Comparative Immunology, Microbiology and Infectious Diseases 40 (2015) 1–6

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Rapid and specific identification of *Brucella abortus* using the loop-mediated isothermal amplification (LAMP) assay

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ARTICLE INFO

Article history:
Received 6 October 2014
Received in revised form 3 March 2015
Accepted 4 March 2015

Keywords:
Brucella abortus
Bst polymerase
Diagnosis
LAMP
Zoonosis

ABSTRACT

A rapid and accurate diagnosis of brucellosis is required to reduce and prevent the spread of disease among animals and the risk of transfer to humans. In this study, a *Brucella abortus*-specific (Ba) LAMP assay was developed, that had six primers designed from the BruAb2_0168 region of chromosome I. The specificity of this LAMP assay was confirmed with *Brucella* reference strains, *B. abortus* vaccine strains, *B. abortus* isolates and phylogenetically or serologically related strains. The detection limit of target DNA was up to 20 fg/μl within 60 min. The sensitivity of the new LAMP assay was equal to or slightly higher than other PCR based assays. Moreover, this Ba-LAMP assay could specifically amplify all *B. abortus* biovars compared to previous PCR assays. To our knowledge, this is the first report of specific detection of *B. abortus* using a LAMP assay. The Ba-LAMP assay can offer a rapid, sensitive and accurate diagnosis of bovine brucellosis in the field.

1. Introduction

Brucellosis is caused by members of the genus *Brucella* and is regarded as a worldwide major zoonotic disease [1]. *Brucella* species are gram-negative, facultative, and intracellular pathogens. There are 10 different species with specific host preferences [2,3]. They are able to infect several animal species as well as humans [4].

Brucellosis can spread through the movement of cattle, by contact with infected livestock, wildlife, or humans, and by the consumption of contaminated foods such as dairy products [5]. For these reasons, the diagnosis of brucellosis needs to occur as quickly as possible. Currently, a variety of diagnostic methods is used and is mainly based on serological methods, bacteriological characterization, and molecular genetics assays. However, they all have some limitations for the diagnosis of brucellosis. First, serological tests are very convenient and cost-effective, and they have comparatively high sensitivity and specificity. However, they are not capable of detecting the early stages of the disease owing to low immunogenicity, and can have false-positive reactions to cross-reactive bacteria [6]. The conventional microbiological methods take longer, are laborious, require a skilled technician for isolation and identification, and carry a risk of exposure to the infections agent. Furthermore, in the case of antibiotic treatment, it is difficult to isolate the pathogen from infected animals. On the contrary, molecular methods can detect pathogens with relatively high rapidity, sensitivity and safety because viable pathogens are not required. Therefore, there is interest in diagnosing brucellosis through molecular methods rather than conventional culture or serology. However, current molecular methods have some limitations due to the high homology of DNA among *Brucella* species [7]. Until now, there has not been a species-specific PCR for all 10 *Brucella* species.

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http://dx.doi.org/10.1016/j.cimid.2015.03.001
0147-9571/

Loop-mediated isothermal amplification (LAMP) was first developed by Notomi et al. [8]. This technique relies on autocycling strand displacement in DNA synthesis performed by the *Bst* DNA polymerase. Amplification can be performed in a water bath or heating block without specific reaction equipment or a PCR machine, and the products have the advantage of being visible to the naked eye after addition of a fluorescent reagent [8,9]. Currently, this method has been applied to various fields, for example, detection of viral and bacterial diseases in fish, animals, and humans, owing to the high specificity, sensitivity, simplicity and rapidity of this technique [9–13]. A LAMP assay for *Brucella* was introduced previously, but it only detects *Brucella* at the genus level using IS711 and the *omp 25* gene, which avoids amplification of *O. anthropi* [9,12,14–17]. However, a LAMP assay to detect only *B. abortus* at the species level has not yet been developed.

Therefore, we designed and evaluated the Ba-LAMP assay to identify and diagnose *B. abortus* specifically. This method could be used as an alternative for or to supplement conventional diagnostic methods and offers rapid and precise diagnoses in the field.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains used in this study were 22 *Brucella* reference strains, 2 *B. abortus* vaccine strains, 20 *B. abortus* field isolates, and 3 non-*Brucella* strains, including serologically cross-reacting strains or phylogenetically related strains (Table 1). All strains were cultured on tryptic soy agar (BD, Detroit, MI) supplemented with 5% bovine serum (GIBCO, Grand Island, NY, USA) and 5% sheep blood agar for 3–5 days at 37 °C and 5% CO_2. *Brucella* field strains were identified on the basis of classical biotyping methods and molecular assays including the differential multiplex-PCR [2,3,18,19].

2.2. DNA extraction and electrophoresis

Genomic DNA of all the strains was extracted using a DNeasy blood and tissue kit (Qiagen Korea Ltd., South Korea) according to the manufacturer's instructions, and was stored at −20 °C until future use. All amplification products were run at 120 volts for 55 min and confirmed by electrophoresis on 1.5% standard agarose gels. A 100-bp ladder (Bioneer, South Korea) was used as a molecular size marker. Gels were stained with ethidium bromide (50 μg/ml), visualized and photographed under UV light.

2.3. Primer design and LAMP assay

The specific target gene was analyzed using CLC Main Workbench software version 6.0 (Insilicogen Inc., South Korea) and the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) for whole-genome or partial sequences of 10 *Brucella* species. The target primer sets were designed at the BruAb2_0168 region (GenBank accession no. AE017224) from *B. abortus* using the Primer Explorer version 4 software program (http://primerexplorer.jp/e/) (Fig. 1). The LAMP assay was carried out in 25 μl of reaction mixture containing 5 pmol each of outer primers F3 and B3, 40 pmol each of inner primers FIP (F1c-F2) and BIP (B1c-B2), 20 pmol each of loop primers LF and LB, 12.5 μl of 2× reaction mixture [40 mM Tris–HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween20, 1.6 M Betaine, 2.8 mM dNTPs], 16 units of the *Bst* DNA polymerase (Elkan chemical, Tochigi, Japan) and 2 μl of genomic DNA. In addition, a fluorescent indicator was added to the reaction mixture using a fluorescent detection reagent kit (Elkan chemical, Tochigi, Japan). The amplification mixture was incubated at 65 °C for 60–120 min, and the reaction was terminated by heating at 80 °C for 2 min. The absorbance of real-time turbidity was measured with a LA-320C (Elkan chemical Co. Ltd, Kyoto, Japan).

Table 1
The results of the specificity of the LAMP assay for the *Brucella* strains used in this study.

Strains	Reference	Ba-LAMP
Brucella species		
B. abortus bv. 1 544	ATCC 23448	+
B. abortus bv. 2 86/8/59	ATCC 23449	+
B. abortus bv. 3 Tulya	ATCC 23450	+
B. abortus bv. 4 292	ATCC 23451	+
B. abortus bv. 5 B3196	ATCC 23452	+
B. abortus bv. 6 870	ATCC 23453	+
B. abortus bv. 9 C68	ATCC 23455	+
B. abortus RB51	–	+
B. abortus S19	–	+
B. canis RM6/66	ATCC 23365	–
B. suis bv. 1 1330	ATCC 23444	–
B. suis bv. 2 Thomsen	ATCC 23445	–
B. suis bv. 3 686	ATCC 23446	–
B. suis bv. 4 40	ATCC 23447	–
B. suis bv. 5 513	NCTC 11996	–
B. ovis 63/290	ATCC 25840	–
B. neotomae 5K33	ATCC 23459	–
B. melitensis bv. 1 16 M	ATCC 23456	–
B. melitensis bv. 2 63/9	ATCC 23457	–
B. melitensis bv. 3 Ether	ATCC 23458	–
B. ceti B1/94	NCTC 12891	–
B. pinnipedialis B2/94	NCTC 12890	–
B. microti CCM4915	BCCN 07-01	–
B. inopinata B01	BCCN 09-01	–
B. abortus	20 isolates	+
Non-*Brucella* organisms		
Ochrobactrum anthropi	Field strain†	–
Esherichia coli O157:H7	Field strain	–
Yersinia enterocolitica O: 9	NCTC 11174	–

2.4. Specificity and sensitivity of LAMP

The specificity of Ba-LAMP was investigated using the genomic DNA from the *Brucella* species and non-*Brucella* strains in Table 1. The amplification was monitored in real-time by measuring turbidity on a LA-320C instrument (Elkan chemical Co. Ltd, Kyoto, Japan) and detected with the naked eye under UV light in a dark field. Additionally, to detect the amplification status of the Ba-LAMP assay and efficacy, PCR products were confirmed through 1.5% agarose gel electrophoresis.

All tests were performed in duplicate. The sensitivity of the Ba-LAMP assay was evaluated by 10-fold serial