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Journal of Microbiological Methods 63 (2005) 36–44
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Evaluation of a loop-mediated isothermal amplification method for rapid detection of channel catfish *Ictalurus punctatus* important bacterial pathogen *Edwardsiella ictaluri*

Hung-Yueh Yeh*, Craig A. Shoemaker, Phillip H. Klesius

United States Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Unit, Post Office Box 952, Auburn, AL 36831-0952, USA

Received 23 November 2004; received in revised form 17 February 2005; accepted 17 February 2005
Available online 31 March 2005

Abstract

Channel catfish *Ictalurus punctatus* infected with *Edwardsiella ictaluri* results in $40–50 million annual losses in profits to catfish producers. Early detection of this pathogen is necessary for disease control and reduction of economic loss. In this communication, the loop-mediated isothermal amplification method (LAMP) that amplifies DNA with high specificity and rapidity at an isothermal condition was evaluated for rapid detection of *E. ictaluri*. A set of four primers, two outer and two inner, was designed specifically to recognize the *eip18* gene of this pathogen. The LAMP reaction mix was optimized. Reaction temperature and time of the LAMP assay for the *eip18* gene were also optimized at 65 °C for 60 min, respectively. Our results show that the ladder-like pattern of bands sizes from 234 bp specifically to the *E. ictaluri* gene was amplified. The detection limit of this LAMP assay was about 20 colony forming units. In addition, this optimized LAMP assay was used to detect the *E. ictaluri eip18* gene in brains of experimentally challenged channel catfish. Thus, we concluded that the LAMP assay can potentially be used for rapid diagnosis in hatcheries and ponds.

Keywords: *Edwardsiella ictaluri*; *Ictalurus punctatus*; LAMP

1. Introduction

Diseases have been a very critical limiting factor in aquaculture. It is estimated that U.S. aquatic industries lose about one billion dollars annually to various diseases (USDA, 2003a). Among them, enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* (Hawke et al., 1981) is the leading disease that hinders the catfish production industry in the southeastern U.S. (Wagner et al., 2002; USDA, 2003b). *E. ictaluri*, a member of the Enterobacteriaceae family, is a facultative anaerobic, rod-shaped, Gram-negative, oxidase-negative, glucose-positive and nitrate-nega-

* Corresponding author. Tel.: +1 334 887 3741; fax: +1 334 887 2983.
E-mail address: yyeh@ars.usda.gov (H.-Y. Yeh).

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doi:10.1016/j.mimet.2005.02.015

tive microorganism (Hawke et al., 1981; Waltman et al., 1986). The factors associated with its pathogenesis are not well defined. Recent studies have showed several potential virulence factors, including extracellular capsular polysaccharide (Stanley et al., 1994; Williams et al., 2003), lipopolysaccharide (Arias et al., 2003; Klesius and Shoemaker, 1997, 1999; Lawrence et al., 2001, 2003), outer membrane proteins (Baldwin et al., 1997; Skirpstunas and Baldwin, 2003) and chondroitinase (Cooper et al., 1996).

To prevent these diseases effectively, good management practices such as rapid early and accurate detection of the pathogen are necessary for disease control and reduction of economic loss (Bader et al., 2003). Traditionally, isolation and biochemical speciation are the primary tools for detection and identification of this microorganism. These procedures take at least 48 h by which time the diseases may have already spread throughout the fish population (Bilodeau et al., 2003; Klesius, 1994). Recently, powerful nucleic acid-based real-time PCR for rapid detection of *E. ictaluri* was developed (Bilodeau et al., 2003) and its application for rapid diagnosis seems to be promising. However, PCR inherits its intrinsic disadvantages, such as (1) T*aq* DNA polymerase is often inactivated by inhibitors present in biological samples (e.g. Abu Al-Soud and Rådström, 2000; Abu Al-Soud et al., 2000; de Franchis et al., 1988; Lantz et al., 2000), (2) high-cost, sophisticated instruments for amplification and detection of the amplified products are required, and (3) minimal 1 h are needed for the procedure. Thus, a rapid, simple cost-effective assay is needed to complement the current methods.

We evaluated the loop-mediated isothermal amplification method (LAMP) that is also nucleic acid-based amplification and was developed by Notomi et al. (2000). The principle of LAMP is based on autocycling strand displacement DNA synthesis in the presence of exonuclease-negative *Bst* DNA polymerase under isothermal condition within 1 h (Notomi et al., 2000). Because four specific primers that recognize six different sequences on the DNA target are used, LAMP amplifies DNA in high specificity. Since it was published in 2000, the LAMP method has been tested in diagnosis of viruses (Fukuta et al., 2003a,b; Hong et al., 2004; Ihira et al., 2004; Kono et al., 2004; Parida et al., 2004; Poon et al., 2004; Yoshikawa et al., 2004), bacteria (Enosawa et al., 2003; Iwamoto et al., 2003; Maruyama et al., 2003; Savan et al., 2004), protozoa (Kuboki et al., 2003) and fungus (Endo et al., 2004).

In this communication, we applied this recently developed loop mediated isothermal amplification method (Notomi et al., 2000) to detect the channel catfish important pathogen—*E. ictaluri*. Initially, we designed the primers and optimized the LAMP assay for detection of *E. ictaluri*. Second, the specificity and sensitivity of the primers in the LAMP assay for detection of *E. ictaluri* were determined. Finally, the specific and sensitive LAMP assay for detection of the pathogen from experimentally challenged channel catfish was tested.

2. Materials and methods

2.1. Bacterial strains and DNA preparation

Bacteria and their sources used in this study are listed in Table 1. These bacteria were cultured according to a standard protocol (Arias et al., 2003; Bader et al., 2003). Bacterial genomic DNA was prepared by lysis of the bacterial pellet in 100 μl of lysis buffer (20 mM Tris–HCl, pH 8.0, 2 mM EDTA, pH 8.0 and 1.2% Triton X-100) that was boiled for 10 min (Iwamoto et al., 2003).

2.2. Design of LAMP DNA oligonucleotide primers

To design the species-specific LAMP DNA oligonucleotide primers, we first searched the nucleic acid sequences of *E. ictaluri* deposited in the GenBank database, and used the BLAST program (Altschul et al., 1997) to choose the species-specific gene sequences. The accession number of the sequence was AF037441. The sequence was further analyzed by the PrimerExplorer V1 software program (http://venus.netlaboratory.com/partner/lamp/pevl.html) to have the LAMP primers: F3 (Forward outer primer), B3 (Backward outer primer), FIP (Forward inner primer) and BIP (Backward inner primer) (Table 2, See Ref. Notomi et al., 2000 for nomenclature of primers). In addition, each internal primer that recognizes both sense and anti-sense strands of the target DNA was connected by a TTTT spacer (Notomi et al., 2000). The LAMP primers were synthesized

Table 1
Bacterial strains used in this study

Bacterial species	Medium	Growth temperature
Edwardsiella ictaluri ATCC 33202	Brain heart infusion broth	28 °C
Edwardsiella ictaluri AL93-58	Brain heart infusion broth	28 °C
Edwardsiella ictaluri AL93-75	Brain heart infusion broth	28 °C
Edwardsiella ictaluri S94-1034	Brain heart infusion broth	28 °C
Edwardsiella ictaluri S94-1051	Brain heart infusion broth	28 °C
Edwardsiella tarda AU98-24	Brain heart infusion broth	37 °C
Flavobacterium columnare ATCC 23463	Shieh medium	28 °C
Flavobacterium columnare ARS-1	Shieh medium	28 °C
Flavobacterium columnare ALG-522	Shieh medium	28 °C
Flavobacterium aquatile ATCC 11947	Shieh medium	28 °C
Flavobacterium johnsoniae ATCC 17061	Shieh medium	28 °C
Tenacibaculum maritimum ATCC 43398	Shieh medium+1.5% NaCl	28 °C
Salmonella choleraesuis	Brain heart infusion broth	37 °C
Vibrio vulnificus CECT 61	Brain heart infusion broth	28 °C
Escherichia coli ATCC 25922	Brain heart infusion broth	37 °C
Pseudomonas aeruginosa ATCC 27853	Brain heart infusion broth	37 °C
Enterococcus faecalis	Brain heart infusion broth	37 °C
Streptococcus iniae ARS 58B	Brain heart infusion broth	28 °C

commercially by Sigma-Genosys (The Woodlands, TX) or MWG Biotech (High Point, NC).

2.3. LAMP reaction

The LAMP reaction was carried out in a 25-μl reaction mixture containing the following reagents with final concentrations: 1 × reaction mix with 6 mM $MgSO_4$ (New England Biolabs, Beverly, MA), 0.8 M betaine (USB Corporation, Cleveland, OH), 1.0 mM dNTP (USB Corporation, Cleveland, OH), 0.2 μM each of F3 and B3 primers, 1.6 μM each of FIP and BIP, 0.32 U/μl *Bst* DNA polymerase (New England Biolabs, Beverly, MA) and appropriate amount of template genomic DNA. The reaction was carried out at 65 °C for 1 h and inactivated at 80 °C for 10 min (Notomi et al., 2000). The amplified products (3 μl/well) were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

2.4. Detection of *E. ictaluri* in experimentally challenged catfish by LAMP assay

E. ictaluri infected channel catfish were prepared by intraperitoneal injection (Bilodeau et al., 2003) of the AL93-75 isolate. Uninfected fish were inoculated with brain heart infusion broth. After 24 h post infection, brains (3 mm × 3 mm × 3 mm) were aseptically excised from both uninfected and infected fish. The DNA templates were prepared by using a DNeasy tissue kit (Qiagen, Valencia, CA). At the same time, a standard direct plating method was used to recover the pathogen from tissue. About 0.5 μg DNA was used in LAMP assay.

Table 2
Sequence of LAMP primers used for specific amplification of the *Edwardsiella ictaluri* gene[a]

Primer[b]	Type	Length	Sequence
F3	Forward outer	18-mer	5′-TAA GAC TCC AGC CCT CGG-3′
B3	Backward outer	18-mer	5′-TTC CCT CGC TGG AAG TGG-3′
FIP	Forward inner (F1C+TTTT+F2)	44-mer (F1C:22-mer, F2:18-mer)	5′-GCC CGC AGG AAA CCA TTG ATT T *TTTT* CCG CCT TAC CGC TCT GAT-3′
BIP	Backward inner (B1C+TTTT+B2)	44-mer (B1C:20-mer, B2:20-mer)	5′-GAG GCC CCG GAG CAG TCA TA *TTTT* GCG ATA AGT TCG CCT TCT GT-3′

[a] GenBank accession no.: AF037441.
[b] For nomenclature see Ref. Notomi et al., 2000.

3. Results and discussion

3.1. Optimized LAMP assay condition for E. ictaluri detection

We initially optimized and standardized the LAMP assay for *E. ictaluri* detection by using two outer and two inner primers from *eip18* gene (Table 2) and DNA template from the AL93-75 isolate. The *eip18* gene were chosen because of its specificity to *E. ictaluri*. The specific amplification generated the ladder-like pattern of bands on agarose gel, sizes ranging from 234 bp up to the loading wells (Fig. 1), confirming the specific amplification. No amplification was observed in either distilled water or lysis buffer samples.

3.1.1. Effect of Mg^{++} concentration

Because free Mg^{++} availability affects primer annealing and DNA polymerase activity (Saiki et

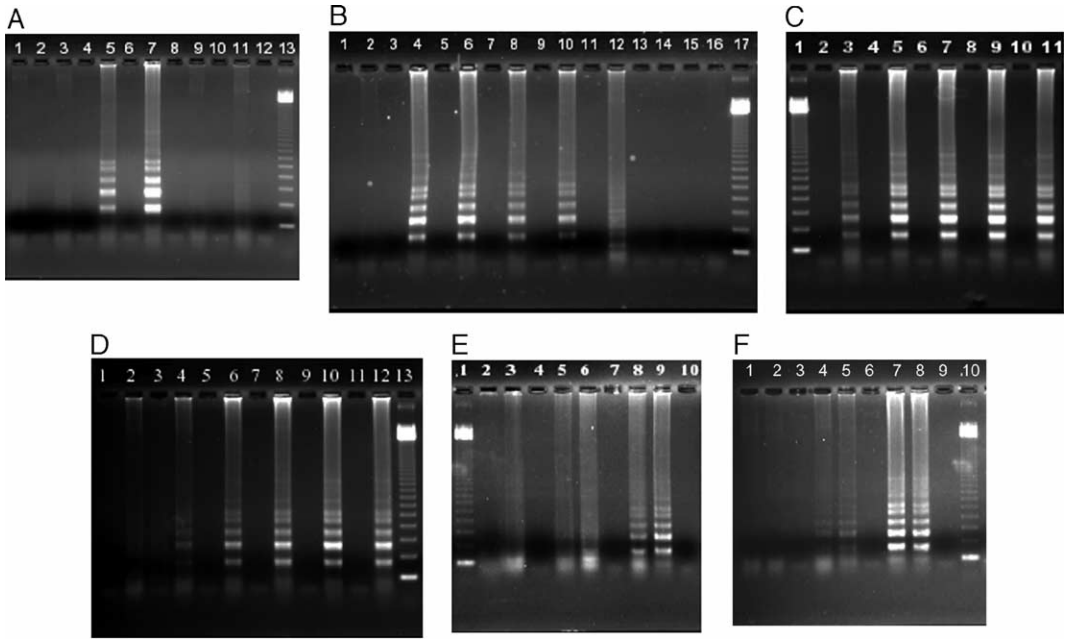

Fig. 1. Optimization of the LAMP reaction detection of *E. ictaluri*. (A) Effect of $MgSO_4$ concentrations on the LAMP reaction: lanes 1 and 2, 0 mM $MgSO_4$; lanes 3 and 4, 2 mM $MgSO_4$; lanes 5 and 6, 4 mM $MgSO_4$; lanes 7 and 8, 6 mM $MgSO_4$; lanes 9 and 10, 8 mM $MgSO_4$; lanes 11 and 12, 10 mM $MgSO_4$; lane 13; 123 bp molecular weight standards (Invitrogen). Lanes 1, 3, 5, 7, 9 and 11, with DNA template in the reaction; lanes 2, 4, 6, 8 10 and 12, without DNA template in the reaction. (B) Effect of deoxynucleotide triphosphate concentrations on the LAMP reaction: lanes 1 and 2, 1.4 mM each nucleotide; lanes 3 and 4, 1.2 mM each nucleotide; lanes 5 and 6, 1.0 mM each nucleotide; lanes 7 and 8, 0.8 mM each nucleotide; lanes 9 and 10, 0.6 mM each nucleotide; lanes 11 and 12, 0.4 mM each nucleotide; lanes 13 and 14, 0.2 mM each nucleotide; lanes 15 and 16, 0 mM each nucleotide; lane 17, 123 bp molecular weight standards. Lanes 1, 3, 5, 7, 9, 11, 13 and 15, without DNA template in the reaction; lanes 2, 4, 6, 8, 10, 12, 14 and 16 with DNA template in the reaction. (C) Effect of ratio of outer and inner primers on the LAMP reaction: lanes 2 and 3, 1 : 1 ratio; lanes 4 and 5, 1 : 2 ratio; lanes 6 and 7, 1 : 4 ratio; lanes 8 and 9, 1 : 8 ratio; lanes 10 and 11, 1 : 10 ratio; lane 1, 123 bp molecular weight standards. Lanes 2, 4, 6, 8 and 10, without DNA template in the reaction; lanes 3, 5, 7, 9 and 11, with DNA template in the reaction. (D) Effect of betaine concentrations on the LAMP reaction: lanes 1 and 2, 0 M betaine; lanes 3 and 4, 0.2 M betaine; lanes 5 and 6, 0.4 M betaine; lanes 7 and 8, 0.6 M betaine; lanes 9 and 10, 0.8 M betaine; lanes 11 and 12, 1.0 M betaine; lane 13, 123 bp molecular weight standards. Lanes 1, 3, 5, 7, 9 and 11, without DNA template in the reaction; lanes 2, 4, 6, 8, 10 and 12, with DNA template in the reaction. (E) Effect of temperature on the LAMP reaction: lanes 2–4, amplification at 60 °C; lanes 5–7, amplification at 63 °C; lanes 8–10, amplification at 65 °C; lane 1, 123 bp molecular weight standards. Lanes 2, 5 and 8, with DNA template from AL93-75 isolate in the reaction; lanes 3, 6 and 9, with DNA template from S94-1034 isolate in the reaction; lanes 4, 7 and 10, without DNA template in the reaction. (F) Effect of reaction length on the LAMP reaction: lanes 1–3, amplification for 30 min; lanes 4–6, amplification for 45 min; lanes 7–9, amplification for 60 min; lane 10, 123 bp molecular weight standards. Lanes 1, 4 and 7, with DNA template from AL93-75 isolate in the reaction; lanes 2, 5 and 8, with DNA template from S94-1034 isolate in the reaction; lanes 3, 6 and 9, without DNA template in the reaction.

al., 1998), the effect of Mg^{++} concentrations ranging from 0 to 10 mM on the LAMP reaction was determined. The Mg^{++} concentration at 6 mM gave the optimal amplification (Fig. 1A). This concentration falls in the published ranges between 4 mM and 8 mM (e.g. Kuboki et al., 2003; Notomi et al., 2000).

3.1.2. Effect of deoxynucleotide triphosphate concentration

It is known that the deoxynucleotide triphosphate concentration affects the specificity of DNA polymerase amplification (Innis et al., 1988). The LAMP reaction in the presence of various concentrations of deoxynucleotide triphosphate was tested. The deoxynucleotide triphosphate concentrations ranging from 0.6 to 1.2 mM amplified the target DNA, but at 1.0 mM gave the maximal reaction product (Fig. 1B). This concentration is much lower than those reported for bacteria by others (Enosawa et al., 2003; Iwamoto et al., 2003; Maruyama et al., 2003; Savan et al., 2004).

3.1.3. Effect of primer ratio

Because wide ranges of primer ratio that may affect the LAMP sensitivity (Ihira et al., 2004) have been used in the LAMP reaction (Enosawa et al., 2003; Iwamoto et al., 2003; Kuboki et al., 2003; Nagamine et al., 2002a; Notomi et al., 2000; Parida et al., 2004; Yoshikawa et al., 2004), the effect of ratios of outer primers and inner primers on the LAMP reaction was determined. As shown in Fig. 1C, the *eip18* DNA target was amplified in the primer ratios ranging from 1 : 1 to 1 : 10. In separate experiments the ratios below 1 : 8 gave inconsistent results. Therefore, the primer ratio of 1 : 8 was used for the following experiments.

3.1.4. Effect of betaine concentration

Betaine has been used in DNA amplification (Abu Al-Soud and Rådström, 2000; Frackman et al., 1998). The LAMP reaction in the presence of different concentrations of betaine was tested. As shown in Fig. 1D, we observed that increasing betaine concentrations increased the LAMP reaction products and the concentration at 0.8 M gave the maximal amplification product. How betaine acts in the reaction is not fully understood. Two mechanisms have been suggested: (1) Betaine makes DNA templates accessible for DNA polymerase (Henke et al., 1997), and (2) it is able to destabilize GC-rich DNA sequences (Rees et al., 1993). Some reports (Nagamine et al., 2001, 2002a,b; Notomi et al., 2000) used higher concentration of betaine than our study. These differences may be due to amplification of different DNA sequences (Henke et al., 1997).

3.1.5. Effect of temperature

Although the *Bst* DNA polymerase has the optimal activity at 65 °C, several reports showed this enzyme can amplify DNA templates at lower temperatures in the LAMP reaction (Endo et al., 2004; Iwamoto et al., 2003; Parida et al., 2004; Poon et al., 2004; Yoshikawa et al., 2004). The effect of temperature on the LAMP reaction was determined. As shown in Fig. 1E, LAMP reaction temperature at 65 °C generated ladder-like pattern products, but no such typical pattern product was detected at 63 and 60 °C.

3.1.6. Effect of reaction length

Several reports (Iwamoto et al., 2003; Savan et al., 2004; Yoshikawa et al., 2004) have demonstrated that amplified products can be detected less than 60 min in the LAMP assay. We varied the LAMP reaction lengths from 30 to 60 min. As shown in Fig. 1F, we observed that increasing the length of the LAMP reaction increased the *eip18* DNA target yield. Amplification was initially detected at 45 min, and reached maximal at 60 min.

On the basis of the above analyses, the LAMP assay condition was optimized in a 25-μl reaction mixture as follows: 1 × ThermoPol buffer with 6 mM $MgSO_4$, 0.8 M betaine, 1.0 mM each deoxynucleotide triphosphate, 0.2 μM each of F3 and B3 primers, 1.6 μM each of FIP and BIP, 0.32 U/μl *Bst* DNA polymerase and appropriate amount of template genomic DNA. The amplification was carried out at 65 °C for 1 h.

3.2. Specificity of eip18 gene in LAMP detection of E. ictaluri

To determine the specificity of the primers of the *E. ictaluri eip18* gene, 12 species of bacteria that are likely associated with channel catfish were tested. Each bacterial species was cultured overnight and harvested by centrifugation. The DNA templates were prepared by lysing bacterial pellets in the lysis buffer.

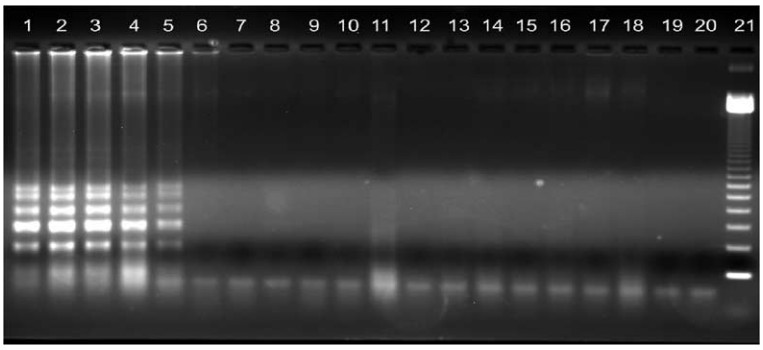

Fig. 2. Specificity of *eip18* gene for the LAMP detection of *E. ictaluri*. Lanes 1, *E. ictaluri* AL93-75; 2, *E. ictaluri* AL93-58; 3, *E. ictaluri* S94-1034; 4. *E. ictaluri* S94-1051; 5, *E. ictaluri* ATCC 33202; 6, *E. tarda*; 7, *Pseudomonas aeruginosa*; 8, *Enterococcus faecalis*; 9, *Salmonella choleraesuis*; 10, *Vibrio vulnificus*; 11, *Escherichia coli*; 12, *Streptococcus iniae*; 13, *Flavobacterium columnare* ATCC 23463; 14, *F. columnare* ARS-1; 15, *F. columnare* ALG-522; 16, *F. aquatile* ATCC 11947; 17, *Tenacibaculum maritimum* ATCC 43398; 18, *F. johnsoniae* ATCC 17061; 19, distilled water; 20, lysis buffer; 21, 123 bp molecular weight standards.

As shown in Fig. 2, we observed that the primers only amplified the *E. ictaluri eip18* gene (Fig. 2, lanes 1–5), but not other bacterial species (Fig. 2, lanes 6–18). High molecular weight bands were seen in some non-*E. ictaluri* lanes that may be due to excessive DNA templates used in the LAMP reaction, but not due to non-specific amplification. Distilled water and lysis buffer were used as negative controls and no amplification was observed (Fig. 2, lanes 19–20). This result suggests that these primers are specific for detection of *E. ictaluri*, and is consistent with other studies of the specificities of LAMP assays in bacterial detection (Enosawa et al., 2003; Iwamoto et al., 2003; Maruyama et al., 2003).

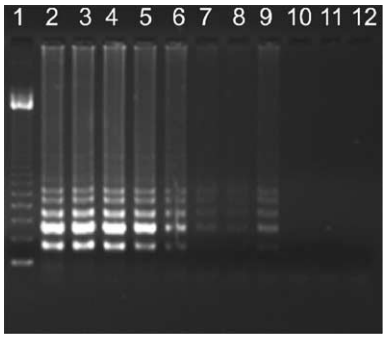

Fig. 3. Sensitivity of *eip18* gene for the LAMP detection of *E. ictaluri*. Lanes: 1, 123 bp molecular weight standards; 2, 1.79×10^8 CFU; 3, 1.79×10^7 CFU; 4, 1.79×10^6 CFU; 5, 1.79×10^5 CFU; 6, 1.79×10^4 CFU; 7, 1.79×10^3 CFU; 8, 1.79×10^2 CFU; 9, 1.79×10^1 CFU; 10, 1.79×10^0 CFU; 11, 1.79×10^{-1} CFU; 12, distilled water.

3.3. Sensitivity of eip18 gene in LAMP detection of *E. ictaluri*

To determine the sensitivity of the *eip18* gene primers in LAMP detection of *E. ictaluri*, we tested serial 10-fold dilutions of the pathogen that were previously quantitated by direct plating. As shown in Fig. 3, the detection limit of the LAMP assay for *eip18* gene was around 20 CFU/ml, which is equivalent to 76 fg of *E. ictaluri* genomic DNA (Bilodeau et al., 2003). This detection limit by the

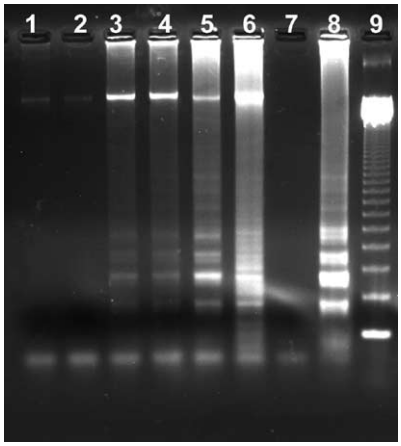

Fig. 4. Detection of the *E. ictaluri eip18* gene in brain samples from experimentally challenged channel catfish. Lanes: 1 and 2, uninfected fish brains; lanes 3–6, infected fish brains; lane 7, distilled water; lane 8, *E. ictaluri* AL93-58 lysate; lane 9, 123 bp molecular weight standards.

LAMP assay is comparable to that by real-time PCR assay (Bilodeau et al., 2003).

3.4. Detection of eip18 gene of E. ictaluri in infected channel catfish organ

The LAMP assay condition optimized above was carried out by using pure culture lysates. For rapid diagnosis of *E. ictaluri* in the hatcheries and field, this LAMP assay must be able to detect the pathogen gene in infected fish organs, such as brain. Fish were infected with the *E. ictaluri* AL93-75 isolate. Brains were aseptically excised, and DNA was isolated by using a DNeasy tissue kit (Qiagen, Valencia, CA). As shown in Fig. 4, *eip18* gene of *E. ictaluri* was detected by the LAMP assay in the infected brain samples, but not the samples from uninfected fish. It is noted that a different banding pattern was observed (Fig. 4) in LAMP assay from the fish samples. Kuboki et al. (2003) used the LAMP assay for detection of African trypanosomes and also found the different banding pattern. The banding was later confirmed as the specific LAMP product, not non-specific amplification. The reason may be due to the activity of *Bst* DNA polymerase that undergoes linear target isothermal multimerization and amplification (Hafner et al., 2001; Kuboki et al., 2003). *E. ictaluri* was isolated from brains of the infected channel catfish by direct plating.

In conclusion, the LAMP assay optimized in this study is specific and sensitive for identification of *E. ictaluri* in culture isolates as well as diseased fish samples. In addition, because this assay is simple and does not require sophisticated equipment, the LAMP assay can potentially be used for preliminary screening and surveillance of *E. ictaluri* in hatcheries and the field.

Acknowledgements

We thank Ifayet Johnson-Mayo, Paige Mumma, Dave Carpenter and Curtis Day for excellent technical support, and thank Drs. Covadonga R. Arias, Victor Panangala, Richard Shelby and Jeff Terhune for critical comments on the manuscript.

This study was supported by the CRIS Project No. 6420-32000-011-00D. Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

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