, Japan). The bacteria were harvested into saline, and the suspension was adjusted to an optical density at 600 nm (OD_{600}) of 1.2. Lipopolysaccharide (LPS) was extracted using an LPS extraction kit (iNtRON Biotechnology, Kyungki-Do, Korea) according to the manufacturer's protocol after mixing the bacterial suspension with extraction buffer, followed by incubation at 65°C for 10 min. The dried LPS pellets were dissolved in 10 mM Tris-HCl buffer (pH 8.0) at a concentration of 10 μg/μl and stored at 4°C until use. Live bacteria were handled in a biosafety level 3 laboratory at the NIID.

cELISA. A 96-well flat-bottom microtiter plate (Greiner Bio-One, Frickenhausen, Germany) was coated with LPS antigen in carbonate-bicarbonate buffer (pH 9.6; 2.5 μg/50 μl/well) at 37°C overnight. The wells were rinsed thrice with PBS containing 0.1% Tween 20 (MP Biomedicals, Illkirch, France) (PBST) to remove unbound antigens and were blocked

TABLE 1 Antibodies against *F. tularensis* in patients determined by cELISA, iELISA, and MA[a]

Patient	Blood collection (days after onset of tularemia)	Test: cELISA (% inhibition)	iELISA (OD)	MA (titer)
P1	11	25.8	0.60	<10
	36	79.7	1.91	320
P2	12	54.1	1.84	20
	37	74.5	2.65	320
P3	37	84.9	2.99	>1,280
	83	80.4	3.07	>1,280
P4	1	9.9	0.27	<10
	13	43.4	0.79	40
P5	9	47.9	1.01	<10
	25	77.3	2.15	80
P6	80	78.2	2.54	320
P7	21	82.8	2.57	640
P8	ND	42.7	1.02	80
P9	87	75.8	2.66	320
P10	13	43.2	0.89	<10
P11	59	41.1	1.18	160
P12	ND	36.1	0.79	<10
P13	8	79.2	2.49	320
P14	78	54.8	2.44	>1,280
P15	ND	59.7	1.64	>1,280
P16	42	71.0	2.15	640
	89	53.4	1.68	320
P17	13	42.0	2.84	<10
	241	−1.5	1.31	40
P18	16	83.0	2.36	40
	23	94.2	2.86	160
	30	90.3	2.83	160
	59	86.1	3.34	80
	185	68.7	2.42	40
P19	16	66.6	0.95	40
	23	65.3	1.19	160
	30	66.2	1.18	160
	59	79.0	1.62	160
	185	60.2	1.01	40

[a] A total of 34 serum samples of 19 patients were used. Sera of patients P1 to P5, P16, and P17 are paired samples obtained at the indicated days after the onset of tularemia symptoms. Patients P18 and P19 represent the five sets of samples obtained for several days after symptom onset. ND, not documented. Shaded numbers are values below the cutoff level in each test.

with PBST containing 3% (wt/vol) skim milk (150 μl/well) at 37°C for 1 h. For all subsequent steps, PBST containing 1% (wt/vol) skim milk was used as dilution buffer. After three washes with PBST, 50 μl of diluted sample serum was added to the antigen coated wells in duplicate, and the plates were incubated at 37°C for 90 min. Pooled patient sera ($n = 10$) and pooled healthy human sera ($n = 5$) were also added as positive and negative controls during each test. After the wells were washed three times with PBST, biotin-labeled anti-LPS MAb (M14B11) (21) (50 μl/well, 1:5,000 dilution) was added, and the plates were further incubated at 37°C for 60 min. The biotin labeling of M14B11 (isotype IgG2a; 3.4 mg of IgG/ml and a biotin/IgG coupling ratio of 4/67) was performed at a com-

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mercial laboratory (T. K. Craft, Maebashi, Japan). After three washes, streptavidin-peroxidase (Thermo Scientific, Rockford, IL) (50 μl/well, 1:5,000 dilution) was added to each well, and the plates were incubated at 37°C for 60 min. After three washing steps, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) enzyme substrate (SureBlue Reserve, TMB microwell peroxidase substrate; KPL, Gaithersburg, MD) was added to each well, and the plates were incubated at 37°C for 30 min. Finally, 100 μl of stop solution (1 N HCl) was added, and the OD values were measured at 450 nm using an iMark microplate reader (Bio-Rad, Hercules, CA). The percent inhibition was calculated using the following formula: $\{1 - [(OD_{sample} - OD_{background})/(OD_{MAb} - OD_{background})]\} \times 100$, where OD_{sample} and OD_{MAb} are the absorbances observed in the presence and in the absence of samples, respectively, and $OD_{background}$ was obtained in the absence of sample or labeled MAb.

MA test. Portions (25 μl) of 2-fold serial dilutions of serum were mixed with an equal volume of formalin-inactivated *F. tularensis* subsp. *holarctica* (Yama) whole-cell suspension (OD_{560} = 1.0) in a 96-well round-bottom microtiter plate (IWAKI, Tokyo, Japan). The reactions in the plates were observed 18 h after incubation at 37°C for agglutination. Agglutination titers were expressed as reciprocals of the highest serum dilution showing agglutination with the antigen. Agglutination at dilutions of 1:10 or higher were considered MA positive.

iELISA. The LPS solution was diluted 1:800 in carbonate-bicarbonate buffer (pH 9.6). Ninety-six-well microtiter plates (Greiner Bio-One) were coated with 50 μl of antigen at 37°C overnight. The wells were washed with PBST and blocked with 150 μl of PBST containing 3% (wt/vol) skim milk. After another washing step, 50 μl of patient serum samples, diluted 1:500 in PBST containing 1% (wt/vol) skim milk, were added, followed by incubation at 37°C for 1 h. After the plates were washed three times, 50 μl of horseradish peroxidase (HRP)-conjugated goat anti-human immunoglobulin G (IgG) (ICN Pharmaceuticals, Cappel, OH), diluted 1:8,000 in PBST containing 1% (wt/vol) skim milk, was added, followed by incubation at 37°C for 1 h. Colorimetric development and measurement of the OD values was performed as described above in the cELISA protocol.

WB. Western blot (WB) analyses were performed as described previously (7) using whole bacterial cell lysates and purified LPS as antigens. After SDS-PAGE, antigens were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA), and the membranes were saturated with PBST containing 3% (wt/vol) skim milk for 1 h. After being washed with PBST, the membranes were incubated with samples diluted 1:1,000 with PBST containing 1% (wt/vol) skim milk for 1 h. After three washes with PBST for 5 min, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (H+L; Zymed Laboratories, Inc., CA) or HRP-conjugated goat anti-human IgG (ICN Pharmaceuticals) at a dilution of 1:8,000 for 1 h. The antigens were visualized by soaking the membranes in 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) and hydrogen peroxide visualization solution. Samples were considered to contain specific antibodies when the typical LPS ladder-like banding pattern was observed.

Statistical analysis. Diagnostic efficiency of the assay in terms of sensitivity and specificity were determined initially by receiver operating characteristics (ROC) and two-graph-ROC (TG-ROC) curves using Stat-Flex software (Artech Co., Ltd., Osaka, Japan) (22, 23). An optimal cutoff value was estimated by comparing a range of sensitivity and specificity values for a range of cutoff values. The relationship between results for patients and healthy humans determined by cELISA and iELISA were evaluated by linear regression analysis using Microsoft Excel software for Windows.

RESULTS

Target antigens recognized by MAb and antibodies induced by infection. WB was performed to determine antigens predominantly recognized by serum samples of infected humans and animals (Fig. 1). Regardless of whether LPS or whole-cell antigen was targeted, typical LPS ladder-like banding patterns were observed in the serum samples of patients with tularemia and mice that had recovered from experimental infection. These reactions were similar to that of the MAb M14B11 recognizing LPS. No band was obtained from healthy human serum samples.

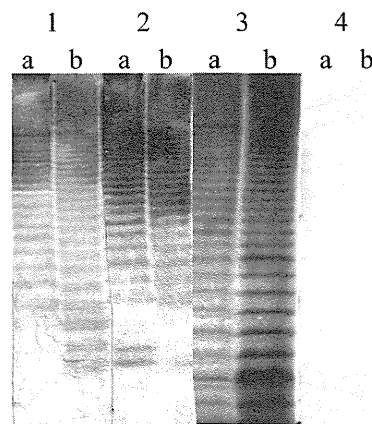

FIG 1 Western blot analysis of target antigens recognized by MAb and antibodies induced by infection. MAb M14B11 (lane 1) and sera from an *F. tularensis*-infected mouse (lane 2) and a patient with tularemia (lane 3) were reacted with *F. tularensis* whole-cell lysate (a) or purified LPS antigens (b). Sera obtained from healthy human donors (lane 4) were also included.

Development of cELISA. Optimization of LPS concentration and dilution of antibodies were performed by checkerboard titration (data not shown). The composition of blocking and dilution buffers and incubation times for serum samples were also determined by preliminary experiments (data not shown). After optimization of the test conditions, samples in which the presence or absence of *F. tularensis* antibodies was known were subjected to cELISA; the representative results are shown in Fig. 2. The reaction of MAb to LPS was inhibited by serum samples of patients with tularemia in a dose-dependent manner (Fig. 2a). Serum samples of hyperimmune rabbits and mice infected with *F. tularensis* also inhibited MAb reaction to LPS (Fig. 2b and c). When the serum samples of three tularemia patients were tested, all serum samples were shown to inhibit MAb reactions at dilutions up to 1:1,000. No significant inhibition was observed in serum samples of healthy individuals (Fig. 2a).

Serum samples of rabbits immunized with *F. tularensis* subsp. *holarctica* and subsp. *tularensis* exhibited 100 and 88% inhibition of MAb reaction, respectively, at a dilution of 1:125 and thereafter exhibited decreasing percent inhibitions with increasing serum dilutions (Fig. 2b). Thus, the level of inhibition was directly proportional to the amount of antibody in the samples. No inhibition of MAb reaction was observed with normal serum obtained from two different rabbits.

Furthermore, serum samples obtained from convalescent mice experimentally infected with attenuated *F. tularensis* subsp. *tularensis* and mice immunized with formalin-fixed *F. tularensis* subsp. *holarctica* exhibited clear inhibition of MAb reaction at dilutions up to 1:1,000, whereas the sera of mice immunized with *F. novicida* exhibited only slight inhibition (ca. 11%) at a dilution of 1:125. The plasma or sera of normal mice did not exhibit inhibition of MAb reaction at any of the dilutions tested (Fig. 2c).

Inhibition of MAb reaction was consistently achieved by both patient sera and immune and infected animal sera even at higher

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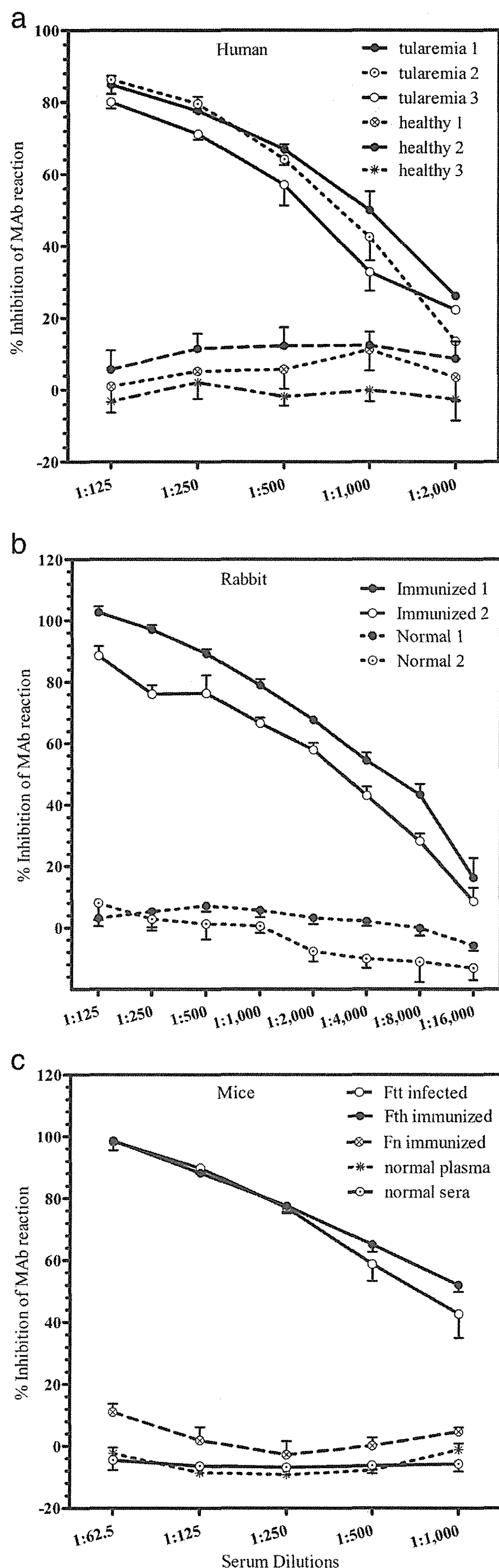

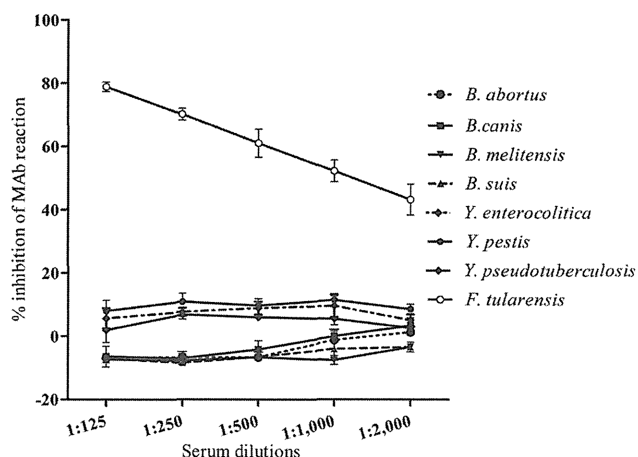

FIG 3 Inhibition of binding of *F. tularensis* LPS-specific MAb by rabbit antisera against various bacterial species. Serial dilutions of the rabbit antisera against *B. abortus*, *B. canis*, *B. melitensis*, *B. suis*, *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis* were negative by cELISA, whereas antisera against *F. tularensis* were positive, with clear inhibition of MAb binding. Error bars indicate the standard deviations from four-well replications for each serum sample.

dilutions, whereas inhibition caused by normal serum never exceeded 20%, even at lower dilutions. Immune serum diluted 1:125 exhibited the highest levels of inhibition, whereas normal serum at this dilution exhibited the least inhibition. We also determined whether cELISA designed for *F. tularensis* cross-reacted with other bacterial species, particularly, *Brucella* spp. and *Yersinia* spp. None of the rabbit immune serum samples against *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis* exhibited significant inhibition (<10%) at a dilution of 1:125 (Fig. 3).

Determination of cutoff values for cELISA and iELISA. cELISA was performed to detect specific antibodies against *F. tularensis* in 34 serum samples collected from 19 patients and 50 healthy individuals. The percent inhibition values for each serum sample at a dilution of 1:100 were plotted (Fig. 4a), and ROC and TG-ROC curves were plotted (Fig. 4b and c). The area under the ROC curve was 0.98559, indicating that the assay had an excellent ability to discriminate between patients and healthy donors (Fig. 4b). The TG-ROC analysis initially identified the cutoff value for discrimination of patients and healthy donors to be 25.8% inhibition (Fig. 4c). With this value, cELISA had a sensitivity of 91.1%

FIG 2 Inhibition of binding of MAb targeting the *F. tularensis* LPS by sera of humans, rabbits, and mice positive for *F. tularensis* antibodies in MA and iELISA. (a) Serial dilutions of three MA-positive (tularemia 1, 2, and 3) and three MA-negative (healthy 1, 2, and 3) human serum samples were examined for their ability to inhibit MAb binding. (b) Serum samples of four rabbits were subjected to cELISA. Two rabbits (immunized 1 and 2) were immunized with *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis*, respectively, whereas two rabbits (normal 1 and 2) were unimmunized. (c) Serum obtained from a mouse infected with attenuated *F. tularensis* subsp. *tularensis* (Ftt infected), a mouse immunized with formalin-fixed *F. tularensis* subsp. *holarctica* (Fth immunized), and a mouse immunized with formalin-fixed *F. novicida* (Fn immunized) were subjected to cELISA. Plasma and serum of a normal mouse were also tested as negative controls. The error bars in each figure indicate standard deviations from four-well replications for each serum sample.

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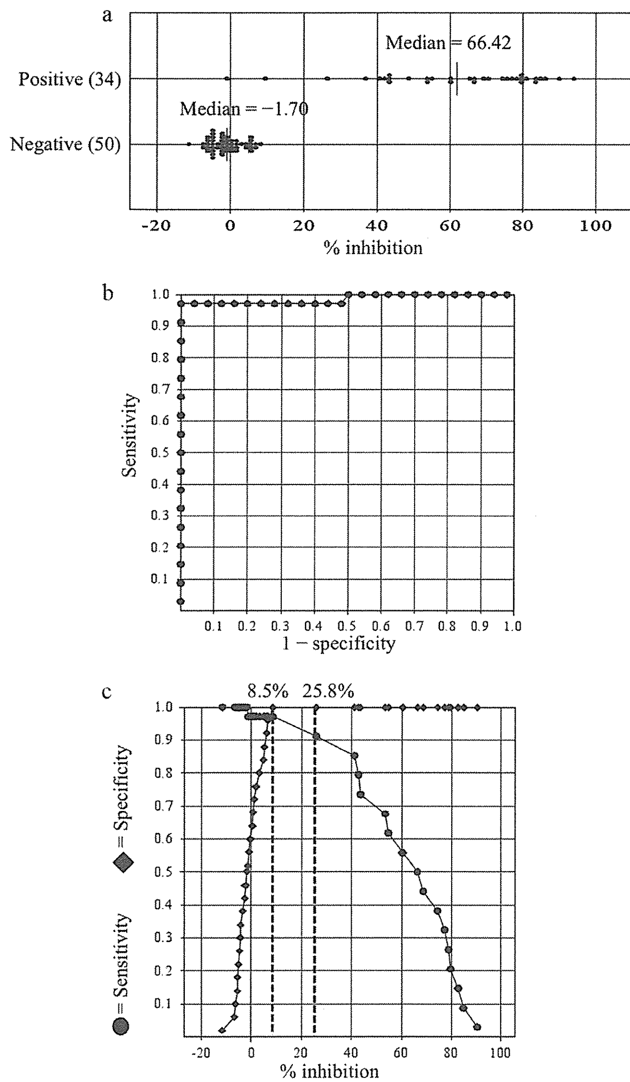

FIG 4 Distribution of percent inhibition values and ROC and TG-ROC analyses of cELISA specific for *F. tularensis* LPS. (a) The percent inhibition value of each serum sample at a dilution of 1:100 was plotted for serum samples obtained from patients with tularemia and healthy donors. (b and c) The ROC curve (b) and two-graph-ROC (TG-ROC) curve (c) were analyzed and drawn using Stat Flex software. The area under the ROC curve is 0.98559, which indicates the test has a good probability of distinguishing between patients with tularemia and healthy individuals (see panel b). The graphs show the relationship between the sensitivity and specificity of cELISA for each cutoff percent inhibition. The dashed vertical lines indicate the cutoff values at 8.5 and 25.8% inhibition, respectively (see panel c). The most optimal sensitivity (97.0%) and specificity (100%) are obtained when the cutoff value is set at 8.5% inhibition.

and a specificity of 100%. To increase the sensitivity of the assay without decreasing the specificity, the cutoff value was decreased to 8.5% inhibition (Fig. 4c). As a result, the sensitivity increased from 91.1 to 97.0%. Antibodies against *F. tularensis* were successfully detected in samples of patients with tularemia at a cutoff value of 8.5% inhibition, except for one serum sample. The cutoff value and diagnostic performance of iELISA were determined similarly. As a result, iELISA was considered positive at a cutoff

TABLE 2 Sensitivity and specificity of MA relative to cELISA[a]

	MA (no. of samples)		
cELISA	Positive	Negative	Total
Positive	27	6	33
Negative	1	50	51
Total	28	56	84

[a] Relative sensitivity = 27 of 33 (81.8%); relative specificity = 50 of 51 (98%). Positive, human serum with a positive MA titer with agglutination at dilutions of ≥1:10; negative, human serum with no agglutination in MA.

OD of 0.61 at which the sensitivity and the specificity were 94.1 and 98.0%, respectively (data not shown).

Sensitivity and specificity of cELISA, iELISA, and MA. cELISA, iELISA, and MA were performed simultaneously on 84 human serum samples. The results for the 34 serum samples collected from 19 patients are summarized in Table 1. Only one serum sample was found to be false negative by cELISA, whereas the number of false-negative samples by iELISA and MA were 2 and 6, respectively (Table 1). Similarly, among the 50 healthy human serum samples, only one sample was found to be false-positive by iELISA, and no sample was found to be false positive by cELISA and MA (data not shown). In comparison to cELISA, the sensitivity and specificity of MA were 81.8 and 98.0%, respectively (Table 2). In addition, when cELISA was compared to iELISA, the relative sensitivity and specificity of cELISA were 93.9 and 96.1%, respectively (Table 3).

Correlation between cELISA and iELISA. The results of cELISA and iELISA using the 84 human serum samples were analyzed to determine whether the two assays were statistically correlated. Linear regression analysis showed a significant linear correlation between cELISA percent inhibition values and OD values determined by iELISA ($R^2 = 0.82$, $r = 0.91$) (Fig. 5).

Persistence of *F. tularensis*-specific antibodies in patients. Two patients were evaluated by cELISA and iELISA to determine the level and persistence of disease-specific antibodies between 19 to 188 days after exposure to *F. tularensis* (Fig. 6). The antibodies were detected on day 19, and thereafter their levels increased or remained constant until 62 days after exposure. However, antibody levels in the sera of both patients decreased moderately at 188 days. cELISA and iELISA demonstrated similar patterns of antibody persistence in both patients.

DISCUSSION

Human tularemia is diagnosed on the basis of clinical findings and laboratory tests that include serological and molecular methods (11, 13, 14, 17). However, variations in sensitivity and specificity

TABLE 3 Sensitivity and specificity of cELISA relative to iELISA[a]

	cELISA		
iELISA	Positive	Negative	Total
Positive	31	2	33
Negative	2	49	51
Total	33	51	84

[a] Relative sensitivity = 31 of 33 (93.9%); relative specificity = 49 of 51 (96.1%). Positive, human serum with an iELISA cutoff OD of >0.61; negative, human serum with an iELISA cutoff OD of ≤0.61.

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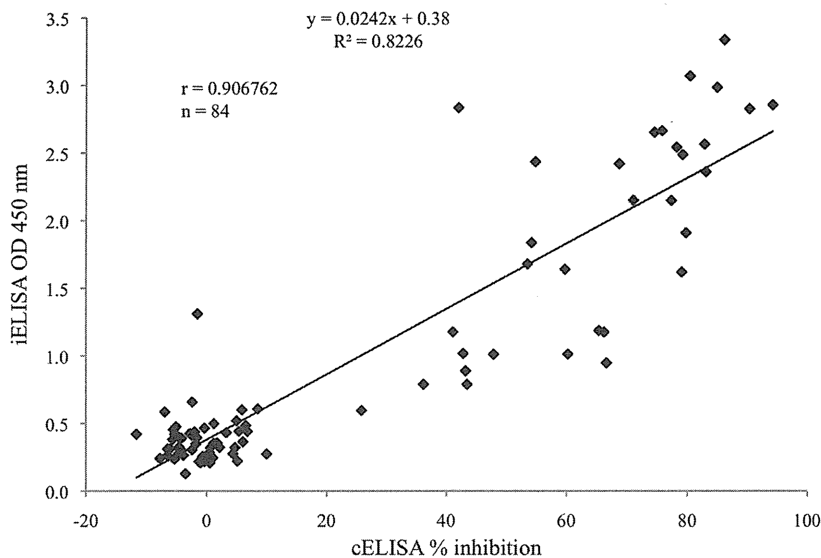

FIG 5 Correlation between cELISA and iELISA for detection of *F. tularensis*-specific antibodies. A scatter plot of the percent inhibition obtained by cELISA and OD values obtained by iELISA for serum samples of patient ($n = 34$) and healthy human donor ($n = 50$). Linear regression analysis showed that a significant linear correlation was observed between cELISA and iELISA with a correlation coefficient of 0.91.

of different assays during surveillance studies sometimes lead to misdiagnosis of the disease. The cELISA developed here is a simple, specific, and sensitive test that is applicable to both humans and animals for detection of antibodies against *F. tularensis*.

Our cELISA is based on the measurement of competition between the test sample and MAb for LPS, the major antigen of *F. tularensis* (14, 24, 25). Similar assays using MAb against LPS have been described for *Brucella* species (26, 27). The typical ladder-like bands observed on WB demonstrated that both MAb and *F. tularensis*-infected human and mice serum contained antibodies predominantly recognizing the LPS antigen (Fig. 1). In cELISA, the human and animal serum samples that were positive by MA and iELISA clearly inhibited MAb binding to LPS (Fig. 2). Serum samples of tularemia patients with MA titers of 1:160 (tularemia 1 and 2 in Fig. 2a) and 1:40 (tularemia 3 in Fig. 2a) positively inhibited MAb binding up to a dilution of 1:1,000, whereas the serum samples of healthy donors did not. This result shows that cELISA is useful for detecting *F. tularensis* antibodies and is more sensitive than MA.

In previous studies, the possible difference in antigenic structure of LPS between *F. tularensis* subsp. *tularensis* and subsp. *holarctica* were suggested (28). In the present study, we used anti-LPS MAb (M14B11) (21), which recognized both *F. tularensis* subsp. *tularensis* and subsp. *holarctica* in the cELISA. Furthermore, anti-

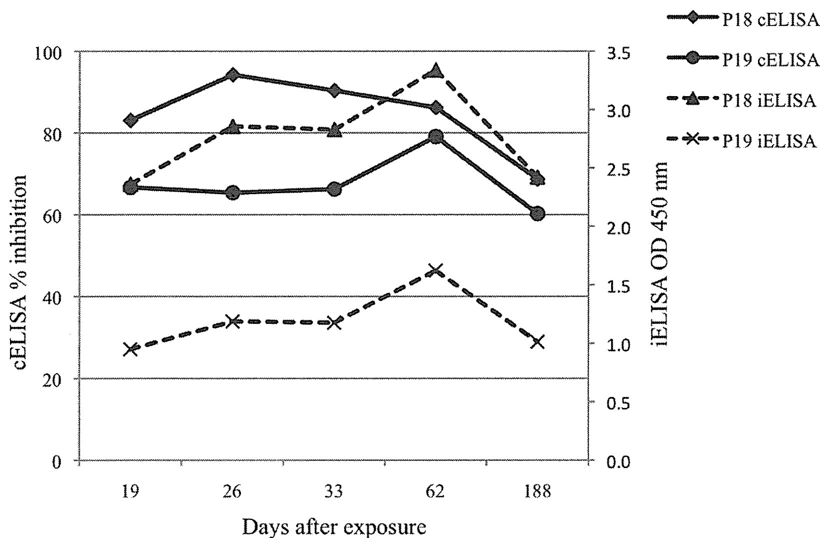

FIG 6 Kinetics of antibody levels in patients with tularemia over a 6-month period. *F. tularensis*-specific antibodies were measured by cELISA and iELISA in two patients with tularemia (P18 and P19, Table 1) at the indicated days after exposure to *F. tularensis*. The percent inhibition values in cELISA and OD values in iELISA are shown on the left and right sides of the *y* axis, respectively.

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