(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

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.

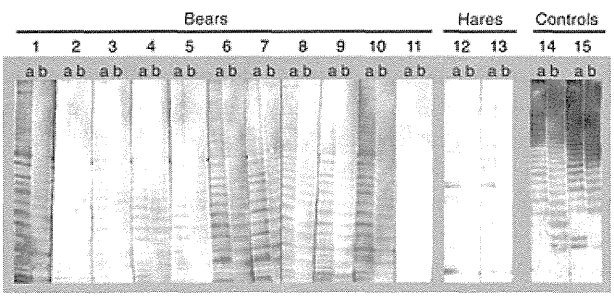


Fig. 2. Detection of antibodies to *Francisella tularensis* antigen by western blot. Sera from 10 black bears (sheets 1–10) reacted specifically with whole cell lysate (a) and purified LPS (b). Thirteen other samples tested positive in the screening MA did not show any positive reaction with either antigens 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

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

	Male		Female		Unknown		Total	
Age	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.,

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.

Acknowledgements

The authors are grateful to Dr Mikiko Aoki (Iwate University, Iwate), Prof. Toshio Tsubota (Hokkaido University), Dr Atsushi Katayama (Wildlife Management Office, Hyogo), Dr Sachiko Nakamura (University of Hyogo, Hyogo) and Ms Rumiko Nakashita (Tokyo Metropolitan University, Tokyo) for providing samples from Japanese black bears. The authors also thank the volunteer hunters and Dai-Nippon Ryo-Yu-Kai (Japanese Hunters association) for hunting Japanese hares and providing the samples. This study was supported by the Health and Labour Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare in Japan.

References

Al Dahouk, S., K. Nockler, H. Tomaso, W. D. Splettstoesser, G. Jungersen, U. Riber, T. Petry, D. Hoffmann, H. C. Scholz, A. Hensel, and H. Neubauer, 2005: Seroprevalence of brucellosis, tularemia, and yersiniosis in wild boars (*Sus scrofa*) from north-eastern Germany. *J. Vet. Med. B Infect. Dis. Vet. Public Health* 52, 444–455.

Berrada, Z. L., H. K. Goethert, and S. R. Telford 3rd, 2006: Raccoons and skunks as sentinels for enzootic tularemia. *Emerg. Infect. Dis.* 12, 1019–1021.

Binninger, C. E., J. J. Beecham, L. A. Thomas, and L. D. Winward, 1980: A serologic survey for selected infectious diseases of black bears in Idaho. *J. Wildl. Dis.* 16, 423–430.

CDC, 2008: Public health consequences of a false-positive laboratory test result for Brucella – Florida, Georgia, and Michigan, 2005. *MMWR Morb. Mortal. Wkly. Rep.* 57, 603–605.

Chomel, B. B., R. W. Kasten, G. Chappuis, M. Soulier, and Y. Kikuchi, 1998: Serological survey of selected canine viral pathogens and zoonoses in grizzly bears (Ursus arctos horribilis) and black bears (*Ursus americanus*) from Alaska. *Rev. Sci. Tech.* 17, 756–766.

De Swart, R. L., Y. Nur, A. Abdallah, H. Kruining, H. S. El Mubarak, S. A. Ibrahim, B. Van Den Hoogen, J. Groen, and A. D. Osterhaus, 2001: Combination of reverse transcriptase PCR analysis and immunoglobulin M detection on filter paper blood samples allows diagnostic and epidemiological studies of measles. *J. Clin. Microbiol.* 39, 270–273.

Ellis, J., P. C. Oyston, M. Green, and R. W. Titball, 2002: Tularemia. *Clin. Microbiol. Rev.* 15, 631–646.

Francis, E., and D. Moore, 1926: Identity of Ohara's disease and tularemia. *J. Am. Med. Assoc.* 86, 1329–1332.

Frölich, K., J. Wisser, H. Schmüser, U. Fehlberg, H. Neubauer, R. Grunow, K. Nikolaou, J. Priemer, S. Thiede, W. J. Streich, and S. Speck, 2003: Epizootiologic and ecologic investigations of European brown hares (*Lepus europaeus*) in selected populations from Schleswig-Holstein, Germany. *J. Wildl. Dis.* 39, 751–761.

Fujita, O., M. Tatsumi, K. Tanabayashi, and A. Yamada, 2006: Development of a real-time PCR assay for detection and quantification of *Francisella tularensis*. *Jpn. J. Infect. Dis.* 59, 46–51.

Fujita, O., A. Uda, A. Hotta, A. Okutani, S. Inoue, K. Tanabayashi, and A. Yamada, 2008: Genetic diversity of *Francisella tularensis* subspecies holarctica strains isolated in Japan. *Microbiol. Immunol.* 52, 270–276.

Grebenchtchikov, N., C. G. Sweep, A. Geurts-Moespot, A. Piffanelli, J. A. Foekens, and T. J. Benraad, 2002: An ELISA avoiding interference by heterophilic antibodies in the measurement of components of the plasminogen activation system in blood. *J. Immunol. Methods* 268, 219–231.

Hopla, C. E., 1974: The ecology of tularemia. *Adv. Vet. Sci. Comp. Med.* 18, 25–53.

Hotta, A., A. Uda, O. Fujita, K. Tanabayashi, and A. Yamada, 2007: Preparation of monoclonal antibodies for detection and identification of *Francisella tularensis*. *Clin. Vaccine Immunol.* 14, 81–84.

Magnarelli, L., S. Levy, and R. Koski, 2007: Detection of antibodies to *Francisella tularensis* in cats. *Res. Vet. Sci.* 82, 22–26.

Mörner, T., and E. Addison, 2001: Tularemia. Iowa State University Press, Iowa, USA.

Mörner, T., G. Sandström, R. Mattsson, and P. O. Nilsson, 1988: Infections with *Francisella tularensis* biovar palaearctica in hares (*Lepus timidus*, *Lepus europaeus*) from Sweden. *J. Wildl. Dis.* 24, 422–433.

Ohara, Y., T. Sato, H. Fujita, T. Ueno, and M. Homma, 1991: Clinical manifestations of tularemia in Japan – analysis of 1,355 cases observed between 1924 and 1987. *Infection* 19, 14–17.

Ohara, Y., T. Sato, and M. Homma, 1996: Epidemiological analysis of tularemia in Japan (yato-byo). *FEMS Immunol. Med. Microbiol.* 13, 185–189.

Park, C. H., A. Nakanishi, H. Hatai, D. Kojima, T. Oyamada, H. Sato, N. Kudo, J. Shindo, O. Fujita, A. Hotta, S. Inoue, and K. Tanabayashi, 2009: Pathological and microbiological studies of Japanese Hare (*Lepus brachyurus* angustidens) naturally infected with *Francisella tularensis* subsp. holarctica. *J. Vet. Med. Sci.* 71, 1629–1635.

Porsch-Ozcurumez, M., N. Kischel, H. Priebe, W. Splettstosser, E. J. Finke, and R. Grunow, 2004: Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clin. Diagn. Lab. Immunol.* 11, 1008–1015.

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.

Shoemaker, D., A. Woolf, R. Kirkpatrick, and M. Cooper, 1997: Humoral immune response of cottontail rabbits naturally infected with Francisella tularensis in southern Illinois. *J. Wildl. Dis.* 33, 733–737.

Tärnvik, A., G. Sandström, and A. Sjöstedt, 1996: Epidemiological analysis of tularemia in Sweden 1931–1993. *FEMS Immunol. Med. Microbiol.* 13, 201–204.

Tsubota, T., N. Mizoguchi, and I. Kita, 1998: Ecological and physiological studies of the Japanese black bear, *Ursus thibetanus* japonicus. *Jpn. J. Zoo Wildl. Med.* 3, 17–24.

Uda, A., K. Tanabayashi, O. Fujita, A. Hotta, Y. Yamamoto, and A. Yamada, 2007: Comparison of whole genome amplification methods for detecting pathogenic bacterial genomic DNA using microarray. *Jpn. J. Infect. Dis.* 60, 355–361.

Yamazaki, K., and O. Inaba, 2009: Possible expansion of Japanese black bear distribution in the southern part of the Abukuma Mts. (Ibaraki, Fukushima and Tochigi Prefectures), central part of Japan. *Mammalian Science* 49, 257–261.

CVI
Journals.ASM.org

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

Neekun Sharma,[a,b] Akitoyo Hotta,[a] Yoshie Yamamoto,[a] Osamu Fujita,[a] Akihiko Uda,[a] Shigeru Morikawa,[a,b] Akio Yamada,[a,b] Kiyoshi Tanabayashi[a]

Department of Veterinary Science, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan[a]; The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan[b]

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-

Received 23 August 2012 Returned for modification 5 October 2012
Accepted 22 October 2012
Published ahead of print 31 October 2012
Address correspondence to Kiyoshi Tanabayashi, ktana@nih.go.jp.

doi:10.1128/CVI.00516-12

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 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-

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.

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 StatFlex 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.

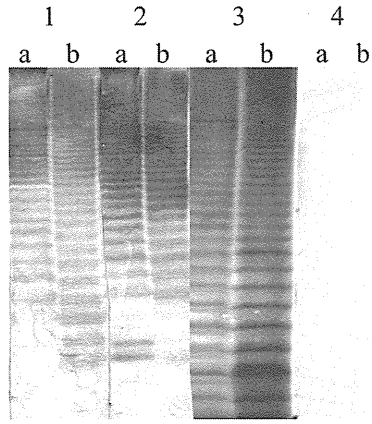


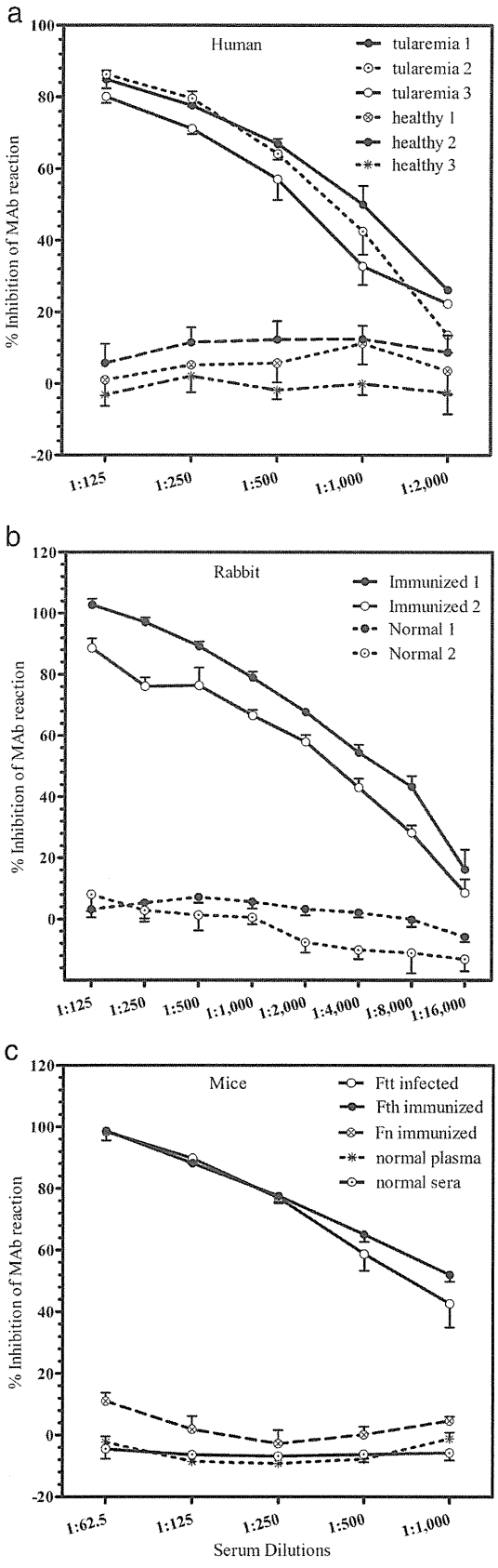
FIG 1 Western blot analysis of target antigens recognized by MAb and antibodies induced by infection. MAb M14B11 (lane 1) and sera from an *F. tularensis*-infected mouse (lane 2) and a patient with tularemia (lane 3) were reacted with *F. tularensis* whole-cell lysate (a) or purified LPS antigens (b).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



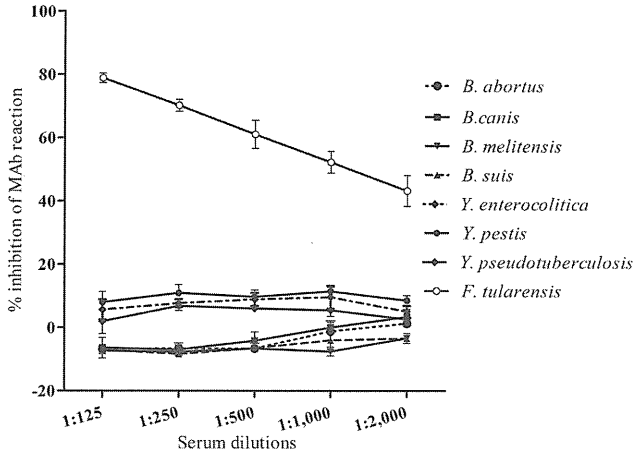


FIG 3 Inhibition of binding of *F. tularensis* LPS-specific MAb by rabbit antisera against various bacterial species. Serial dilutions of the rabbit antisera against *B. abortus*, *B. canis*, *B. melitensis*, *B. suis*, *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis* were negative by cELISA, 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.

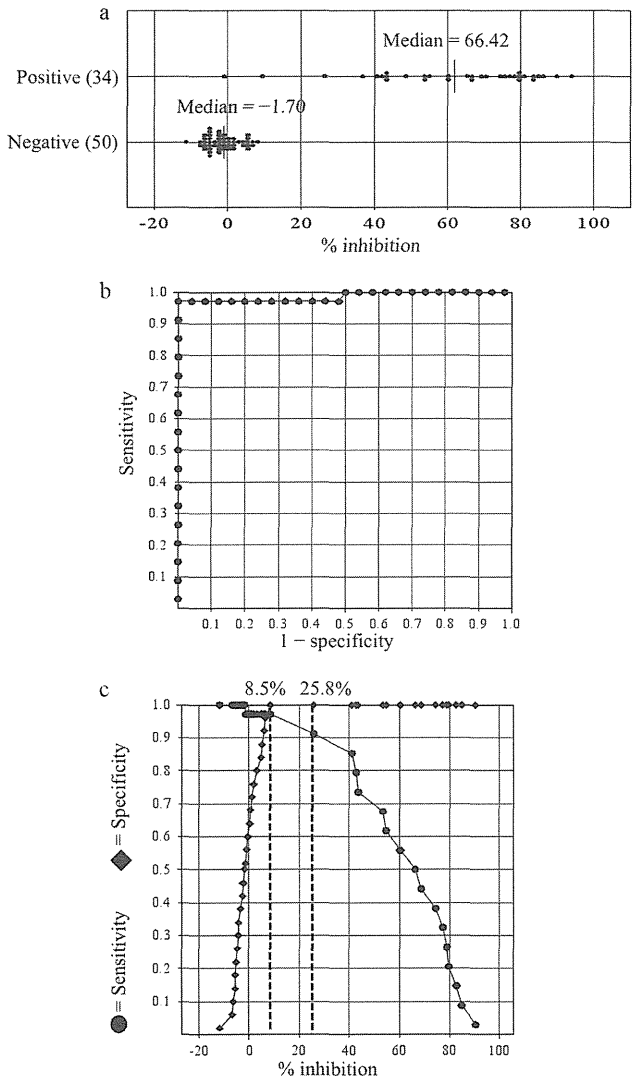


FIG 4 Distribution of percent inhibition values and ROC and TG-ROC analyses of cELISA specific for *F. tularensis* LPS. (a) The percent inhibition value of each serum sample at a dilution of 1:100 was plotted for serum samples obtained from patients with tularemia and healthy donors. (b and c) The ROC 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.

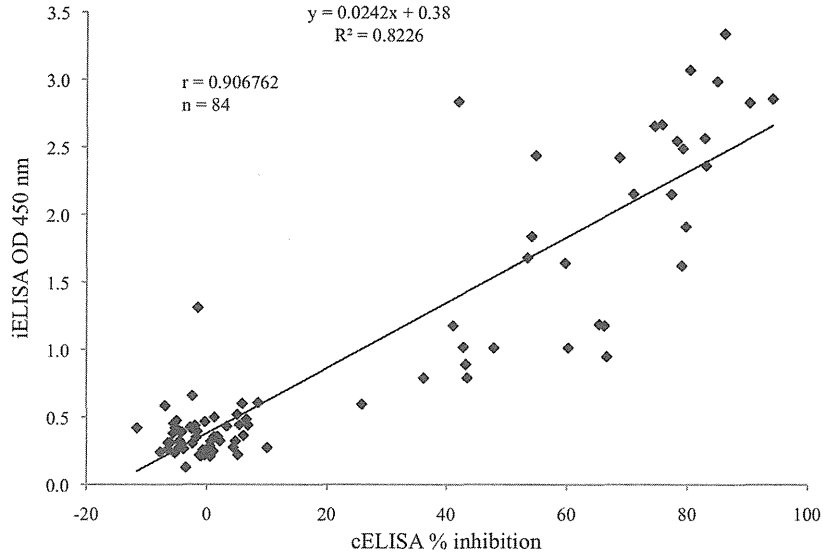


FIG 5 Correlation between cELISA and iELISA for detection of *F. tularensis*-specific antibodies. A scatter plot of the percent inhibition obtained by cELISA and OD values obtained by iELISA for serum samples of patient (n = 34) and healthy human donor (n = 50). Linear regression analysis showed that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

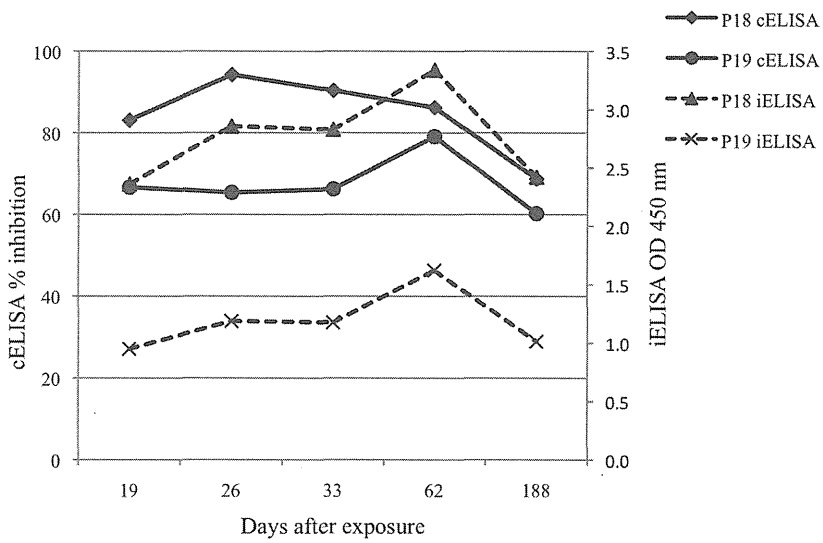


FIG 6 Kinetics of antibody levels in patients with tularemia over a 6-month period. *F. tularensis*-specific antibodies were measured by cELISA and iELISA in two patients with tularemia (P18 and P19, Table 1) at the indicated days after exposure to *F. tularensis*. The percent inhibition values in cELISA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

bodies against both *F. tularensis* subsp. *tularensis* and subsp. *holarctica* in rabbit and mouse sera were detected by cELISA (Fig. 2b and c). Although we could not test convalescent human patients sera infected with *F. tularensis* subsp. *tularensis* due to unavailability in Japan, the result in the present study indicated that the cELISA is capable to detect antibodies induced after both *F. tularensis* subsp. *tularensis* and subsp. *holarctica* infections. On the other hand, when serum sample of a mouse immunized with *F. novicida* were tested, 11% inhibition was observed (Fig. 2c). This slight inhibition may be due to the common antigenic epitopes shared by *F. novicida* and *F. tularensis* since they are genetically closely related (29). In addition, no serum samples of rabbits immunized with *Brucella* spp. and *Yersinia* spp. interfered with the specific binding of MAb to LPS (Fig. 3). These results indicate that MAb-based cELISA is highly specific for *F. tularensis* without any cross-reactions with other microorganisms.

The results of cELISA of serum samples of tularemia patients and healthy donors were evaluated. ROC analysis showed that cELISA had sensitivities of 91.1 and 97.0% at cutoff values of 25.8% inhibition and 8.5% inhibition, respectively (Fig. 4). Two serum samples of patients at day 1 (P4) and day 11 (P1) after onset of symptoms were negative by cELISA at a cutoff value of 25.8%. These sera were also negative by iELISA and MA (Table 1), and thus it was conceivable that these acute-phase sera did not contain detectable levels of antibodies against *F. tularensis*. However, when the cutoff value was decreased to 8.5% inhibition, these two acute-phase sera became positive, indicating that cELISA with a cutoff value of 8.5% is more sensitive in detecting antibodies against *F. tularensis* than iELISA and MA. In contrast, serum sample of the patient (P17) obtained 8 months (241 days) after recovery was negative even at a cutoff value of 8.5% inhibition, although the serum was positive by iELISA and MA. The paired serum of the same patient (P17) obtained 2 weeks after disease onset was clearly positive by both cELISA and iELISA, but negative by MA. These results might suggest that LPS antibodies recognizing the specific epitope of the MAb M14B11 in patients did not persist for several months, whereas the antibodies recognizing the other epitopes on LPS persisted for more than 8 months. The testing of large numbers of serum samples from both acute and convalescent phases of tularemia patients might have improved the precise interpretation of data. For the 50 healthy human serum samples, cELISA exhibited 100% specificity at both cutoff values of 25.8 and 8.5% inhibition (Fig. 4c). Therefore, the cutoff value of 8.5% inhibition was considered to be suitable for sensitive and specific detection of *F. tularensis* antibodies in human serum samples based on the ROC analysis.

The sensitivity of cELISA and iELISA were comparable, whereas MA was less sensitive in detecting *F. tularensis* antibodies (Tables 2 and 3). All of these results were comparable to those of previously reported cELISA methods that detected antibodies against *F. tularensis* outer membrane protein antigens with a sensitivity and specificity of 95.7 and 96%, respectively (30). However, it is noteworthy that LPS is a more suitable antigen for detecting an antibody response elicited in the early phase after *F. tularensis* infection as well as antibodies that persist for several years (10, 24).

In the present study, we also found, in agreement with a previous report, that antibodies against *F. tularensis* persist for months after infection (16). Although we could not test how long the antibodies persist, a moderately decreasing antibody level was observed after 6 months (Fig. 6), and this pattern was the same in serum samples of two different patients with comparable antibody levels in cELISA and iELISA. Indeed, the correlation between cELISA and iELISA was high ($R^2 = 0.8226$) (Fig. 5). These data suggest that the results obtained for detection of antibodies against *F. tularensis* by the novel cELISA are reliable and consistent with those obtained by iELISA.

Our cELISA method based on the inhibition of binding of MAb specific to the *F. tularensis* LPS is highly sensitive and specific. This method is well suited as a routine, confirmatory laboratory test for tularemia in humans. It can overcome the problems associated with conventional serological assays such as low sensitivity and specificity in MA or requirements of species-specific secondary antibodies in iELISA or indirect immunofluorescence assay. Therefore, our MAb-based cELISA is expected to be advantageous and useful for serosurveillance of various wild animals such as bears, wild boars, fox, raccoon dogs, and even birds, except for the highly susceptible animals such as some species of rodents or lagomorphs since they die before producing antibodies to *F. tularensis*. A seroepidemiological study of various wild animals with this novel cELISA is ongoing. We believe this cELISA method will help facilitate the surveillance of tularemia in humans and wild animals, leading to better understanding of the ecology and epidemiology of tularemia.

ACKNOWLEDGMENTS

We thank H. Fujita, Ohara Research Laboratories, Ohara General Hospital, Fukushima, Japan, for providing serum samples of patients and healthy blood donors. We also thank K. Imaoka (Department of Veterinary Science, NIID) for providing rabbit sera immunized with *Brucella* spp. and *Yersinia* spp.

This study was supported in part 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 (H22-Shinkou-Ippan-010). N.S. was supported by a Tokyu Foundation Scholarship for Inbound Students.

REFERENCES

1. McCoy GW, CC. 1912. *Bacterium tularense*, the cause of a plaguelike disease of rodents. Public Health Bull. 53:17–23.
2. Ellis J, Oyston PC, Green M, Titball RW. 2002. Tularemia. Clin. Microbiol. Rev. 15:631–646.
3. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763–2773.
4. Oyston PC, Sjostedt A, Titball RW. 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat. Rev. Microbiol. 2:967–978.
5. Hopla CE. 1974. The ecology of tularemia. Adv. Vet. Sci. Comp. Med. 18:25–53.
6. Hansen CM, Vogler AJ, Keim P, Wagner DM, Hueffer K. 2011. Tularemia in Alaska, 1938–2010. Acta Vet. Scand. 53:61.
7. Hotta A, Tanabayashi K, Yamamoto Y, Fujita O, Uda A, Mizoguchi T, Yamada A. 2012. Seroprevalence of tularemia in wild bears and hares in Japan. Zoonoses Public Health 59:89–95.
8. Maurin M, Pelloux I, Brion JP, Del Bano JN, Picard A. 2011. Human tularemia in France, 2006–2010. Clin. Infect. Dis. 53:e133–141.
9. World Health Organization Epidemic and Pandemic Alert and Response. 2007. WHO guidelines on tularemia. World Health Organization, Geneva, Switzerland.
10. Eliasson H, Olcen P, Sjostedt A, Jurstrand M, Back E, Andersson S. 2008. Kinetics of the immune response associated with tularemia: com-

parison of an enzyme-linked immunosorbent assay, a tube agglutination test, and a novel whole-blood lymphocyte stimulation test. Clin. Vaccine Immunol. 15:1238–1243.
11. **Splettstoesser WD, Tomaso H, Al Dahouk S, Neubauer H, Schuff-Werner P.** 2005. Diagnostic procedures in tularaemia with special focus on molecular and immunological techniques. J. Vet. Med. B Infect. Dis. Vet. Public Health 52:249–261.
12. **Versage JL, Severin DD, Chu MC, Petersen JM.** 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.
13. **Porsch-Ozcurumez M, Kischel N, Priebe H, Splettstosser W, Finke EJ, Grunow R.** 2004. Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. Clin. Diagn. Lab. Immunol. 11:1008–1015.
14. **Schmitt P, Splettstosser W, Porsch-Ozcurumez M, Finke EJ, Grunow R.** 2005. A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. Epidemiol. Infect. 133:759–766.
15. **Bevanger L, Maeland JA, Kvan AI.** 1994. Comparative analysis of antibodies to *Francisella tularensis* antigens during the acute phase of tularemia and eight years later. Clin. Diagn. Lab. Immunol. 1:238–240.
16. **Koskela P, Salminen A.** 1985. Humoral immunity against *Francisella tularensis* after natural infection. J. Clin. Microbiol. 22:973–979.
17. **Sato T, Fujita H, Ohara Y, Homma M.** 1990. Microagglutination test for early and specific serodiagnosis of tularemia. J. Clin. Microbiol. 28:2372–2374.
18. **Brown SL, McKinney FT, Klein GC, Jones WL.** 1980. Evaluation of a safranin-O-stained antigen microagglutination test for *francisella tularensis* antibodies. J. Clin. Microbiol. 11:146–148.
19. **Behan KA, Klein GC.** 1982. Reduction of *Brucella* species and *Francisella tularensis* cross-reacting agglutinins by dithiothreitol. J. Clin. Microbiol. 16:756–757.
20. **Bevanger L, Maeland JA, Naess AI.** 1988. Agglutinins and antibodies to *Francisella tularensis* outer membrane antigens in the early diagnosis of disease during an outbreak of tularemia. J. Clin. Microbiol. 26:433–437.
21. **Hotta A, Uda A, Fujita O, Tanabayashi K, Yamada A.** 2007. Preparation of monoclonal antibodies for detection and identification of *Francisella tularensis*. Clin. Vaccine Immunol. 14:81–84.
22. **Greiner M, Sohr D, Gobel P.** 1995. A modified ROC analysis for the selection of cutoff values and the definition of intermediate results of serodiagnostic tests. J. Immunol. Methods 185:123–132.
23. **Zweig MH, Campbell G.** 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin. Chem. 39:561–577.
24. **Carlsson HE, Lindberg AA, Lindberg G, Hederstedt B, Karlsson KA, Agell BO.** 1979. Enzyme-linked immunosorbent assay for immunological diagnosis of human tularemia. J. Clin. Microbiol. 10:615–621.
25. **Viljanen MK, Nurmi T, Salminen A.** 1983. Enzyme-linked immunosorbent assay (ELISA) with bacterial sonicate antigen for IgM, IgA, and IgG antibodies to *Francisella tularensis*: comparison with bacterial agglutination test and ELISA with lipopolysaccharide antigen. J. Infect. Dis. 148: 715–720.
26. **Meegan J, Field C, Sidor I, Romano T, Casinghino S, Smith CR, Kashinsky L, Fair PA, Bossart G, Wells R, Dunn JL.** 2010. Development, validation, and utilization of a competitive enzyme-linked immunosorbent assay for the detection of antibodies against *Brucella* species in marine mammals. J. Vet. Diagn. Invest. 22:856–862.
27. **Weynants V, Gilson D, Cloeckaert A, Denoel PA, Tibor A, Thiange P, Limet JN, Letesson JJ.** 1996. Characterization of a monoclonal antibody specific for *Brucella* smooth lipopolysaccharide and development of a competitive enzyme-linked immunosorbent assay to improve the serological diagnosis of brucellosis. Clin. Diagn. Lab. Immunol. 3:309–314.
28. **Gunn JS, Ernst RK.** 2007. The structure and function of *Francisella* lipopolysaccharide. Ann. N. Y. Acad. Sci. 1105:202–218.
29. **Broekhuijsen M, Larsson P, Johansson A, Bystrom M, Eriksson U, Larsson E, Prior RG, Sjostedt A, Titball RW, Forsman M.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J. Clin. Microbiol. 41:2924–2931.
30. **Bevanger L, Maeland JA, Naess AI.** 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.

Sekizuka et al. BMC Microbiology 2012, 12:72
http://www.biomedcentral.com/1471-2180/12/72

BMC Microbiology

RESEARCH ARTICLE Open Access

Corynebacterium ulcerans 0102 carries the gene encoding diphtheria toxin on a prophage different from the *C. diphtheriae* NCTC 13129 prophage

Tsuyoshi Sekizuka[1], Akihiko Yamamoto[2], Takako Komiya[2], Tsuyoshi Kenri[2], Fumihiko Takeuchi[1], Keigo Shibayama[2], Motohide Takahashi[2,3], Makoto Kuroda[1] and Masaaki Iwaki[2*]

Abstract

Background: *Corynebacterium ulcerans* can cause a diphtheria-like illness, especially when the bacterium is lysogenized with a *tox* gene-carrying bacteriophage that produces diphtheria toxin. Acquisition of toxigenicity upon phage lysogenization is a common feature of *C. ulcerans* and *C. diphtheriae*. However, because of a lack of *C. ulcerans* genome information, a detailed comparison of prophages has not been possible between these two clinically important and closely related bacterial species.

Results: We determined the whole genome sequence of the toxigenic *C. ulcerans* 0102 isolated in Japan. The genomic sequence showed a striking similarity with that of *Corynebacterium pseudotuberculosis* and, to a lesser extent, with that of *C. diphtheriae*. The 0102 genome contained three distinct prophages. One of these, ΦCULC0102-I, was a *tox*-positive prophage containing genes in the same structural order as for *tox*-positive *C. diphtheriae* prophages. However, the primary structures of the individual genes involved in the phage machinery showed little homology between the two counterparts.

Conclusion: Taken together, these results suggest that the *tox*-positive prophage in this strain of *C. ulcerans* has a distinct origin from that of *C. diphtheriae* NCTC 13129.

Keywords: Bacteriophage, Toxin gene, Horizontal gene transfer, Diphtheria, Zoonosis

Background

A diphtheria-like infectious disease caused by *Corynebacterium ulcerans* is increasing in clinical importance in developed countries and is now regarded as "diphtheria" in Europe [1,2]. Infection with *C. ulcerans* occurs in a wide range of hosts, including cats, dogs, pigs, cows, and whales [3-9]. The first clearly documented case of zoonotic transmission involved a dog, as reported by Lartigue et al. [5]. This is in contrast to the causative agent of classical diphtheria, *C. diphtheriae*, whose host species is thought to be limited to humans [10]. Nevertheless, the two species share a common feature: upon lysogenization of *tox*-encoding bacteriophages, they become toxigenic and are able to produce the potent diphtheria toxin [1,10]. This toxin is known to contribute to disease progression, occasionally leading to death. It is encoded by a single gene designated *tox*, situated inside prophages lysogenized in the bacterial genome of *C. diphtheriae* [11]. The prophages are capable of induction, by ultraviolet light or DNA-damaging agents such as mitomycin C, and yield β-, δ-, ω- and other functional bacteriophage particles [12]. Some types of bacteriophages can infect both *C. diphtheriae* and *C. ulcerans* [13-16]. Furthermore, the *C. ulcerans tox* gene is also encoded in a genome region surrounded by phage attachment (*att*) sites conserved between the two species [7,16]. The nucleotide sequences of *C. ulcerans tox* genes were published by Sing et al. They showed some

* Correspondence: miwaki@nih.go.jp
[2]Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan
Full list of author information is available at the end of the article

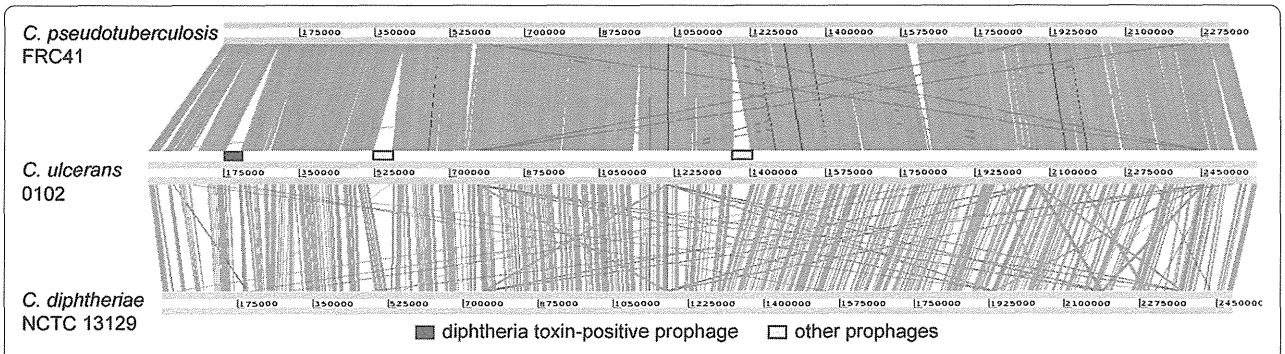


Figure 1 Schematic genome comparison. *C. ulcerans* 0102 (middle) with *C. pseudotuberculosis* FRC41 (top) and *C. diphtheriae* NCTC 13129 (bottom) using a BLASTN homology search visualized by the ACT program. The red and blue bars between chromosomal DNA sequences represent individual nucleotide matches in the forward and reverse directions, respectively. BLASTN match scores less than 200 are not shown. A blue box and two yellow boxes represent a *tox*-positive prophage and other prophages on the chromosome of *C. ulcerans* 0102, respectively.

diversity in the genetic sequence among *C. ulcerans* strains, in contrast to the highly conserved *C. diphtheriae tox* gene [17,18].

In 2003, the nucleotide sequence of the whole genome of *C. diphtheriae* strain NCTC13129 was reported [19]. The sequence information revealed some striking features of the bacterial genome, such as the presence of as many as 13 pathogenicity islands (PAIs) [19], uncommon among *C. diphtheriae* strains [20]. The presence of a *tox*-positive prophage flanked by the *att* regions was confirmed and supported the findings of previous reports [21]. Despite comparable clinical importance, the genomic sequence of toxigenic *C. ulcerans* has not yet been reported. In the present study, we determined the nucleotide sequence of the toxigenic *C. ulcerans* isolate 0102 genome, obtained in 2001 from the pharyngeal pseudomembrane of a 52-year-old woman presenting with a sore throat and fever. This was the first toxigenic *C. ulcerans* infection reported in Japan. This patient had been living with nearly 20 cats before the onset of illness [22]. Details of the bacteriological characteristics of the isolate have been described elsewhere [23]. Our analysis was especially directed towards the structure of the *tox*-positive prophage because of its unexpectedly novel structure.

Results

Genome sequence and genomic information for *C. ulcerans* 0102

To determine the complete genome sequence of *C. ulcerans* 0102, obtained short reads were assembled into five contigs by *de novo* assembly. Each gap was filled by direct PCR and sequencing. A circular chromosome sequence of *C. ulcerans* 0102 represents 2,579,188 bp, with a G + C content of 53.4% (Additional file 1) and corresponds to the predicted restriction fragment profiles obtained by PFGE analysis (Additional file 2). The chromosome possesses 2,349 coding sequences, 51 tRNA genes, and 4 *rrn* rRNA operons.

Comparative genome analysis of three pathogenic *Corynebacterium* spp

Pair-wise sequence alignment revealed a highly conserved synteny among pathogenic *Corynebacterium* spp. (*C. pseudotuberculosis* FRC41, *C. ulcerans* 0102, and *C. diphtheriae* NCTC 13129; Figure 1). No significant genome rearrangements, such as inversion or transposition events, were observed among the three species, in accordance with previous findings [24]. The sequence similarity suggests that the chromosomes of *C. ulcerans* 0102 and *C. pseudotuberculosis* FRC41 are highly similar compared with that of *C. diphtheriae* NCTC 13129 (Figure 1). Once again, this is in accordance with previous findings in other *C. ulcerans* strains [24]. Similarly, a neighbor-joining phylogenetic tree, based on the partial sequence of *rpoB*, indicates that *C. ulcerans* 0102 is closely related with *C. pseudotuberculosis*, but clearly distinguishable from the *C. diphtheriae* clade (Additional file 3). Three prophages, ΦCULC0102-I, -II, -III, were identified in *C. ulcerans* 0102. One of the prophages, ΦCULC0102-I, carries *tox*, the gene encoding the diphtheria toxin (Figure 1).

The *tox*-positive prophage of *C. ulcerans* 0102

The ΦCULC0102-I prophage of *C. ulcerans* 0102 is integrated into $tRNA^{Arg}$ (CULC0102_t08) (Figure 2), suggesting that the integration site is identical to that in the *C. diphtheriae* NCTC 13129 corynephage. In contrast, the recently reported *C. ulcerans* 809 and *C. pseudotuberculosis* FRC41 genomes possess a phage-related integrase (*intC*) and a nitric oxide reductase (*nor*) gene, respectively, instead of a prophage (Figure 2). Putative attachment sequences were similar between both prophages carrying the *tox* genes (Additional file 4).

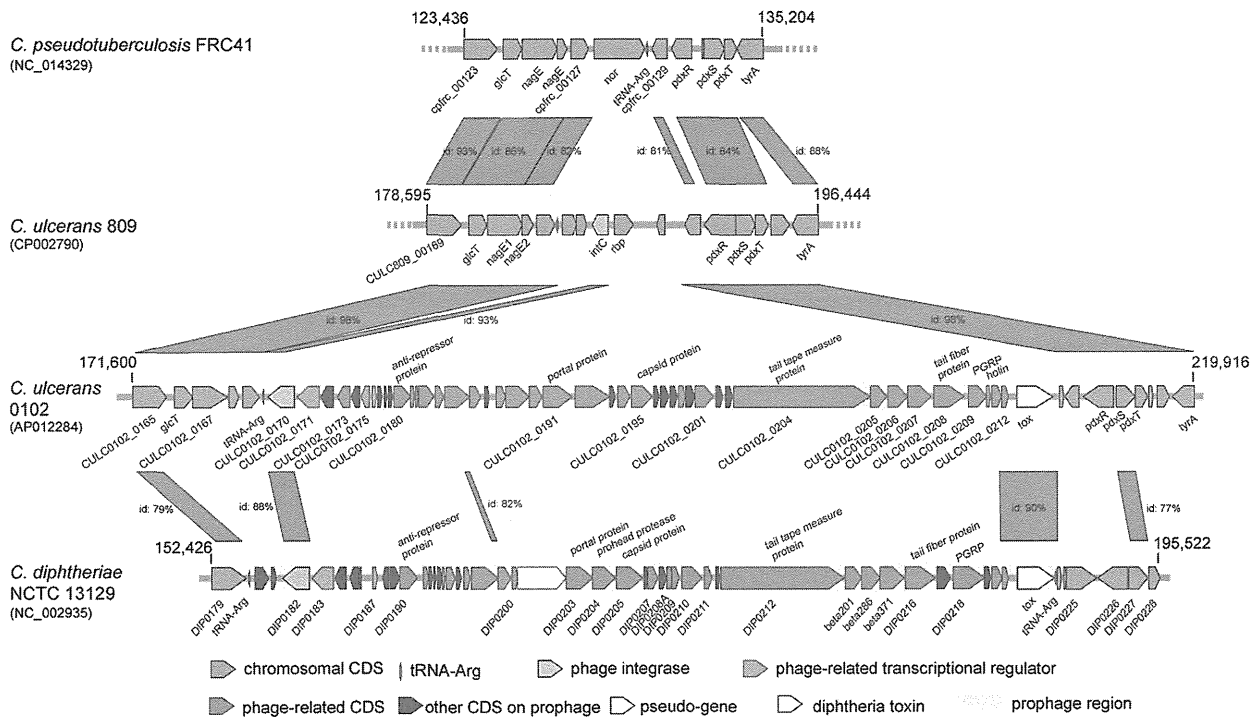


Figure 2 Schematic representation and comparative analysis of *tox*-positive prophages and flanking regions. The *tox*-positive prophage and flanking regions of *C. ulcerans* 0102 and *C. diphtheriae* NCTC13129 are shown. The corresponding region of *C. pseudotuberculosis* FRC41 and *C. ulcerans 809* is also shown. Boxes indicate individual coding regions with colors assigned to their functions. GenBank accession numbers are given in parentheses.

The two *tox*-positive prophages share the same structural features, with genes aligned in an 'integrase - packaging - head - tail - lysis - toxin' orientation (Figure 2). Pair-wise alignment of the prophages indicates a high similarity in the region encoding the putative integrase, the 3′-ends of CULC0102_0211 and CULC0102_0212, *tox*, and the attachment sites (Figure 2). The major phage machineries encoded in the internal phage region showed low similarity at the nucleotide and amino acid levels (less than 18%) between *C. ulcerans* 0102 and *C. diphtheriae* NCTC13129.

Discussion

Whole-genome sequencing has revealed that the *C. ulcerans* 0102 genome is composed of 2,579,188 bp with a G + C content of 53.4%. These values are similar to those recently reported for *C. ulcerans* strains 809 (2,502,095 bp, 53.3% G + C) and BR-AD22 (2,606,374 bp, 53.4% G + C) [24]. *C. ulcerans* 0102 shares many common features with the two previously reported strains, including 12 virulence factors. Strain 0102 is distinctive with respect to the features of prophages integrated in its genome. It possesses a unique *tox*-positive prophage, ΦCULC0102-I, in its chromosome (Figure 1 and Additional file 1). In the same position of the recently reported *C. ulcerans* 809 genome exists a remnant phage-related integrase (*intC*) gene [24] (Figure 2). The *C. ulcerans* 0102 prophage differs from the corresponding prophage in *C. diphtheriae*. Although the integrase and *tox* gene sequences of ΦCULC0102-I showed high similarity to those of the corynephage encoding *tox* in *C. diphtheriae* NCTC 13129, the major phage machinery genes in ΦCULC0102-I are distinct from those in other corynephages in *C. diphtheriae* (Figure 2). This suggests that *C. ulcerans* 0102 did not immediately acquire the *C. diphtheriae tox*-positive corynephage.

There are many possible explanations for the origins of these two prophages that are *tox*-positive but obviously different. One of the simplest explanations we can postulate is outlined in Figure 3. Generally, bacterial prophages are duplicated by excision from chromosomal DNA and subsequent concatenation at both ends of the *att* sites (Figure 3A). This duplication step indicates that two highly homologous regions, *int* and *tox*, could be in close proximity and adjacent to the *att* site concatenation. It could be speculated that homologous recombination between two prophages may facilitate the acquisition of the *tox* gene in *C. ulcerans* 0102 from an unknown *tox*-positive prophage (Figure 3B) [25]. Horizontal gene transfer is one of the major mechanisms of foreign gene acquisition by bacteria, as reviewed by Ochman et al. [26]. Liu et al. have

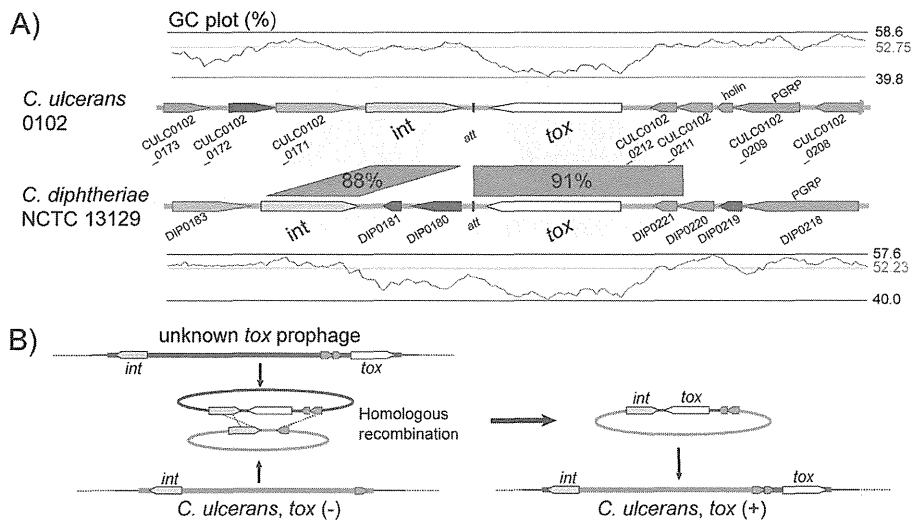


Figure 3 Schema of the diphtheria toxin acquisition hypothesis. (A) Pair-wise comparison of regions with high similarity between *C. ulcerans* and *C. diphtheriae*. These structures of putative phages are constructed by connecting attachment sites. The plots above and below represent the GC content calculated with a window size of 500 bp. **(B)** Schematic representation of how diphtheria toxin has been acquired in *C. ulcerans*.

demonstrated that horizontally transferred genes are often disabled and become pseudogenes. In these cases the genes are no longer beneficial to the recipients [27]. Non-toxigenic *C. diphtheriae* (CD450, CD119, CD448, and CD443 strains) carry *tox* pseudogenes that are relatively similar to the *tox* genes of *C. ulcerans* (Additional file 5), suggesting that horizontal gene transfer among *Corynebacterium* spp. might occur. Consistent with previous findings [7,17,18,28], tthe *tox* gene in *C. ulcerans* 0102 is not identical to that of *C. diphtheriae* (Additional file 5); phylogenetic analysis of *tox* showed greater heterogeneity among *C. ulcerans* isolates than that for *C. diphtheriae* isolates (Additional file 5).

The *C. diphtheriae tox* gene is highly conserved among temporally and geographically diverse strains [29], therefore greater variation in *tox* genes from *C. ulcerans* isolates suggests that this strain might have acquired the *tox* gene before *C. diphtheriae*.

In a recent report, whole genome sequence analysis of non-toxigenic *C. ulcerans* 809 and BR-AD22 [24], the β-corynephage-like truncated integrases (CULC809_00176 and CULC22_00173) are located adjacent to the $tRNA^{Arg}$ gene, similar to ΦCULC0102-I in *C. ulcerans* 0102 and *C. diphtheriae*. The $tRNA^{Arg}$ gene (CULC0102_t08) appears to be a 'hotspot' for the acquisition of ΦCULC0102-I-like prophages by homologous integrase.

The whole genome sequences of *C. ulcerans* 809 and BR-AD22 contain possible virulence factors, such as corynebacterial protease (CP40), phospholipase D (Pld), neuraminidase (NanH), venom serine protease (Vsp1), trypsin-like serine protease (TspA), Rpf interacting protein (RpfI), cell wall-associated hydrolase (CwlH), and five surface-anchored proteins (SpaB–F) [24]. The SpaA-type pilin, encoded by the *spaABC-srtA* gene cluster, is considered to play a crucial role in adhesion of *C. diphtheriae* [30]. The gene encoding the shaft protein of SpaA-type pilin (*spaA*) was absent in *C. ulcerans* 0102, a feature consistent with previous findings in *C. ulcerans* 809 and BR-AD2 [24]. As SpaB and SpaC proteins, which are assumed to be present in all three *C. ulcerans* strains, can contribute to host-cell adhesion in the absence of SpaA [30], this may imply a common mechanism of cell adhesion by *C. ulcerans* [24].

The *C. ulcerans* 809 strain was isolated from a patient with a rapid fatal pulmonary infection. The 809 strain-unique virulence factor (shiga toxin-like ribosome-binding protein, Rbp) is located adjacent to the truncated integrase (CULC809_00176) and corresponds to the integrase of ΦCULC0102-I. It appears that virulence factors have been acquired as a cassette gene in the ΦCULC0102-I-like prophage. It is intriguing to note that the 0102 strain does not carry the 809 strain-unique virulence factors (Rbp and the additional venom serine protease, Vsp2), but instead carries the *tox* gene on ΦCULC0102-I, which resulted in a diphtheria-like illness in a 52-year-old woman.

Isolates of *C. ulcerans* are generally obtained from a diverse range of animals, including humans. Isolation of a human pathogen *C. diphtheriae* from animals has been reported previously, although it is rare [31]. The *tox* gene might be frequently transmitted through common

prophages with the aid of the highly homologous regions among *Corynebacterium* spp., including *C. diphtheriae* and *C. ulcerans* isolated from animal sources.

Conclusions

Toxigenic *C. ulcerans* is an emerging pathogen that can be transmitted from animals to humans [5]. In the host organism, as well as in *C. diphtheriae*, the *tox* gene [18] is encoded by prophages. Through genome sequencing, we have identified a novel structure in a *tox*-positive *C. ulcerans* prophage with no significant sequence homology to those in *C. diphtheriae*. This suggests distinct origins of the prophages and thus may also explain the difference in the primary structures of their *tox* genes. The *tox*-positive bacteriophages may increase the dissemination risk of toxigenic *C. ulcerans* isolates, therefore, *C. ulcerans* isolates from both human and animal sources should be investigated further to determine the level of variation.

Methods

This research was not carried out on humans. No experimental research on animals was carried out.

Bacterial strain

The toxigenic *C. ulcerans* isolate 0102 was obtained in 2001 as a human clinical isolate [22,23].

Preparation of genomic DNA

Genomic DNA was isolated by conventional methods, using phenol extraction and ethanol precipitation from heat-killed bacterial cells propagated in brain-heart infusion liquid medium.

Short-read DNA sequencing using an Illumina Genome Analyzer IIx

DNA libraries of the ~600 bp insert length of *C. ulcerans* 0102 were prepared using a genomic DNA Sample Prep Kit (Illumina, San Diego, CA, USA). DNA clusters were generated on a slide using a Cluster Generation Kit (ver. 4) on an Illumina Cluster Station (Illumina), according to the manufacturer's instructions. Sequencing runs for 80-mer short reads were performed using an Illumina Genome Analyzer IIx (GA IIx) and TruSeq SBS kit v5. Fluorescent images were analyzed using the Illumina base-calling pipeline RTA2.6/SCS2.8 to obtain FASTQ-formatted sequence data.

De novo assembly of short DNA reads and gap-closing

The 80-mer reads were assembled (parameters k64, n51, c32.1373) using ABySS-pe v1.2.0 [32]. Predicted gaps were amplified with a specific PCR primer pair, followed by Sanger DNA sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Validation of the complete genome sequence using short-read mapping and pulsed-field gel electrophoresis (PFGE)

To validate the genome sequence, 40–mer short reads were re-aligned with the sequence using Maq software (ver. 0.7.1) and the *easyrun* Perl-command [33]. Read alignment was inspected using the MapView graphical alignment viewer [34]. PFGE analysis was performed to validate the predicted restriction fragment profiles from the complete genome sequence, according to De Zoysa et al. [35]. Bacterial cells were lysed with lysozyme and protease [36], embedded in plugs, digested with the restriction endonuclease *Sfi*I (New England Biolabs, Ipswitch, MA, USA) and electrophoresed in a CHEF DRII apparatus (Bio-Rad, Hercules, CA, USA) at 11°C with a pulse time of 5–20 s for the first 20 h and 1–5 s for the following 18 h.

Annotation and pair-wise alignment analysis

Gene prediction from the complete sequence was performed using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; http://www.ncbi.nlm.nih.gov/genomes/static/pipeline.html). Several of the suggested errors were revised manually. Pseudogenes that were identified by PGAAP were checked using the read-mapping correction described above. Genomic information, such as nucleic acid variations and circular representation, was analyzed using IMC-GE software (Insilicobiology, Yokohama, Japan). A BLASTN homology search [37] was performed for the whole chromosome sequences of *C. pseudotuberculosis* FRC41 (accession no. NC_014329), *C. ulcerans* 0102, and *C. diphtheriae* NCTC 13129 (accession no. NC_002935). Aligned images of the homologous regions were visualized with the ACT program [38].

Phylogenetic analysis

Phylogenetic analyses of all nucleotide sequences were conducted using the neighbor-joining method with 1,000-times bootstrapping in ClustalW2 [39]. FigTree ver. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) software was used to display the generated tree.

Nucleotide sequence accession numbers

The complete chromosome sequence for the *C. ulcerans* 0102 strain has been deposited in the DNA Data Bank of Japan (DDBJ; accession no. AP012284).

Additional files

Additional file: 1 Circular representation of the *C. ulcerans* 0102 genome. From the outside inward, the outer circle 1 indicates the size in base pairs (Mb). The red bars on Circle 2 show prophage region. Circles 3 and 4 show the positions of CDS transcribed in clockwise and anticlockwise directions, respectively. The dark blue bars on circle 5 indicate ribosomal DNA loci. Circle 6 shows a plot of G+C content (in a 20 kb window). Circle 7 shows a plot of GC skew ([G - C]/[G+C]; in a 20 kb window).

Additional file: 2 PFGE analysis of *C. ulcerans* 0102 with four restriction enzyme digestions.

Additional file: 3 Jukes-Cantor-derived phylogenetic tree based on the partial *rpoB* gene region among *Corynebacterium* isolates with 1,000-fold bootstrapping. Scale bar indicates number of substitutions per site. The number at each branch node represents the bootstrapping value. GenBank accession nos. given in parentheses.

Additional file: 4 Alignment of the nucleotide sequences of attachment site common regions among *C. ulcerans* 0102 and *C. diphtheriae* NCTC 13129. The red characters show regions annotated as $tRNA^{Arg}$.

Additional file: 5 Phylogenetic tree based on the tox genes among toxgenic and nontoxigenic *Corynebacterium* spp. using the Neighbor-joining method with 1,000-fold bootstrapping. Scale bar indicates number of substitutions per site. The number at each branch node represents the bootstrapping value. GenBank accession nos. given in parentheses.

Competing interests
The authors declare that they have no competing interests.

Acknowledgments
The authors are grateful to Akio Hatanaka, Atsuhiro Tsunoda and Kenji Ooe for the 0102 clinical isolate. This work was supported by grants for Research on Emerging and Re-emerging Infectious Diseases (H23 Shinko-Ippan-007 and H22-Shinko-Ippan-010), from the Ministry of Health, Labour and Welfare, Japan.

Author details
[1]Laboratory of Bacterial Genomics, Pathogen Genomics Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. [2]Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. [3]Present address: Pharmaceutical and Medical Devices Agency, Tokyo, Japan.

Authors' contributions
TS and FT carried out the genome sequencing studies, participated in the sequence alignment and drafted the manuscript. TKo carried out maintenance, quality control and propagation of the bacterial strain for genome sequencing. AY and TKe participated in the design of the study. MT and KS conceived of and participated in coordination of the study, respectively. MK and MI coordinated the study, and drafted and finalized the manuscript. All authors read and approved the final manuscript.

Received: 10 January 2012 Accepted: 14 May 2012
Published: 14 May 2012

References
1. Bonnet JM, Begg NT: **Control of diphtheria: guidance for consultants in communicable disease control.** *Commun Dis Public Health* 1999, **2**:242–249.
2. European Centre for Disease Prevention and Control: **Diphtheria.** In *Surveillance Report: Annual epidemiological report on communicable diseases in Europe 2010.* 2010:133–135.
3. Dias AASO, Silva FC, Pereira GA, Souza MC, Camello TCF, Damasceno JALD, Pacheco LGC, Miyoshi A, Azevedo VA, Hirata R, *et al*: ***Corynebacterium ulcerans*** **isolated from an asymptomatic dog kept in an animal shelter in the metropolitan area of Rio de Janeiro, Brazil.** *Vector Borne Zoonotic Dis* 2010, **10**:743–748.
4. Katsukawa C, Kawahara R, Inoue K, Ishii A, Yamagishi H, Kida K, Nishino S, Nagahama S, Komiya T, Iwaki M, Takahashi M: **Toxigenic *Corynebacterium ulcerans* Isolated from the domestic dog for the first time in Japan.** *Jpn J Infect Dis* 2009, **62**:171–172.
5. Lartigue M-F, Monnet X, Le Flèche A, Grimont PAD, Benet J-J, Durrbach A, Fabre M, Nordmann P: ***Corynebacterium ulcerans*** **in an immunocompromised patient with diphtheria and her dog.** *J Clin Microbiol* 2005, **43**:999–1001.
6. Schuhegger R, Schoerner C, Dlugaiczyk J, Lichtenfeld I, Trouillier A, Zeller-Peronnet V, Busch U, Berger A, Kugler R, Hörmansdorfer S, Sing A: **Pigs as source for toxigenic *Corynebacterium ulcerans*.** *Emerg Infect Dis* 2009, **15**:1314–1315.
7. Seto Y, Komiya T, Iwaki M, Kohda T, Mukamoto M, Takahashi M, Kozaki S: **Properties of corynephage attachment site and molecular epidemiology of *Corynebcterium ulcerans* isolated from humans and animals in Japan.** *Jpn J Infect Dis* 2008, **61**:116–122.
8. De Zoysa A, Hawkey PM, Engler K, George R, Mann G, Reilly W, Taylor D, Efstratiou A: **Characterization of toxigenic *Corynebacterium ulcerans* strains isolated from humans and domestic cats in the United Kingdom.** *J Clin Microbiol* 2005, **43**:4377.
9. Yoshimura Y, Yamamoto A, Komiya T: **A case of axillary lymph node abscess caused by percutaneous infection of *Corynebacterium ulcerans* through scratch by a pus-discharging cat, June 2010 (in Japanese).** *Infect Agents Surveillance Rep* 2010, **31**:331.
10. Murphy JR: **Chapter 32 *Corynebacterium diphtheriae*.** In *Medical Microbiology*. 4th edition. Edited by Baron S. Galveston: University of Texas Medical Branch at Galveston; 1996.
11. Pappenheimer AM Jr, Gill DM: **Diphtheria. Recent studies have clarified the molecular mechanisms involved in its pathogenesis.** *Science* 1973, **182**:353–358.
12. Rappuoli R, Michel JL, Murphy JR: **Integration of corynebacteriophages: tox^+, $xtox^+$ and $gtox^+$ into two attachment sites on the *Corynebacterium diphtheriae* chromosome.** *J Bacteriol* 1983, **153**:1202–1210.
13. Ishii-Kanei C, Uchida T, Yoneda M: **Isolation of a cured strain from *Corynebacterium diphtheriae* PW8.** *Infect Immun* 1979, **25**:1081–1083.
14. Cianciotto NP, Groman NB: **Extended host range of a β-related corynebacteriophage.** *FEMS Microbiol Lett* 1996, **140**:221–225.
15. Oram M, Woolston JE, Jacobson AD, Holmes RK, Oram DM: **Bacteriophage-based vectors for site-specific insertion of DNA in the chromosome of Corynebacteria.** *Gene* 2007, **391**:53–62.
16. Cianciotto N, Rappuoli R, Groman N: **Detection of homology to the beta bacteriophage integration site in a wide variety of *Corynebacterium* spp.** *J Bacrteriol* 1986, **168**:103–108.
17. Sing A, Bierschenk S, Heesemann J: **Classical diphtheria caused by *Corynebacterium ulcerans* in Germany: amino acid sequence differences between diphtheria toxins from *Corynebacterium diphtheriae* and *C. ulcerans*.** *Clin Infect Dis* 2005, **40**:325–326.
18. Sing A, Hogardt M, Bierschenk S, Heesemann J: **Detection of differences in the nucleotide and amino acid sequences of diphtheria toxin from *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* causing extrapharyngeal infections.** *J Clin Microbiol* 2003, **41**:4848–4851.
19. Cerdeño-Tárraga A-M, Efstratiou A, Dover LG, Holden MTG, Pallen M, Bentley SD, Besra GS, Churcher C, James KD, De Zoysa A, *et al*: **The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129.** *Nucl Acids Res* 2003, **31**:6516–6523.
20. Iwaki M, Komiya T, Yamamoto A, Ishiwa A, Nagata N, Arakawa Y, Takahashi M: **Genome organization and pathogenicity of *Corynebacterium diphtheriae* C7(–) and PW8 strains.** *Infect Immun* 2010, **78**:3791–3800.
21. Cianciotto N, Serwold-Davis T, Groman N, Ratti G, Rappuoli R: **DNA sequence homology between *att*B-related sites of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, *Corynebacterium glutamicum*, and the *att*P site of gamma-corynephage.** *FEMS Microbiol Lett* 1990, **66**:299–301.
22. Hatanaka A, Tsunoda A, Okamoto M, Ooe K, Nakamura A, Miyakoshi M, Komiya T, Takahashi M: ***Corynebacterium ulcerans*** **diphtheria in Japan.** *Emerg Infect Dis* 2003, **9**:752–753.
23. Komiya T, Seto Y, De Zoysa A, Iwaki M, Hatanaka A, Tsunoda A, Arakawa Y, Kozaki S, Takahashi M: **Two Japanese *Corynebacterium ulcerans* isolates from the same hospital: ribotype, toxigenicity and serum antitoxin titre.** *J Med Microbiol* 2010, **59**:1497–1504.

24. Trost E, Al-Dilaimi A, Papavasiliou P, Schneider J, Viehoever P, Burkovski A, Soares SC, Almeida SS, Dorella FA, Miyoshi A, *et al*: **Comparative analysis of two complete *Corynebacterium ulcerans* genomes and detection of candidate virulence factors.** *BMC Genomics* 2011, **12**:383.
25. Brüssow H, Canchaya C, Hardt W-D: **Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion.** *Microbiol Mol Biol Rev* 2004, **68**:560–602.
26. Ochman H, Lawrence JG, Groisman EA: **Lateral gene transfer and the nature of bacterial innovation.** *Nature* 2000, **405**:299–304.
27. Liu Y, Harrison PM, Kunin V, Gerstein M: **Comprehensive analysis of pseudogenes in prokaryotes: widespread gene decay and failure of putative horizontally transferred genes.** *Genome Biol* 2004, **5**:r64.
28. Katsukawa C, Komiya T, Yamagishi H, Ishii A, Nishino S, Nagahama S, Iwaki M, Yamamoto A, Takahashi M: **Prevalence of *Corynebacterium ulcerans* in dogs in Osaka, Japan.** *J Med Microbiol* 2012, **61**:266–273.
29. Nakao H, Mazurova IK, Glushkevich T, Popovic T: **Analysis of heterogeneity of *Corynebacterium diphtheriae* toxin gene, *tox*, and its regulatory element, *dtxR*, by direct sequencing.** *Res Microbiol* 1997, **148**:45–54.
30. Mandlik A, Swierczynski A, Das A, Ton-That H: ***Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells.** *Mol Microbiol* 2007, **64**:111–124.
31. Hall AJ, Cassiday PK, Bernard KA, Bolt F, Steigerwalt AG, Bixler D, Pawloski LC, Whitney AM, Iwaki M, Baldwin A, *et al*: **Novel *Corynebacterium diphtheriae* in domestic cats.** *Emerg Infect Dis* 2010, **16**:688–691.
32. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJM, Birol İ: **ABySS: a parallel assembler for short read sequence data.** *Genome Res* 2009, **19**:1117–1123.
33. Li H, Ruan J, Durbin R: **Mapping short DNA sequencing reads and calling variants using mapping quality scores.** *Genome Res* 2008, **18**:1851–1858.
34. Bao H, Guo H, Wang J, Zhou R, Lu X, Shi S: **MapView: visualization of short reads alignment on a desktop computer.** *Bioinformatics* 2009, **25**:1554–1555.
35. De Zoysa A, Efstratiou A, George RC, Jahkola M, Vuopio-Varkila J, Deshevoi S, Tseneva GY, Rikushin Y: **Molecular epidemiology of *Corynebacterium diphtheriae* from northwestern Russia and surrounding countries studied by using ribotyping and pulsed-field gel electrophoresis.** *J Clin Microbiol* 1995, **33**:1080–1083.
36. Murrey BE, Singh KV, Heath JD, Sharma BR, Weinstock GM: **Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites.** *J Clin Microbiol* 1990, **28**:2059–2063.
37. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**:403–410.
38. Carver T, Berriman M, Tivey A, Patel C, Böhme U, Barrell BG, Parkhill J, Rajandream M-A: **Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database.** *Bioinformatics* 2008, **24**:2672–2676.
39. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, *et al*: **Clustal W and Clustal X version 2.0.** *Bioinformatics* 2007, **23**:2947–2948.

doi:10.1186/1471-2180-12-72
Cite this article as: Sekizuka *et al.*: *Corynebacterium ulcerans* 0102 carries the gene encoding diphtheria toxin on a prophage different from the *C. diphtheriae* NCTC 13129 prophage. *BMC Microbiology* 2012 **12**:72.