110 kDa, respectively (Huang *et al.*, 2004; Kudva *et al.*, 2005). The structure and function of these exosporium components of *B. anthracis* spores have not yet been fully elucidated, and further study, such as 2D electrophoresis in combination with MS, is required. The identification of these surface-exposed antigens may provide useful information for the development of new and more effective vaccines for human and animal use.

The exosporium molecules may play a role in the interaction of spores with the infected host. Macrophages are major effectors of the host immune system against bacterial infection. The entry of the spore into macrophages is a critical step for *B. anthracis* infection. *B. anthracis* spores are able to escape from phagolysosomes, and can germinate and multiply within the macrophage cytoplasm (Dixon *et al.*, 2000). There may be an association between macrophages and exosporium components of *B. anthracis* spores (Dixon *et al.*, 2000; Guidi-Rontani *et al.*, 2001). In this study, we demonstrated that anti-BA-spore IgG inhibited the germination of spores in macrophages, which suggests that the binding of the antibody to spores opsonizes the pathogen, and promotes phagocytosis and subsequent killing by macrophages. Our data also suggest the possibility that the spore-associated proteins detected by the anti-BA-spore IgG may have important roles in germination, and escape of spores from macrophages. The exact mechanisms by which *B. anthracis* spores germinate, and escape from macrophages, are not well characterized. The immunogenic spore proteins shown in this study are potentially involved in the germination process and/or escape from killing by macrophages. Taken together, the results indicate that the anti-BA-spore IgG may be useful in controlling the early phase of anthrax infection, and thus contribute to averting the toxaemia and bacteraemia that result from the massive replication of bacteria.

Mendelson *et al.* (2005) have demonstrated that intramuscularly administered spores disperse to other organs immediately, and that clearance of viable spores from the spleen takes at least 60 days p.i. This explains the long-term protection and sustained titres of neutralizing antibodies achieved by spore vaccination. However, the persistent survival of virulent spores in the body may be a cause of concern for clinicians, and for patients who are exposed to *B. anthracis* spores. To date, antibiotics are the only treatment available for anthrax infection, but they cannot help in the late stage of anthrax infection. Anti-BA-spore IgG prolonged life in infected mice, and decreased mortality (Fig. 6). These results suggest that a combination therapy of antibiotics and anti-BA-spore IgG in cases of possible exposure may be advantageous in inhibiting germination, and clearing remaining spores from the body.

The live spore vaccine is effective; however, there are safety concerns. The vaccine can occasionally result in necrosis at the injection site, or death of treated animals (Turnbull, 1991). A PA-based vaccine is a promising strategy in anthrax prophylaxis, and anti-PA antibody has a definitive role in protective immunity; however, the PA-based vaccine does not provide protection against all virulent strains of *B. anthracis* (Welkos & Friedlander, 1988). Accumulated evidence suggests that other virulence factors are required to optimize the efficacy of anthrax vaccines (Hahn *et al.*, 2005). Considering the pathology of *B. anthracis*, an ideal vaccine would confer protection against spores, bacilli and toxins in a comprehensive manner. Although further investigation should be carried out, the data presented here support the idea that a vaccine with combined action against the major virulent component PA and the spore-specific somatic antigens is a safer option, and may offer full protection against anthrax.

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Rapid Genome Sequencing of RNA Viruses

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We developed a system for rapid determination of viral RNA sequences whereby genomic sequence is obtained from cultured virus isolates without subcloning into plasmid vectors. This method affords new opportunities to address the challenges of unknown or untypeable emerging viruses.

Over the past few years, global migration has led to emerging infectious diseases that pose substantial risks to public health. To prevent potential outbreaks, early detection of infectious pathogens is necessary. In particular, the recent outbreak of severe acute respiratory syndrome (SARS) provided important lessons on how unknown viruses should be detected rapidly. Thus, a standardized and qualified system is required for rapid nucleic acid sequence determination for newly emerging viruses.

Recently, we developed a new method for detecting RNA viruses. This method, based on cDNA representational difference analysis (cDNA RDA), uses 96 hexanucleotides that are not suitable for priming ribosomal RNAs but that normally prime most of the genome of an RNA virus as primers for reverse transcription in cDNA RDA (*1*). However, the RDA method with a cloning step requires at least 1 week for the determination of the nucleic acid sequence.

The Method

Our new system for rapid determination of viral RNA sequence (RDV) uses whole-genome amplification and direct sequencing techniques (Figure 1). The RDV method comprises 6 procedures: 1) effective destruction of cellular RNA and DNA for semipurification of viral particles, 2) effective elimination of DNA fragments by using a prefiltration column system and elution of small amounts of RNA, 3) effective synthesis of first- and second-strand cDNAs, 4) construction and amplification of a cDNA library, 5) construction of a second cDNA library, and 6) direct sequencing using optimized primers. The RDV method enables a broad range of partial nucleotide sequences within the entire viral RNA genome to be obtained within 2 days without cloning into plasmids.

To eliminate contaminating cellular RNA and DNA from the samples, 0.001 μg of RNase A (Qiagen, Hilden, Germany) and 1 μL (2 U) of Turbo DNA-free DNase I (Ambion, Austin, TX, USA) with 1× Turbo DNA-free buffer were incubated at 37°C for 30 min under conditions that prevented destruction of viral RNA in the viral particles. The RNA in the viral particles was then extracted within 30 min by using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). We confirmed that DNA was effectively eliminated by this RNA extraction kit.

In accordance with the Invitrogen manual, cDNA was synthesized, by using random hexamers (Takara Bio Inc., Kyoto, Japan) and Superscript III (Invitrogen, Carlsbad, CA, USA) lacking RNase H activity, at 50°C for 1 h. Then 60 U of RNase H (Takara Bio Inc.) added before synthesis of second-strand cDNA at 50°C for 1 h. In accordance with the manual, a whole genome amplification system (WGA; Sigma-Aldrich, Saint Louis, MO, USA), which was developed for amplification of genomic DNA, was used to amplify viral double-stranded cDNA. This process was

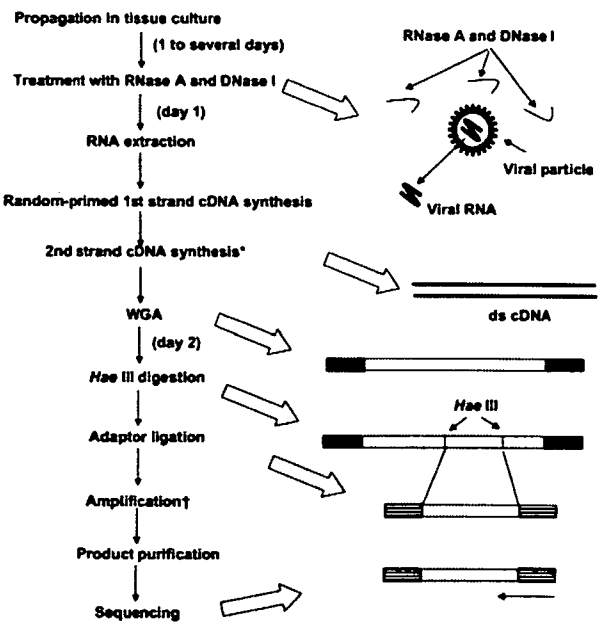

Figure 1. Overall scheme of the rapid determination of viral RNA sequence method. *By adding RNase H; WGA, whole genome amplification; †With specially designed primer sets as shown in Figure 2.

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performed within 90 min. Instead of the Taq polymerase recommended in the kit, we used 1.25 U of AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the PCR products. Primers were provided in the WGA kit, but no information regarding their sequences was obtained. The reaction mixture was heated at 95°C for 9 min (for activation of AmpliTaq Gold), followed by 70 cycles of amplification using Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 68°C for 1 min, primer extension at 72°C for 5 min, and denaturation at 94°C for 1 min.

The 1st cDNA library was digested with 40 U of *Hae*III (Takara Bio Inc.) at 37°C for 30 min. DNA was purified by using the MonoFas DNA isolation system (GL Science, Tokyo, Japan), and a blunt *EcoR*I-*Not*I-*Bam*HI adaptor (10 pmol; Takara Bio Inc.) was ligated at 16°C for 30 min by using DNA Ligation Kit, Mighty Mix (Takara Bio Inc.). The second cDNA library was amplified by PCR with specially designed primer sets in which 6 nucleotides composed of CC (*Hae*III-digested sequence) and 4 variable nucleotides were added to the 3′ end of the adaptor sequence (Figure 2). For example, 1 primer set was as follows: forward primer, H1–1: 5′-AATTCGGCGGCCGCG GATCC*CC*GGGG-3′; reverse primer H9–3: 5′-AATT CGGCGGCCGCGGATCC*CC*AGGA-3′ (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics) (Figure 2).

We always used >12 primer sets and 0.83 μmol of each primer per cDNA library. PCR was performed with AmpliTaq Gold Master Mix (Applied Biosystems). The reaction mixture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing and primer extension at 72°C for 30 s and denaturation at 94°C for 30 s. A single band was consistently obtained in ≈50% of the reactions. DNA was purified from the PCR by using MonoFas. Occasionally, we purified DNA fragments from the gels when >2 bands were detected. Direct sequencing was performed with the forward primer, reverse primer, or both.

When the number of viral particles in the sample was high, we omitted the RNase A and DNase I treatments and used the RNeasy Mini Kit (Qiagen) for RNA extraction. We occasionally used a whole transcriptome amplification kit (Rubicon Genomics Inc, Ann Arbor, MI, USA) instead of the WGA kit because both kits yielded similar amplification results.

In preliminary studies that used referential RNA viruses, we attempted to determine the nucleic acid sequences of SARS coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants (10–100 μL) by using the RDV method. The percentages of positive fragments (number of fragments containing viral nucleic acid/total number of

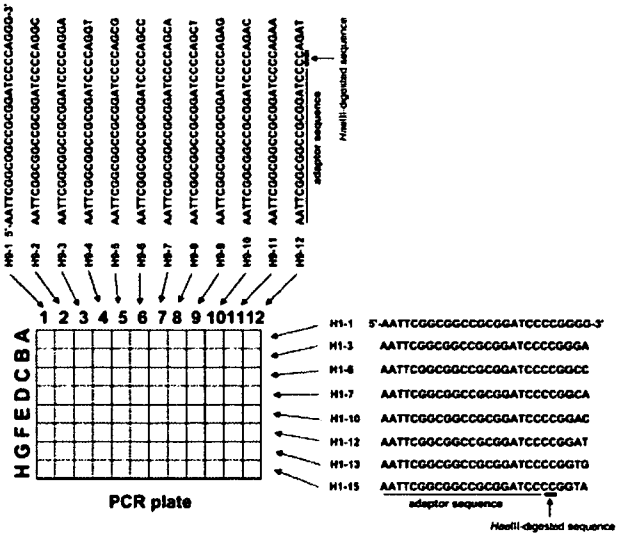

Figure 2. Primers used in rapid determination of viral RNA sequence method.

sequenced fragments) in the reactions for detection of these 5 viruses were 60% (3/5), 45% (5/11), 100% (12/12), 50% (5/10), and 40% (4/10), respectively. As a clinical application, a throat swab specimen from a patient with fever and upper respiratory infection was characterized. Although the specimen exhibited enterovirus-like cytopathic effect by inoculation into HEF and GMK cells when cell culture system for virus isolation was used (*2*), extracted RNA from the supernatant of the cells showed no amplification by reverse transcription–PCR (RT-PCR) when 1 of the conventional primer sets for human enteroviruses was used (*3*,*4*). In the cell culture supernatant analysis by the RDV method, the specimen exhibited amplification of the partial nucleotide sequences of coxsackie A14 virus (nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB275848–AB275853). Thus, the RDV method could detect unidentified cytopathic-effect agents such as enterovirus that could not be detected by RT-PCR when the conventional primer set for enteroviruses was used.

Conclusions

The RDV method is a rapid method for the direct determination of viral RNA sequences without using the cDNA cloning procedure. The limitations of the RDV method are the requirement for cell culture isolate and the large number of steps. However, RDV would be useful for species-independent detection of RNA viruses including unknown or untypeable emerging RNA viruses. Furthermore, with minor modifications, this method would also be applicable to the detection of DNA viruses and bacteria.

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Method

Rapid Multiplex Immunofluorescent Assay to Detect Antibodies against *Burkholderia pseudomallei* and Taxonomically Closely Related Nonfermenters

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SUMMARY: An indirect immunofluorescent assay to detect antibodies against the lipopolysaccharide (LPS) of *Burkholderia pseudomallei* and taxonomically closely related species was developed with the Luminex system. LPSs of *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Burkholderia thailandensis*, *Burkholderia vietnamiensis*, *B. pseudomallei*, and *Burkholderia mallei* were successfully conjugated to Luminex microspheres. Antibodies measured against the LPS of *B. pseudomallei*-conjugated Luminex beads only cross-reacted with those of two genetically closely related species, *B. mallei* and *B. thailandensis* (previously classified as non-pathogenic arabinose-negative *B. pseudomallei*). However, this system could distinguish other closely related species from *B. pseudomallei*. This assay is able to detect significantly high levels of anti-LPS antibodies of *B. pseudomallei* in serum from patients with culture-proven melioidosis.

INTRODUCTION

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei* that is mainly found in Southeast Asia and northern Australia. Outside of these areas, most cases reported involve travelers to the areas of endemicity (1).

B. pseudomallei is listed as a biological risk class III and has been considered a potentially important biological warfare agent of bioterrorism, classified as such in list B by the Centers for Disease Control and Prevention (Atlanta, Ga., USA) (2).

Acute severe pneumonia is often fatal after inhalation of the bacteria. When the disease progresses and acute septicemia develops, mortality can be as high as 60% within 48 h of admission (3). However, the disease is usually chronic, highlighted by symptoms such as localized skin infection, bone abscess, and lung abscess. In many cases, attempts to isolate the pathogen fail because of ongoing antibiotic therapy. The disease has diverse clinical manifestations, ranging from subclinical infection and localized infection to acute fatal septicemia, which may be difficult to differentiate from other infectious diseases (4). Because *B. pseudomallei* is intrinsically resistant to penicillin and gentamicin, the infection requires treatment-specific antibiotics (ceftazidime or carbapenems) that are not generally used as empirical treatments for septicemia in the areas of endemicity (5,6). A critical issue is that delays in microbiological confirmation of the infection may have fatal consequences, although definitive diagnosis requires positive bacterial culture and confirmation of the organisms, a process that usually takes several days, and also often fails. To reach a diagnosis in the early stage, serological assays such as indirect hemagglutination assay (IHA), indirect immunofluorescent assay (IFA), complement fixation tests (CFT), and enzyme-linked immunosorbent assay (ELISA) have been developed (7-11). However, these serological methods are not used for early diagnosis in countries where melioidosis is not endemic.

Antibody detection in these chronic infections is essential, but the antigen of this biosafety level 3 pathogen is not readily available at clinical laboratories or even reference laboratories.

Pseudomonas aeruginosa, *Burkholderia cepacia*, *Burkholderia vietnamiensis*, *Burkholderia thailandensis*, and *Burkholderia mallei* are phenotypically closely related to *B. pseudomallei* (12). In these species, *B. cepacia* and *P. aeruginosa* are dominant opportunistic pathogens in non-endemic areas. We consider that the simultaneous detection of specific antibodies of these species is useful for distinguishing melioidosis and possibly discovering a bioterrorism attack.

To enable the simultaneous screening of *B. pseudomallei* and closely related nonfermenters, we developed a multiplexed IFA based on Luminex xMAP technology (Luminex, Austin, Tex., USA) to detect serum antibodies against the lipopolysaccharide (LPS) of *B. pseudomallei*. The LPS of *B. pseudomallei* is reported to be an important pathogenic factor (13,14). Clinical isolates carried a highly homologous LPS, but the LPS contained two O-antigenic polysaccharides (15). Furthermore, Ho et al. reported that melioidosis patients produced high levels of LPS antibodies (16).

We successfully immobilized the LPSs to Luminex beads and established a flow cytometric assay to measure six anti-

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bodies at the same time for the serodiagnosis of suspected cases of melioidosis.

MATERIALS AND METHODS

Bacterial strains: *P. aeruginosa* (Gifu Type Culture Collection, GTC 002^T), *B. cepacia* (GTC 013^T), *B. thailandensis* (GTC 3P407^T), *B. vietnamiensis* (GTC 436^T), *B. pseudomallei* (GTC 3P056), *B. mallei* (GTC 3P003^T), *Legionella pneumophila* (GTC 745^T), and *Escherichia coli* (GTC 503^T) were used in this study.

P. aeruginosa, *B. cepacia*, *B. thailandensis*, *B. vietnamiensis*, and *E. coli* were grown in brain heart infusion (BHI) broth (Becton Dickinson and Co., Paramus, N.J., USA) at 37°C for 24 h. In addition, *B. pseudomallei* and *B. mallei* were grown on BHI agar containing 10% glycerol at 37°C for 24 to 48 h, and *L. pneumophila* was grown on BCYEα agar (Kyokuto Pharmaceutical, Tokyo, Japan) at 35°C for 48 h.

B. pseudomallei and *B. mallei* were handled in a physical containment level 3 laboratory at Gifu University.

Extraction and purification of LPS: LPSs were extracted using a slight modification of the previously described hot phenol method (17). Briefly, about 2 to 4 g (wet weight) of bacteria was suspended in 35 ml of distilled water and mixed with an equal volume of 90% aqueous phenol. The mixture was treated at 70°C for 15 min with intensive stirring. After cooling on ice for 15 min, the mixture was centrifuged at 10,000 rpm for 10 min at 4°C (KR-20000T; Kubota Corp., Tokyo, Japan). The upper water phase was isolated. Equivalent distilled water was added to the left phenol layer, and the treatment was repeated. For *B. pseudomallei* and *B. thailandensis*, the remaining phenol phases were used for the extraction of LPSs as described below. For other species, the combined water phase was submitted to dialysis using 8,000 MW cutoff dialysis membranes (Spectrum Laboratories, Rancho Dominguez, Calif., USA) and then lyophilized. Reconstituted LPS was purified with DNase I (100 μg/ml) (Sigma, St. Louis, Mo., USA) plus RNase A (400 μg/ml) (Sigma) at 37°C overnight. To remove contaminating protein, proteinase K (500 μg/ml) (Wako Pure Chemical Industries, Osaka, Japan) was added and incubated at 60°C for 1 h. Purified LPS was taken through the additional phenol-water treatment described above, and then subjected to dialysis and lyophilization.

The phenol phases were used for *B. thailandensis* and *B. pseudomallei* LPS extraction. Briefly, the phenol phase was mixed with nine-fold acetone and cooled on ice for 15 min. The mixture was then centrifuged at 3,000 rpm for 10 min at 4°C. The liquid phase was removed and the precipitated LPS phase was resuspended in distilled water. This LPS mixture was dialyzed and then lyophilized. Reconstituted LPS was purified with proteinase K (5 mg/ml) at 60°C for 1 h. To inactivate proteinase K, this mixture was then treated for 5 min at 95°C. Finally, after dialysis and lyophilization, purified LPS was obtained.

Antibodies: The following polyclonal rabbit antibodies raised from whole bacterial cells were obtained commercially (Sigma Genosys, Ishikari, Japan) and were used for immunization: *P. aeruginosa* (GTC 002^T), *B. cepacia* (GTC 013^T), *B. pseudomallei* (GTC 3P056), *E. coli* (GTC 503^T), and *L. pneumophila* (GTC 745^T). Preimmune serum was used as normal serum.

To construct a standard curve, 11 standard reference antisera were prepared serially at increasing five-fold dilutions (from 1:5 to 1:48,828,125) in phosphate buffered saline (PBS), 0.1% bovine serum albumin (BSA), 0.02% Tween 20 and 0.05% sodium azide, pH 7.4 (PBS-TBN). For specificity studies, antisera were diluted to 1:100 in PBS-TBN.

Two culture-proven melioidosis sera from a Japanese patient (18) and a serum kindly provided by Dr. Shu (Centers for Disease Control, Taipei, Taiwan) were used in this study. Five normal human sera were collected from healthy individuals, and were diluted to 1:1000 in PBS-TBN.

R-phycoerythrin conjugate goat anti-rabbit IgG (γ chain specific) and *R*-phycoerythrin conjugate goat anti-human IgG (γ chain specific) (Southern Biotechnology Associates, Birmingham, Ala., USA) were used as secondary antibodies.

Conjugation of LPS to Luminex microspheres: LPSs were conjugated to xMAP® Carboxylated Microspheres (Luminex). Six xMAP® Carboxylated Microspheres with different fluorescent ratios (33997-10704, 33997-11804, 33997-12504, 33997-14204, 33997-16104, and 33997-18204) were used. Each of the LPS samples was reconstituted in water at 250 μg/ml. Dispensed 8×10^5 microspheres were resuspended in 50 μl of 0.1 M 2-(N-morpholino) ethanesulfonic acid, pH 5.0 (MES; Sigma), and then the pellet was dispersed by exposure to sonication and vortex for 20 s. The microspheres were mixed with 150 μl of LPS solution, and 5 μl of fresh 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Pierce, Rockford, Ill., USA) solution (50 mg/ml) was added and well mixed immediately. The solution was incubated for 30 min at room temperature in the dark. Following this, an additional 200 μl of LPS solution and 5 μl of fresh EDC solution were mixed and incubated for 30 min under the same conditions, and this process was repeated twice. After incubation, the microspheres were washed with 500 μl of PBS containing 0.05% Tween 20, pH 7.4 (PBST), by centrifugation (8,000 rpm, 5 min). Following this, the microspheres were twice washed with 1 ml of PBS-TBN. Finally, the microspheres were stored in 100 μl of PBS-TBN at 4°C in the dark.

LPS-conjugated microspheres were prepared for the following six species: *P. aeruginosa*, *B. cepacia*, *B. thailandensis*, *B. vietnamiensis*, *B. pseudomallei*, and *B. mallei*. Multiplex assay was performed using all these bead sets.

ELISA for detection of anti-LPS IgG: The ELISA method for the detection of antibodies to LPS was performed by previously described methods with slight modifications (19). LPSs were diluted in 0.05 M sodium carbonate buffer at a concentration of 5 μg/ml, pH 9.6, and absorbed onto a Nunc-Immuno™ Module plate (Nunc, Wiesbaden, Germany) at 100 μl/well by overnight incubation at 4°C. After incubation, each plate was washed three times in PBST. Antigen-coated plates were then blocked with PBS containing 1% BSA at 100 μl/well for 1.5 h. After another three PBST washes, 20 μl/well of diluted serum sample and 30 μl/well of PBS-TBN (in triplicate) were added to the plates. Following 1 h of incubation at room temperature, the plates were washed for three cycles as described above. Then a 1:5,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) in PBS-TBN was added at 100 μl/well and incubated for 1 h at room temperature. After three washes, 100 μl/well of peroxidase color substrate (3, 3′, 5 5′-tetramethylbenzidine) (Nacalai tesque, Kyoto, Japan) was applied to each plate. The substrate reaction was stopped after 30 min of incubation at 37°C in the dark by the addition of 100 μl of 1 M phosphoric acid. Absorbance values at wavelengths of 450 nm (primary) and

650 nm (reference) were obtained for each sample well.

Luminex assay for detection of anti-LPS IgG: For blocking, a 1.2-μm filter membrane microtiter plate (Millipore Corp., Bedford, Mass., USA) was incubated by adding 100 μl of PBS-TBN to each well for 5 min with shaking. Another 100 μl of PBS-TBN was added to the wells, and then aspiration was performed using a vacuum manifold filtration system (Millipore). The LPS-conjugated microspheres were prepared to a concentration of 100 beads per species per μl. Twenty microliters each of PBS-TBN, microspheres sets, and diluted serum samples were added to the wells, and the plates were incubated for 1 h at 37°C with shaking in the dark. After the incubation and aspiration, the microspheres were washed three times with 200 μl PBS-TBN each time, followed by vacuum aspiration. For detection, 100 μl of secondary antibodies (a 1:200 dilution in PBS-TBN) was added to each well, and the plates were incubated for 30 min at 37°C with shaking in the dark. The microspheres were then washed three times with 200 μl PBS-TBN each, followed by vacuum aspiration, and resuspended in 100 μl of PBS-TBN. Finally, the plates were shaken for 2 min and placed in a Luminex 100 analyzer equipped with an XY platform. A total of 150 microspheres per species were counted to obtain the median fluorescent intensity (MFI) according to the manufacturer's protocol.

RESULTS

Comparison of Luminex microsphere assay with ELISA: To evaluate the performance of the multiplexed Luminex assay for LPS antibodies, we compared the standard curves generated from the Luminex assay with a standardized ELISA (Fig. 1). The Luminex assay had a greater dynamic range than ELISA. ELISA was linear at the 4th range of dilution, while the Luminex assay was linear at the 5th range of dilution. The Luminex assay and ELISA both showed high detection limits at 1:1,953,125 dilutions. Luminex assay and ELISA could be performed within 2 h and 1.5 days, respectively.

Luminex assay for detection of anti-LPS IgG: To investigate the possible interference between the different LPS-conjugated beads, the MFIs generated by the multiplex and

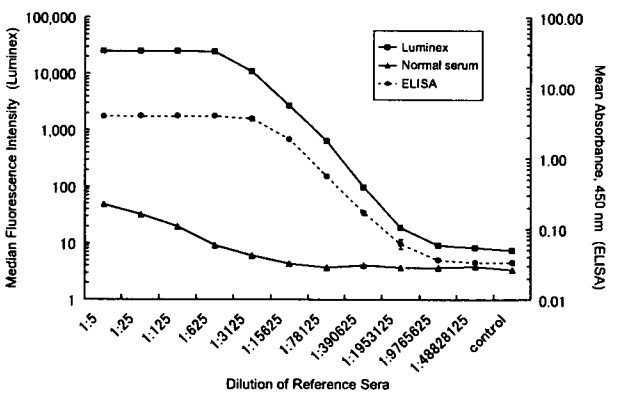

Fig. 1. Comparison of standard curves generated by Luminex assay and ELISA. Standard curves for *B. pseudomallei* are shown. The curves were constructed by plotting the log median fluorescence intensities for Luminex assay (150 microspheres) and mean absorbance values for ELISA (in triplicate) against the dilution factor for the standard serum. Detection limits for these assays are defined by the upper mean + 3 (positive) standard deviation generated from PBS-TBN (in triplicate). Standard curves for normal sera are generated from Luminex assay.

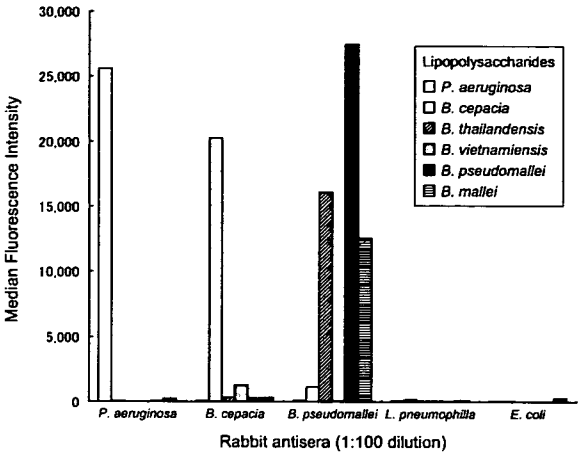

Fig. 2. Binding specificity of polyclonal rabbit antibodies to LPS microspheres. A panel of six LPS isolates from different bacteria was conjugated to xMAP® Carboxylated Microspheres. The complete set of six kinds of microspheres was incubated with polyclonal rabbit antibodies raised from *P. aeruginosa*, *B. cepacia*, *B. pseudomallei*, *L. pneumophila*, and *E. coli*, respectively.

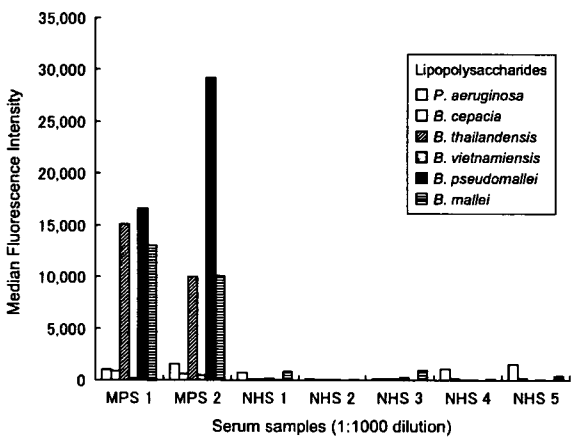

Fig. 3. Detection of *B. pseudomallei* anti-LPS antibodies in serum samples. A panel of six LPS preparations isolates from different bacteria was conjugated to xMAP® Carboxylated Microspheres. The complete set of six kinds of microspheres was incubated either with patient sera with culture-proven melioidosis (MPS 1 and 2) or with normal human sera (NHS 1 to 5), respectively.

monoplex assays were compared. The MFIs from the former were essentially identical to those from the latter in all dilution ranges (data not shown). No interference was observed.

For specificity studies, the complete set of six kinds of microspheres was mixed with each polyclonal rabbit antibody raised from whole bacterial cells (*P. aeruginosa*, *B. cepacia*, *B. pseudomallei*, *L. pneumophila*, and *E. coli*) (Fig. 2). All antisera specifically reacted with the respective LPSs as indicated by the significant increases in fluorescence. Except for the antiserum of *B. pseudomallei*, which recognized three kinds of LPS beads (*B. thailandensis*, *B. pseudomallei*, *B. mallei*), all antisera showed little or no detectable cross-reactivity.

The two culture-proven melioidosis sera tested showed significantly higher levels of anti-LPS antibodies of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* than did the five normal human sera (Fig. 3).

DISCUSSION

Serological assays have been developed for the diagnosis of early-stage melioidosis, and IHA and CFT have been routinely used for the serologic diagnosis of melioidosis in endemic countries. However, both IHA and CFT are based on crude whole-cell preparations or extracts of the bacteria, and therefore, the potential for false positive serodiagnosis cannot be ruled out (20).

The LPS of *B. pseudomallei* is reported to be an important pathogenic factor because the rough mutant has less virulence, and the smooth type induces specific antibody responses during infection (14,16). Previous studies have demonstrated that the detection of anti-LPS antibodies is useful in the diagnosis of various Gram-negative bacterial infections (21-23).

In the present assay, we successfully conjugated LPS to microspheres. LPS-conjugated microspheres could be prepared by a very simple method, as described in Materials and Methods. Microspheres stored at 4°C in the dark were still useful after 12 months. There was no significant difference between the MFIs generated from freshly LPS-conjugated beads and those generated from stored LPS-conjugated beads (data not shown). This long-term stability should make the generation of a diagnostic test kit a real possibility.

While the Luminex assay shows good correlation with ELISA, it is believed to have a number of potential advantages over ELISA for the detection of antibodies (24-26). Our results were consistent with previously reported results showing the Luminex assay greatly reduced sample volumes and reduced the time required, and that it has much greater dynamic range and equal sensitivity compared to ELISA (Fig. 1).

The possibility of interference and cross-reactivity among the bead sets is an important concern for multiplex assays. Our results demonstrated that there was no interference by comparing the MFIs of multiplex and monoplex assays (data not shown). However, cross-reactivity was found when the antiserum of *B. pseudomallei* was applied. This antiserum reacted with three kinds of LPS beads (*B. thailandensis*, *B. pseudomallei*, *B. mallei*) (Fig. 2). This was also observed in the two culture-proven melioidosis clinical samples tested (Fig. 3). However, these clinical samples showed significantly higher levels of anti-LPS antibodies of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* compared to other related nonfermenters. *B. thailandensis* has been classified as arabinose negative and an avirulent *B. pseudomallei* (27). This organism was only reported to be found in soil in Thailand and not in clinical specimens (28).

Previous studies showed that the LPSs from these three species were antigenically indistinguishable (29,30). In comparison with *B. pseudomallei*, *B. thailandensis* was identical to the melioidosis sera in terms of SDS-PAGE profiles and immunoreactivities. Moreover, *B. mallei* has been reported to possess structurally the same forms of LPS (31,32).

B. pseudomallei and *B. mallei* are phylogenetically and genetically identical species. The DNA-DNA relatedness value between these two species has been reported to be more than 80%, and the sequences of 16S rRNA are absolutely identical (12,33,34). *B. mallei* is highly adapted to the host and incurs a loss of motility. Motility-associated flagellar genes of *B. mallei* were partially maintained (35). For these reasons, we consider this cross-reactivity not to be a disadvantage for the primary diagnosis and treatment of melioidosis and glander.

Previous reports demonstrated that *B. thailandensis* could be distinguished from *B. pseudomallei* by monoclonal antibodies specific for the exopolysaccharide antigen present only in *B. pseudomallei* (36,37). The cross-reactivity described above will be dissolved by using these exopolysaccharide-conjugated beads. Furthermore, previous reports demonstrated that other antigens conjugated to microspheres (*Haemophilus influenzae* type b, *Streptococcus pneumoniae* and *Neisseria meningitidis* polysaccharide; and toxoids of *Clostridium tetani* and *Corynebacterium diphtheriae*) were useful for the detection of these antibodies (24,26,38). Biagini et al. reported the benefit of the multiplex measurement of *Bacillus anthracis* anti-protective antigen IgG and anti-lethal factor IgG in serum from confirmed clinical anthrax infection (39).

By using these various antigens, this system would allow simultaneous screening of different pathogens. The Luminex beads system has the potential to measure 100 different antibodies at the same time using two different fluorescence-containing beads with different ratios. Therefore, this multiplex assay could be applied to the screening of a wide range of antibodies of human infectious diseases for patients with fever of unknown etiology, and to the surveillance of seroepidemiology for normal humans.

In conclusion, the simultaneous detection of specific antibodies of *B. pseudomallei*, *P. aeruginosa*, *B. cepacia*, *B. thailandensis*, *B. vietnamiensis*, and *B. mallei* was established, as *P. aeruginosa* and *B. cepacia* are dominant opportunistic pathogens in non-endemic areas, and misidentification of these infections from melioidosis must be avoided.

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ORIGINAL ARTICLE

Rapid detection of *Brucella* spp. by the loop-mediated isothermal amplification method

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Keywords
Brucella spp., brucellosis, detection, LAMP, milk.

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Abstract

Aims: To develop a rapid and sensitive method for detecting *Brucella* spp.
Methods and Results: Two sets of six *Brucella*-specific primers for loop-mediated isothermal amplification (LAMP) were designed from the sequence of the *Brucella abortus BCSP31* gene. The specificity and sensitivity were examined for six *Brucella* species (22 strains) and 18 non-*Brucella* species (28 strains). The LAMP assay was specific to *Brucella* spp. in 35 min at 63°C and sensitive (detected 10 fg of genomic DNA). The assay was also applied for the detection of *Brucella* DNA in contaminated milk and infected mouse organs.
Conclusions: We developed a sensitive and specific LAMP assay for *Brucella* spp., with the test appearing to be useful for the detection of the pathogen from clinical and food samples.
Significance and Impact of the Study: This is the first report of the development of LAMP for the detection of *Brucella* spp. As the LAMP assay can be performed at a constant temperature and its reactivity is directly observed with the naked eye without electrophoresis, our assay should be useful for the diagnosis of brucellosis as well as the detection of the bacteria in environmental or food samples.

Introduction

Brucella spp. are small Gram-negative bacteria that cause brucellosis, resulting in abortion and infertility in numerous domestic and wild animals, and in clinical symptoms such as undulant fever in humans (Corbel 1997). Brucellosis is a major zoonotic disease that poses public health and agricultural economic problems in many countries (Boschiroli *et al.* 2001).

The genus *Brucella* contains six species distinguished by subtle phenotypic and antigenic differences and host specificity: *Brucella abortus* (bovine), *Brucella melitensis* (caprine and ovine), *Brucella ovis* (ovine), *Brucella canis* (canine), *Brucella suis* (porcine) and *Brucella neotomae* (only seen in the desert wood rat) (Corbel 1988; Trujillo *et al.* 1994). Human infections are mainly caused by *Br. abortus*, *Br. melitensis* and *Br. suis*. *Brucella* spp. have also long been considered potential biological weapons (Kortepeter and Parker 1999; Pappas *et al.* 2006). *Brucella melitensis*, *Br. suis* and *Br. abortus* are listed as category B biothreat agents by the Centers for Disease Control and Prevention Strategic Planning Group (Rotz *et al.* 2002).

As the clinical symptoms of brucellosis are nonspecific and show great variability, laboratory diagnosis by bacteriological testing is essential, however, testing is hampered by slow growth of the organisms in culture and their danger to laboratory personnel (Staszkiewicz *et al.* 1991; Yagupsky and Baron 2005). Although serological diagnosis is easy, its specificity is low because of structural similarity of *Brucella* lipopolysaccharide (LPS) with LPS of other bacterial pathogens such as *Yersinia enterocolitica* (Young 1991; Debeaumont *et al.* 2005; Munoz *et al.* 2005). DNA-based methods such as PCR (Baily *et al.* 1992; Herman and De Ridder 1992; Leal-Klevezas *et al.* 1995) are useful for the diagnosis of brucellosis and detection of the pathogen because they are specific, rapid and simple.

Recently, loop-mediated isothermal amplification (LAMP) has been developed as a novel technique that relies on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi *et al.* 2000). The technique is highly specific for the target sequence, because it uses six primers for the amplification of the target gene using an isothermal temperature step (60–65°C) and generates an increase in turbidity in positive samples, allowing detection by visual inspection (Mori *et al.* 2001). The assay has been applied to the identification or detection of various kinds of bacteria, and has high sensitivity and specificity (Iwamoto *et al.* 2003; Maruyama *et al.* 2003; Horisaka *et al.* 2004; Hara-Kudo *et al.* 2005; Kato *et al.* 2005; Ohtsuka *et al.* 2005; Song *et al.* 2005). In this study, we developed a diagnostic method based on the LAMP assay for the detection of *Brucella* spp. and evaluated the sensitivity and specificity of the assay.

Materials and methods

Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. *Brucella* strains were cultured in *Brucella* broth (Becton Dickinson, Sparks, MD, USA) or on *Brucella* broth containing 1·5% agar at 37°C with 5% CO_2 in a BSL3 laboratory. Other strains were cultured in trypticase soy broth (Becton Dickinson) at 37°C (except *Ochrobactrum anthropi*, which was cultured at 30°C). All bacterial pathogens were handled in BSL3 or BSL2 rooms recognized by the Safety Control Committee of Obihiro University of Agriculture and Veterinary Medicine.

Primer design

Two sets of six *Brucella*-specific LAMP primers were designed from the published sequence of the *Br. abortus BCSP31* gene (GenBank accession no. M20404) using the LAMP primer designing software program (Primer Explorer V3) from Eiken Chemical (http://primerexplorer.jp/e/) (Fig. 1, Table 2). The *BCSP31* gene encodes a 31-kDa surface protein found in all *Brucella* species and biovars.

Extraction of bacterial DNA

Brucella abortus was incubated in 2 ml of *Brucella* broth for 24 h at 37°C. Bacterial DNA was extracted using a MORA-Extract kit (Kyokuto Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions. DNA concentration was measured using a spectrophotometer (DU530; Beckman Coulter, Fullerton, CA, USA), and the purity was determined by the ratio of absorbance between 260 and 280 nm, assuming one genome copy corresponds to 3·6 fg of DNA (DelVecchio *et al.* 2002).

LAMP assay

The LAMP assay was performed in 25 μl of reaction mixture containing 40 pmol l^{-1} (each) of FIP and BIP, 5 pmol l^{-1} (each) of F3 and B3, 20 pmol l^{-1} (each) of LF and LB, 1·4 mmol l^{-1} each deoxynucleoside triphosphates, 0·8 mol l^{-1} betain, 20 mmol l^{-1} Tris–HCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $(NH_4)_2SO_4$, 8 mmol l^{-1} $MgSO_4$, 0·1% Tween 20, 8 units of *Bst* DNA polymerase large fragment (New England Biolabs, Berverly, MA, USA), 0·125 μl of YO-PRO-1 iodide (Invitrogen, Carlsbad, CA, USA) and 2 μl of template DNA. The reaction mixture was incubated at 63°C for 35 min with a real-time thermal cycler (ABI 7900HT; Applied Biosystems, Foster City, CA, USA) and then heated to 95°C for 2 min to terminate the reaction. The LAMP amplicon was detected as the level of fluorescence (delta Rn) in real-time when there was an increase in fluorescence intensity caused by the intercalating dye. A total of 2 μl of product was analysed by electrophoresis in 2% agarose gels. To confirm the structures of the amplified products, some of the amplified products were digested with the restriction enzyme (Takara Bio, Shiga, Japan) *Sau*3AI (primer set P-1) or *Eco*RV (primer set P-2) and their sizes were analysed by electrophoresis in 3% agarose gels (Notomi *et al.* 2000).

SYBR Green-based real-time PCR amplification

To examine the sensitivity of our assay, real-time PCR targeting *BCSP31* was performed by a method previously established by Queipo-Ortuno *et al.* (2005). The sequences of primers are described in Table 2. Briefly, a reaction mixture consisting of 2 μl of LightCycler Fast-Start DNA mastermix for SYBR Green I (Roche Diagnostic, Mannheim, Germany), 0·5 μmol l^{-1} each primer, 4 mmol l^{-1} $MgCl_2$ and 2 μl of template DNA in a capillary tube was amplified using a LightCycler (Roche Diagnostic). The amplification conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 9 s. The fluorescence of the PCR product was measured during the 72°C extension step by the detection of the fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analysed with LightCycler software v. 3.5. Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s (hold time) at 95°C, 15 s at 65°C and 0 s (hold time) at 95°C. The melt peak generated represented the specific amplified product.

Table 1 Bacterial strains used in this study and results of LAMP amplification

Source no.	Species (biovar)	Strain	Source	LAMP result P-1	LAMP result P-2
Brucella spp.					
1	*Brucella abortus* (bv1)	544 (ATCC23448)	Our lab.	+	+
2	*Br. abortus* (bv2)	86/8/59 (ATCC23449)	Our lab.	+	+
3	*Br. abortus* (bv3)	Tulya (ATCC23450)	Our lab.	+	+
4	*Br. abortus* (bv4)	292 (ATCC23451)	Our lab.	+	+
5	*Br. abortus* (bv5)	B3196 (ATCC23452)	Our lab.	+	+
6	*Br. abortus* (bv6)	870 (ATCC23453)	Our lab.	+	+
7	*Br. abortus* (bv7)	63/75 (ATCC23454)	Our lab.	+	+
8	*Br. abortus* (bv9)	C68 (ATCC23455)	Our lab.	+	+
9	*Br. abortus*	1119-3	Our lab.	+	+
10	*Br. abortus*	125	Our lab.	+	+
11	*Br. abortus*	45/20	Our lab. (vaccine strain)	+	+
12	*Br. abortus*	99 (NIAH0033)	Our lab.	+	+
13	*Br. abortus*	19 (NIAH1035)	Our lab. (vaccine strain)	+	+
14	*Br. abortus*	3	Our lab. (clinical isolate)	+	+
15	*Brucella canis*	QE13	Our lab. (animal isolate)	+	+
16	*Brucella melitensis* (bv1)	16M (ATCC23456)	Our lab.	+	+
17	*Br. melitensis* (bv2)	63/9 (ATCC23457)	Our lab.	+	+
18	*Brucella neotomae*	5K33 (ATCC23459)	Our lab.	+	+
19	*Brucella ovis*	63/290 (ATCC25840)	Our lab.	+	+
20	*Brucella suis* (bv1)	1330 (ATCC23444)	Our lab.	+	+
21	*Br. suis* (bv2)	Thomsen (ATCC23445)	Our lab.	+	+
22	*Br. suis* (bv4)	40 (ATCC23447)	Our lab.	+	+
Non-*Brucella* spp.					
23	*Bacillus cereus*	JCM2152	Our lab.	–	–
24	*Bacillus subtilis*	UOTO277	Our lab.	–	–
25	*Bacillus thuringiensis*	IAM12077	Our lab.	–	–
26	*Bartonella henselae*	CDCG5436	GTC	–	–
27	*Bartonella quintana*	CIP103739	GTC	–	–
28	*Burkholderia pseudomallei*	GTC3P56	GTC	–	–
29	*Escherichia coli*	O157:H7	Our lab. (clinical isolate)	–	–
30	*Francisella tularensis* subsp. *tularensis*	GTC3P423	GTC	–	–
31	*Mycoplana ramosa*	JCM7822	JCM	–	–
32	*Ochrobactrum anthropi*	JCM10066	JCM	–	–
33	*O. anthropi*	JCM10070	JCM	–	–
34	*O. anthropi*	JCM10072	JCM	–	–
35	*Salmonella enterica* serovar Choleraesuis		Our lab. (animal isolate)	–	–
36	*Salm. enterica* serovar Derby		Our lab. (animal isolate)	–	–
37	*Salm. enterica* serovar Dublin		Our lab. (clinical isolate)	–	–
38	*Salm. enterica* serovar Enteritidis		Our lab. (food isolate)	–	–
39	*Salm. enterica* serovar Oranienburg		Our lab. (food isolate)	–	–
40	*Salm. enterica* serovar Tyhimurium	LT2	Our lab.	–	–
41	*Salm. enterica* serovar Tyhimurium	N491	Our lab.	–	–
42	*Salm. enterica* serovar Typhi		Our lab. (clinical isolate)	–	–
43	*Shigella flexineri*		Our lab. (food isolate)	–	–
44	*Shigella sonnei*		Our lab. (food isolate)	–	–
45	*Staphylococcus aureus*		Our lab. (food isolate)	–	–
46	*Vibrio cholerae*	O1	Our lab. (clinical isolate)	–	–
47	*Wolbachia* spp.		GTC	–	–
48	*Wolbachia* spp.		GTC	–	–

Table 1 Continued

Source no.	Species (biovar)	Strain	Source	LAMP result P-1	LAMP result P-2
49	*Yersinia enterocolitica*	79b (O:9)	Our lab. (animal isolate)	–	–
50	*Yersinia pestis*		GTC	–	–

ATCC: American Type Culture Collection, Rockville, MD, USA; CDC: Centers for Disease Control and Prevention, Atlanta, GA, USA; CIP: Pasteur Institute Collection, Institut Pasteur, Paris, France; GTC: Gifu Type Culture Collection, Department of Microbiology, Gifu University Graduate School of Medicine, Gifu, Japan; JCM: Japan Collection of Micro-organisms, Instituite of Physical and Chemical Research (RIKEN), Tsukuba, Japan; NIAH: National Institute of Animal Health, National Agriculture and food isolate Research Organization (NARO), Tsukuba, Japan.

<P-1>
781 tcgtcgaatg gctcggttgc caatatcaat gcgatcaagt cgggcgctct ggagtccggc

841 tttacgcagt cagacgttgc ctattgggcc tataacggca ccggccttta tgatggcaag
F3 FIP(F2) LF
901 ggcaaggtgg aagatttgcg ccttctggcg acgctttacc cggaaacgat ccatatcgtt
FIP(F1c) BIP(B1c) *Sau*3 AI
961 gcgcgtaagg atgcaaacat caaatcggtc gcagacctga aaggcaagcg cgtttcgctg
LB BIP(B2) B3
1021 gatgagccgg gttctggcac catcgtcgat gcgcgtatcg ttcttgaagc ctacggcctc

<P-2>
961 gcgcgtaagg atgcaaacat caaatcggtc gcagacctga aaggcaagcg cgtttcgctg

1021 gatgagccgg gttctggcac catcgtcgat gcgcgtatcg ttcttgaagc ctacggcctc
F3 FIP(F2) LF
1081 acggaagacg atatcaaggc tgaacacctg aaggcgggac cggcaggcga gaggctgaaa
Eco RV FIP(F1c) BIP(B1c)
1141 gatggtgcgc tggacgccta tttctttgtg ggcggctatc cgacgggcgc aatctcggaa
LB
1201 ctggccatct cgaacggtat ttcgctcgtt ccgatctccg ggccggaagc ggacaagatt
BIP(B2) B3

Figure 1 Primer design for LAMP to detect *Brucella* DNA. Nucleotide sequence of *BCSP31* gene (GenBank accession no. M20404), used to design LAMP primers. Underlining indicates the positions of targeting sequences.

LAMP application for infected tissues and contaminated food

Male A/J Jms mice (9–11 weeks old) were purchased from Japan SLC (Hamamatsu, Japan), and housed in stainless-steel cages with a 12 h light/dark cycle (7 AM–7 PM) in a controlled atmosphere (temperature 22 ± 2°C, humidity 40 ± 2%). The animals were fed a purified diet *ad libitum* and had free access to water. Mice (n = 4) were injected intraperitoneally with $7{\cdot}9 \times 10^7$cells of *Br. abortus* biovar 3 strain, and sacrificed the day after injection by cervical dislocation under ether anaesthesia. The two spleens and two livers were isolated and used for DNA extraction and enumeration of bacteria in organs.

Total DNA from livers (25 mg each) and spleens (10 mg each) was extracted using a DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and suspended in 200 μl of TE buffer. The extracted DNA was serially diluted (1 to 10^{-5}), and 2 μl from each diluted sample was used for the LAMP assay.

For enumeration of bacteria, spleens and livers (100 mg each) were homogenized in 1 ml of sterile distilled water, and plated on *Brucella* broth agar (Becton Dickinson). The CFU of infected organs were as follows: liver 1: $8{\cdot}3 \times 10^7$ CFU per 100 mg; liver 2: $7{\cdot}1 \times 10^8$ CFU per 100 mg; spleen 1: $6{\cdot}6 \times 10^8$ CFU per 100 mg; spleen 2: $9{\cdot}9 \times 10^8$ CFU per 100 mg. Based on these data, 2 μl of undiluted DNA samples contained $2{\cdot}1 \times 10^5$ CFU (liver 1), $1{\cdot}8 \times 10^5$ CFU (liver 2), $6{\cdot}6 \times 10^5$ CFU (spleen 1), $9{\cdot}9 \times 10^5$ CFU (spleen 2) respectively. The protocol was carried out according to the guidelines of the Helsinki declaration and was approved by the Ethics Committee for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine.

For LAMP application in contaminated food samples, we used pasteurized milk purchased from a local shop. Tenfold serial dilutions ($1{\cdot}23 \times 10^9$–$1{\cdot}23 \times 10^1$ CFU ml^{-1}) of an overnight culture of *Br. abortus* 19 vaccine strain were made in PBS. Aliquots (500 μl) of each dilution were added to 4·5 ml of milk, and mixed well

Table 2 Sequences of two sets of primers (P-1 and P-2) used for LAMP and PCR primers

Primer set	Primer	Sequence	Amplicon size (bp)	Reference
P-1	F3	5′-GCTTTACGCAGTCAGACGT-3′	189	This study
	B3	5′-GCTCATCCAGCGAAACGC-3′		
	FIP	5′-AGGCGCAAATCTTCCACCTTGCGCCTATTGGGCCTATAACGG-3′		
	BIP	5′-GGCGACGCTTTACCCGGAAATTCAGGTCTGCGACCGAT-3′		
	LF	5′-CCTTGCCATCATAAAGGCC-3′		
	LB	5′-CGTAAGGATGCAAACATCAA-3′		
P-2	F3	5′-TGGATGAGCCGGGTTCTG-3′	214	This study
	B3	5′-GGAACGAGCGAAATACCGT-3′		
	FIP	5′-GTCCCGGCTTCAGGTGTTCAGATGCGCGTATCGTTCTTGA-3′		
	BIP	5′-GAGAGGCTGAAAGATGGTGCGCAGATGGCCAGTTCCGAGA-3′		
	LF	5′-GTCTTCCGTGAGGCCGTAG-3′		
	LB	5′-GGACGCCTATTTCTTTGTGGG-3′		
PCR	B4	5′-TGGCTCGGTTGCCAATATCAA-3′	223	Baily *et al.* (1992)
	B5	5′-CGCGCTTGCCTTTCAGGTCTG-3′		

FIP, forward inner primer; F3, forward outer primer; BIP, backward inner primer; B3, backward outer primer; LF, forward loop primer; LB, backward loop primer.

($1{\cdot}23 \times 10^{8}$–$1{\cdot}23$ CFU ml^{-1}). One-hundred microlitres of each spiked milk sample was mixed with 900 μl of DNAzol reagent (Invitrogen). The remaining steps were carried out in accordance with the manufacturer's instructions, resulting in 50 μl eluates. A 2 μl volume of the extracted DNA from each samples (corresponding to $4{\cdot}9 \times 10^{5}$–$4{\cdot}9 \times 10^{-2}$ CFU) was used for the LAMP assay with primer set P-1.

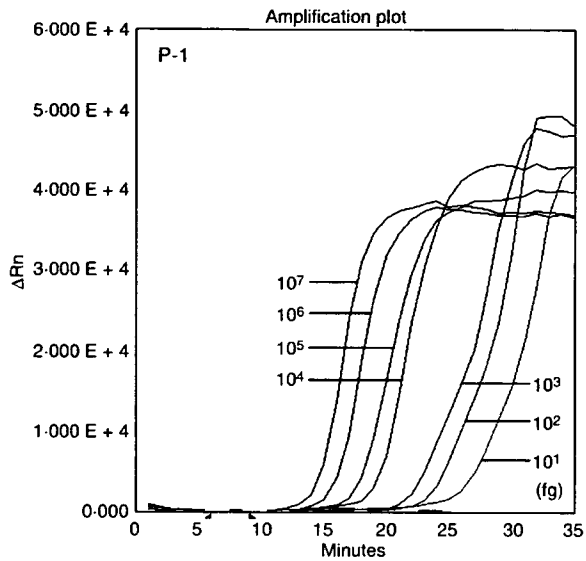
Results

Amplification of *BCSP31* by LAMP

Although the LAMP reaction is terminated at 80°C for 5 min in the general protocol, we terminated the reaction at 95°C for 2 min to save time. We obtained the same results from this modified protocol and the original protocol (data not shown). Using two sets of primers, P-1 and P-2 (Table 2), the LAMP assay successfully amplified the target sequences of *BCSP31* of *Br. melitensis* DNA at 63°C in 35 min (Figs 2 and 3). The detection limit of the LAMP with P-1 was 10 fg, and that with P-2 was 100 fg (Figs 2 and 3). The reaction reached plateau in 30 min using P-1 primers, and in 25 min using P-2 primers (Fig. 2). Although the amplification time was shorter in the LAMP using P-2 primers, the detection sensitivity was 10 times higher in the LAMP using P-1 primers. After amplification, products were also directly observed by the naked eye with a fluorescent detection reagent (Supplementary material, Fig. S1). The ladder-like pattern of bands was confirmed by agarose gel electrophoresis (Fig. 3). This pattern is a characteristic of the LAMP assay and indicates the production of stem-loop DNA with inverted repeats of the target sequence (Notomi *et al.* 2000). To confirm that the amplification products had the expected DNA structures originating from *Brucella* (Fig. 1), the products were digested with restriction enzymes and the sizes of the fragments were analysed by gel electrophoresis. *Sau*3AI digests between B1c and LB for the amplicon of primer set P-1, and *Eco*RV digests between LF and F1c for the amplicon of primer set P-2 (Fig. 1). The sizes of the fragments generated after digestion were in agreement with the theoretically predicted sizes from the expected DNA structures: 135 and 241 bp with *Sau*3AI digestion, and 176 and 250 bp with *Eco*RV digestion (Fig. 4, lanes 17 and 19).

Specificity of the LAMP assay with various kinds of bacterial species

To evaluate the specificity of the LAMP primers, six kinds of *Brucella* species (22 strains) and 18 kinds of other species (28 strains) were tested (Table 1). Significant and specific amplification of DNA was observed after 35 min of incubation in all *Brucella* strains tested, including reference, vaccine strains and clinical isolates, whereas other non-*Brucella* strains showed no amplification (Fig. 4, Table 1). Interestingly, although *O. anthropi* is taxonomically classified as a *Brucella* species (Romero *et al.* 1995) and its amplification has been previously reported with *BCSP31*-targetting PCR assays (Casanas *et al.* 2001; Morata *et al.* 2003), *O. anthropi* DNA was not amplified in our LAMP assay (Fig. 4). These data demonstrate that our LAMP assay was very specific to *Brucella* species.



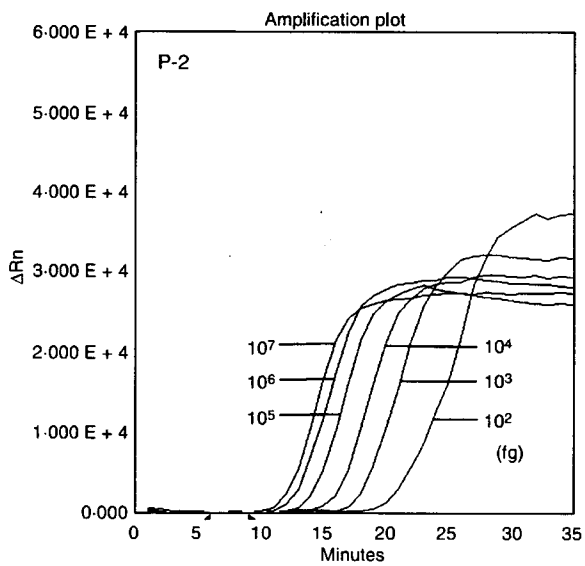


Figure 2 Kinetics of real-time LAMP to detect *Brucella* DNA. Real-time LAMP was performed at 63°C for 35 min using primer sets P-1 and P-2. Serial 10-fold dilutions of template DNA extracted from *Brucella melitensis* 16M were subjected to LAMP and monitored by measuring fluorescence intensity.

Comparison of sensitivity and specificity between LAMP and previously reported real-time PCR

We compared the detection limit of our assay with SYBR Green I-based real time PCR, a method previously established by Queipo-Ortuno *et al.* (2005). As shown in Fig.5, the detection limit of real-time PCR for *Br. melitensis* DNA was 10 fg, as described previously (Queipo-Ortuno

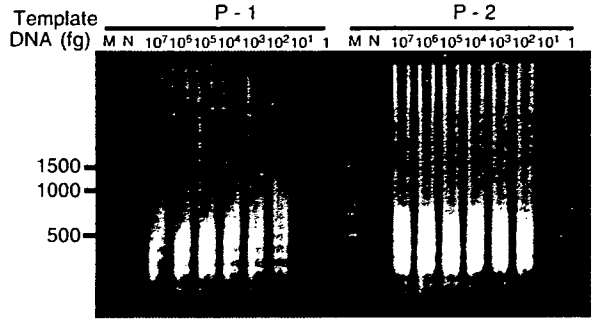


Figure 3 LAMP assays using *Brucella* DNA. Sensitivities of LAMP using primer sets P-1 and P-2 and 10-fold serial diluted *Brucella* DNA were visualized in 2% agarose gel. Lane M, 100-bp ladder (Takara Bio); lane N, negative control with sterilized double-distilled water.

et al. 2005), and was comparable to that of the LAMP assay demonstrated in this study. However, nonspecific amplification was observed for *Vibrio cholerae* O1 at around 42 cycles in real-time PCR, but not in our LAMP assay (Fig. 5).

Practical application of the LAMP assay

Next, we applied the LAMP assay for the detection of *Brucella* spp. in experimentally infected mice. Total DNA of livers and spleens from infected and uninfected mice was extracted and amplified by the LAMP assay using primer set P-1. Figure 6 shows the results from liver 1 and spleen 1. The detection limit for the liver was $2{\cdot}1 \times 10^3$ CFU, and that for the spleen was $6{\cdot}6 \times 10^2$ CFU. In liver 2 and spleen 2, the detection limit was $1{\cdot}8 \times 10^3$ and $9{\cdot}9 \times 10^2$ CFU respectively (data not shown). Thus, the average values of the detection limit for infected liver and spleen were $2{\cdot}0 \times 10^3$ and $8{\cdot}2 \times 10^2$ CFU respectively. On the other hand, the liver and spleen DNA from uninfected mice did not show amplification. The assay was able to detect $4{\cdot}9 \times 10^4$ CFU of *Brucella* DNA extracted from contaminated milk (data not shown).

Discussion

This is the first report of the application of LAMP for the detection of *Brucella* spp. Brucellosis is a zoonotic disease and its importance in public health is increasing in both human and veterinary medicine (Boschiroli *et al.* 2001). Various *Brucella* species affect sheep, goats, cattle, deer, elk, pigs, dogs and several other animals, including marine mammals (Boschiroli *et al.* 2001; Tachibana *et al.* 2006). In humans, the disease manifests as highly diverse symptoms such as fever, malaise and myalgia that develop into a

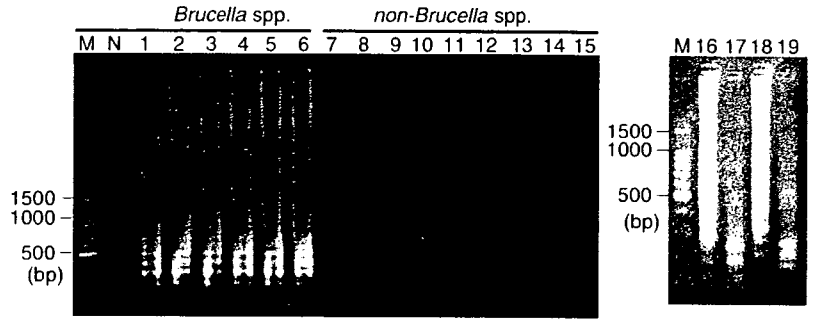


Figure 4 Specificity of LAMP assays among various bacterial DNAs. LAMP using primer set P-1 (except where indicated) was carried out at 63°C for 35 min using 10 ng of total DNA. Lane M, 100-bp ladder; lane N, negative control with sterilized double-distilled water; lane 1, *Brucella abortus* 544; lane 2, *Brucella canis* QE13; lane 3, *Brucella melitensis*16M; lane 4, *Brucella neotomae* 5K33; lane 5, *Brucella ovis* 63/290; lane 6, *Brucella suis* 1330; lane 7, *Bacillus cereus* JCM2152; lane 8, *Bartonella quintana* CIP103739; lane 9, *Burkholderia pseudomallei* GTC3P56; lane 10, *Escherichia coli* O157:H7; lane 11, *Francisella tularensis* subsp. *tularensis* GTC3P423; lane 12, *Ochrobactrum anthropi* JCM10066; lane 13, *Salmonella* Tyhimurium LT2; lane 14, *Shigella sonnei*; lane 15, *Yersinia enterocolitica* O:9; lane 16, *Br. melitensis* (P-1); lane 17, LAMP product from lane 16 after *Sau*3AI digestion; lane 18, *Br. melitensis* (P-2); lane 19, LAMP product from lane 18 after *Eco*RV digestion. (lane 1–15: 2% agarose gel, lane 16–19: 3% agarose gel.).

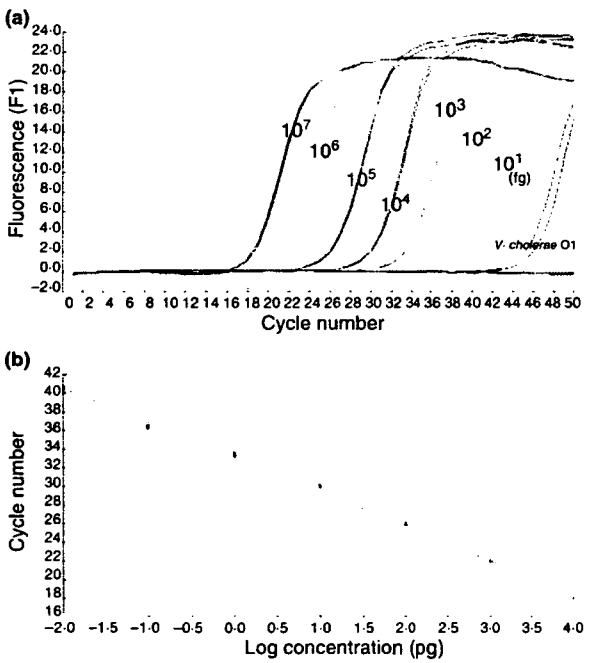


Figure 5 Kinetics of LightCycler assay to detect *Brucella* DNA. Dilutions of *Brucella melitensis* 16M genomic DNA were used as a template for each reaction. (a) A 10-fold dilution series (from 10 ng to 1 fg) were analysed in duplicate and monitored by measuring fluorescence intensity. (b) Logarithmic standard curve of (a). Cp values are plotted against decreasing concentrations of *Brucella* DNA. The slope is −3·46 cycles/log10 and the correlation coefficient is 0·99.

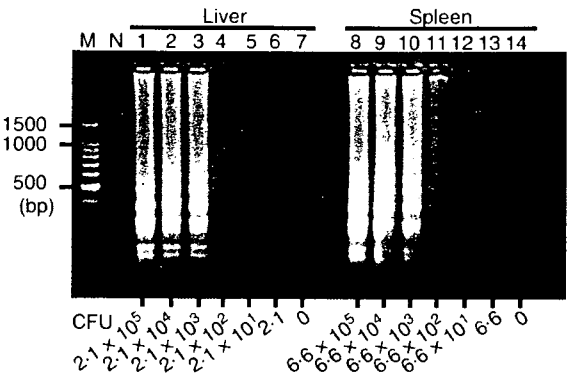


Figure 6 LAMP amplification from infected tissues. Total DNA of livers and spleens from infected and uninfected mice was examined using the LAMP assay. LAMP using a primer set P-1 was shown in the figure. A 10-fold dilution series was analysed in duplicate. Lane M, 100-bp ladder; lane N, negative control with sterile distilled water; lane 1, 8, no dilution; lane 2, 9, 10–1 dilution; lane 3, 10, 10–2 dilution; lane 4, 11, 10–3 dilution; lane 5, 12, 10–4 dilution; lane 6, 13, 10–5 dilution; lane 7, uninfected mice liver (no dilution); lane 14, uninfected mice spleen (no dilution).

chronic illness affecting various organs and tissues. Outbreaks of brucellosis in laboratory workers have also been reported (Grammont-Cupillard *et al.* 1996; Fiori *et al.* 2000; Noviello *et al.* 2004). As drug therapy is prolonged and effective antibiotics are limited, a reliable and early diagnosis of brucellosis is of major importance for initiating adequate therapy. Although convenient serological diagnostic methods such as Rose Bengal tests for the detection of *Brucella*-specific antibodies are available, their usefulness is limited by a high prevalence of *Brucella*-specific antibodies in epidemic areas of brucellosis, low sensitivity in the acute phase, and cross-reactions with other Gram-negative bacteria such as *Y. enterocolitica* O:9 (Young 1995; Munoz *et al.* 2005). In addition, the growth of

Brucella species is slow and fastidious, and therefore their culture and phenotypic identification are difficult (Klietmann and Ruoff 2001). Furthermore, Batchelor *et al.* (1992) suggested that commercial biochemical identification systems may also produce misleading results, because of the slow growth rate of *Brucella* spp. Finally, there is concern about the use of *Brucella* spp. as a potential biological weapon (Kortepeter and Parker 1999; Pappas *et al.* 2006). Overall, a rapid, specific, simple and safe detection system for *Brucella* spp. needs to be established.

The LAMP assay is advantageous because of its simple operation, rapid reaction and easy detection (Notomi *et al.* 2000). A simple and inexpensive apparatus such as a water bath or heat block that provides a constant temperature of *c.* 63°C is sufficient for the assay, and, unlike PCR, the reactivity is directly observed with the naked eye negating the need for electrophoretic analysis. Moreover, the LAMP assay can be performed on site, as special equipment such as a thermal cycler is not required.

Using our LAMP assay, 10 fg of *Brucella* DNA was successfully amplified within 35 min, and was estimated to correspond to 2·8 genome copies per reaction (DelVecchio *et al.* 2002). The sensitivity of *Brucella* LAMP was almost equal to that of real-time PCR previously reported by Queipo-Ortuno *et al.* (2005). However, nonspecific amplification was observed in *V. cholerae* O1 when amplified for more than 42 cycles in real-time PCR, but not in the LAMP. Therefore, the specificity of the LAMP assay was superior to that of real-time PCR. This nonspecific amplification could be recognized by the melt curve analysis (data not shown). When melt curve analysis was included, real-time PCR took about 50 min, while the *Brucella* LAMP can be finished within 35 min.

We also evaluated the LAMP assay for the detection of *Brucella* in infected specimens and in contaminated food samples. In the infected spleen, LAMP detected as few as $8{\cdot}2 \times 10^2$ CFU of *Br. abortus*. These results suggest that the LAMP assay would be useful for rapid diagnosis of brucellosis at an early stage of the infection as well as for the detection of environmental contamination by a low number of bacteria.

The *Brucella* LAMP method developed in this study is a rapid, sensitive and highly specific method that can be substituted for PCR or real-time PCR assays. This is a useful method for clinical diagnosis and surveillance of brucellosis.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1 The detection of *Brucella* by turbidity in LAMP assay.

Sensitivities of LAMP using 10-fold serial diluted *Brucella* DNA were shown. Loopamp Fluorescent Detection Reagent (Eiken chemical) was used in accordance with the manufacture's instruction. The reactivity is directly observed with the naked eye without electrophoresis.

This material is available as part of the online article from http://www.blackwell.synergy.com.

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System for simultaneous detection of 16 pathogens related to urethritis to diagnose mixed infection

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Objective: Urethritis is not always caused by a single pathogen, and isolation of more than two pathogens from one patient is not uncommon. We developed a method to simultaneously detect 16 pathogens related to urethritis.

Methods: We designed specific primers used for amplification of urethritis pathogens in our 16-well microplate assay. Sixteen microliters of each reaction mixture containing template DNA was added to each well to amplify 16 pathogens simultaneously.

Results: After we evaluated the specificity and sensitivity of this microplate polymerase chain reaction method, we used it to detect pathogens in clinical samples. Of 163 clinical samples, 49.7% (81/163) were positive for specific pathogens, and 6.7% (11/163) showed mixed infection. A specific pathogen was not identified in 43.6% (71/163) of cases.

Conclusions: We developed a 16-well microplate assay with 16 specific primers to identify pathogens associated with urethritis.

Key words: microplate, real-time polymerase chain reaction (RT–PCR), sexually transmitted diseases (STD), urethritis.

Introduction

Chlamydia trachomatis is a common causative agent of non-gonococcal urethritis (NGU) and sexually transmitted diseases (STD) in heterosexual men.[1] The number of NGU patients has increased recently, whereas the number of gonococcal urethritis (GU) patients has decreased.[2,3] *Mycoplasma genitalium, Mycoplasma hominis* and *Ureaplasma urealyticum* are currently considered to be pathogens of NGU.[4–6] Other pathogens, such as *Treponema pallidum, Haemophilus ducreyi, Trichomonas vaginalis,* human herpes simplex virus (HSV) and human papilloma virus (HPV), are recognized as pathogens related to STD.

Polymerase chain reaction (PCR) has recently been applied to the diagnosis of urethritis.[7–9] The pathogens related to urethritis are thought to easily cause mixed infection. However, there is little data regarding methods to detect multiple urethritis pathogens simultaneously. In this study, we developed a method to simultaneously detect 16 pathogens related to urethritis.

Materials and methods

Experimental design

The specific primers used for amplification of urethritis pathogens in our 16-well microplate assay are listed in Table 1. These primers were determined by the online Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/) and DNASIS Pro 2 (Hitachi Software Engineering, Tokyo, Japan). The detection limit, sensitivity, specificity, melting temperature (Tm) and standard curve of each primer were determined by real-time (RT) PCR on an iCycler iQ (Software Version 3.0A, Bio-Rad, Hercules, CA, USA) with SYBR Green I (Molecular Probes, Eugene, OR, USA). PCR products were analyzed by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet (UV) transillumination to confirm the size of the expected PCR product for each primer pair.

Real-time PCR

Real-time PCR was performed with an iCycler iQ (Bio-Rad). Our original 16-well microplate and cover are shown in Figure 1. Reactions were carried out in a total volume of 20 μL containing 2.0 μL of 4.0 pmol/μL, forward primer, 2.0 μL of 4.0 pmol/μL reverse primer, 1.6 μL of 2.5 pmol/μL $MgCl_2$, 1.6 μL of 2.5 pmol/μL dNTP mixture (TaKaRa Biomedical, Shiga, Japan), 0.66 μL of 10 × SYBR Green I, 0.1 μL of 5 U/μL Ex Taq Hot Start Version (TaKaRa Biomedical), 2.0 μL of 10 × PCR Buffer (TaKaRa Biomedical), 10 μg bovine serum albumin (BSA; Cohn Fraction V, pH 7.0, Wako Pure Chemical, Osaka, Japan), distilled water and 2 μL template DNA solution. Amplification conditions were one cycle of 95°C for 2 min followed by 55 cycles of 95°C for 20 sec, 55°C for 30 sec and 72°C for 40 sec followed by one cycle at 72°C for 10 min. Reactions were held at 4°C.

Plasmid construction

The amplicon of the target gene was inserted into pGEM-T Easy (Promega Corporation, Madison, WI, USA). We assigned a Gifu Genetic Strain (GGS) number to each plasmid (Table 2). The plasmid was purified with a QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany), and the concentration of DNA was quantified with a NanoDrop ND-1000 Spectrophotometer (version 3.0, NanoDrop Technologies, Wilmington, DE, USA).

Standard curves, detection limits and Tm

We made serial dilutions (1×10^0 to 1×10^{-7} ng/μL) of these plasmids and calculated the slope and intercept of the standard curve and the correlation coefficient for each pathogen (Table 2). Each Tm was determined from the melting curve.

Clinical specimens

We analyzed 163 clinical specimens from patients diagnosed with urethritis at the Toyota Memorial Hospital in 2003 (Table 3). All

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