A Quantitative Loop-Mediated Isothermal Amplification Assay for *Flavobacterium psychrophilum* in River-Water Samples Containing DNA Amplification Inhibitors

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**Abstract:** In this study, we assessed the utility of a quantitative loop-mediated isothermal DNA amplification (LAMP) assay for *Flavobacterium psychrophilum* in natural river water. Direct addition of serial dilutions of DNA standard to the DNA samples extracted from each river water enabled the precise estimation of the amount of *F. psychrophilum*-DNA despite the presence of inhibitors. This is a novel method for attenuating the inhibitory effects of unknown substances in natural water samples.

**Key words:** *Flavobacterium psychrophilum*; Bacterial cold-water disease; Inhibition; Loop-mediated isothermal amplification assay

DNA amplification assays such as polymerase chain reaction (PCR) are effective tools for detecting various unculturable pathogens occurring in natural environments (Bilodeau et al. 2003; Adams and Thompson 2006; Luis Balcazar et al. 2007; Fukui and Sawabe 2008). Rapid and precise detection of such pathogens allows us to develop measures to counter disease outbreaks, thus reducing the use of chemical therapeutic agents such as antibiotics. However, the presence of polymerization inhibitors in samples obtained from natural environments hampers accurate DNA amplification.

Molecular biological methods are liable to be rendered unsuccessful for detecting microorganism from natural environments due to the inhibitors present in the natural samples. Various factors such as body fluids, reagents encountered in clinical and forensic science, food constituents, and environmental compounds have been reported to inhibit PCR (Kreader 1997; Wilson 1997; Abu Al-Soud and Radstrom 1999, 2000, 2001; Kramer et al. 2002; Braid et al. 2003; Guy et al. 2003; Abu Al-Soud et al. 2005; Jiang et al. 2005). Some moderators of PCR inhibition such as bovine serum albumin (BSA), T4 gene 32 protein, betaine, and polyvinylpyrrolidone have been reported previously (Panaccio and Lew 1991; Baskaran et al. 1996; Kreader 1996; Henke et al. 1997; Abu Al-Soud and Radstrom 1999, 2000, 2001; Kramer et al. 2002; Braid et al. 2003; Guy et al. 2003; Abu Al-Soud et al. 2005; Jiang et al. 2005). Most of these approaches focus on the attenuation and/or removal of these inhibitors.

In our previous paper (Fujiwara-Nagata and Eguchi 2009), we reported the loop-mediated isothermal amplification (LAMP) assay for...
rapid and simple detection of *Flavobacterium psychrophilum*. The non-culturability of *F. psychrophilum* derived from the natural environment is a significant difficulty when using conventional media. The *parE*-LAMP assay solved the natural environment detection problem. However, this method and other DNA amplification techniques still had a precise quantification weakness due to the polymerization inhibitors in natural river water that cause underestimation of the target DNA.

In this study, we report the development of a new method to attenuate the inhibitors of DNA amplification. Conventional approaches (Panaccio and Lew 1991; Baskaran et al. 1996; Kreader 1996; Henke et al. 1997; Abu Al-Soud and Radstrom 1998, 2000, 2001; Kramer et al. 2002; Brajd et al. 2003; Guy et al. 2003; Abu Al-Soud et al. 2005; Jiang et al. 2005) directed at removing or reducing inhibitors are difficult to employ, because it is impossible to identify the type and number of inhibitors present in a given natural freshwater sample and determine the most suitable method for their removal. Our method enabled precise quantification of target microbes without elimination of inhibitors from individual DNA samples, and involved the addition of serial dilutions of purified target DNA as internal standards in each sample.

**Materials and Methods**

**Bacterial strain and culture conditions**

*F. psychrophilum* is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome that affect salmonids in various water regions of the world. *F. psychrophilum* strain SG990302 was used in our experiments. This strain was isolated from diseased ayu *Plecoglossus altivelis* in Lake Biwa, Shiga Prefecture, in 1999, and cultured in modified cytophaga broth at 15°C with shaking at 160 rpm (Aoki et al. 2007).

**Site description**

The Chinaigawa river (total length, approximately 9.5 km), which flows into Lake Biwa in Shiga, Japan, was chosen as the model river. Water samples were collected from sites upstream (approximately 7.9 km from the lake), midstream (approximately 5.3 km from the lake), and downstream (approximately 0.3 km from the river mouth). The samples were collected between June 2004 and March 2005. From June to July 2004, there was a peak in the number of ayu coming up the river from the lake. The incidence of BCWD among these fish was significant downstream, judging from visual observations. From September to October 2004, ayu spawned all at once at the downstream site of the model river and died after spawning. Spawned ayu and dead ayu revealed various symptoms such as BCWD and fungal skin disease. The ayu had disappeared from the model river in November 2004.

**Sample preparation**

Each 500-ml sample of water was filtered through an autoclaved 0.22-μm Durapore membrane filter (Millipore, MA, USA; diameter, 25 mm). Bacterial cells retained on the filters were resuspended in 2 ml sterilized phosphate-buffered saline (PBS; 8.0 g NaCl, 2.9 g Na₂HPO₄/12H₂O, 0.2 g KCl, 0.2 g KH₂PO₄, and 1 l MilliQ water; pH 7.4). This bacterial suspension was concentrated to 200 μl by centrifugation (18,750 × g for 20 min at 4°C), and 200 μl of DNA was extracted by using a Generation Capture Column kit (Gentra Systems, MN, USA).

**Quantitative loop-mediated isothermal amplification (qLAMP) of DNA**

The quantitative loop-mediated isothermal amplification (LAMP) method amplifies the *parE* gene of *F. psychrophilum* (Fujiwara-Nagata and Eguchi 2009). Briefly, *parE* qLAMP was performed using a reaction mixture (25 μl) and a LAMP primer set (FIP-17, BIP-17, F3-17, and B3-17) targeting the *parE* gene of *F. psychrophilum* (Fujiwara-Nagata and Eguchi 2009). *parE* DNA was PCR-amplified from the genomic DNA extracted from *F. psychrophilum* SG990302 and used as the internal standard (Fujiwara-Nagata and Eguchi 2009). Serial dilutions of purified *parE* DNA, ranging from 2.0 × 10¹ to 2.0 × 10⁷
copies per reaction for natural samples and 1.0 \times 10^2 \text{ to } 1.0 \times 10^7 \text{ copies per reaction for a MilliQ water control, were used as the internal standard for the parE qLAMP reaction. As a negative control, we added sterilized MilliQ water instead of the internal standard parE DNA. LAMP primer identifies the indigenous target and the exogenous parE DNA. Gene copy numbers corresponding to the purified parE DNA dilutions were plotted against the time points when the turbidity resulting from the production of magnesium pyrophosphate exceeded a threshold value (threshold time: T_t = 0.02).

**Results and Discussion**

The inhibitors present in the samples collected from the model river delayed the parE qLAMP amplification time (Fig. 1) by as much as 14 min, the longest delay, in the February 2005 downstream sample. However, the delay did not affect the correlation coefficient (Fig. 1). Extracted natural DNA and serial dilutions of purified parE DNA were added to the parE qLAMP reaction mixture. For the MilliQ water control, only serial dilutions of purified parE DNA were added (closed circle and thick lines in Fig. 1). The amplification time was longer in the former mixture than in the MilliQ water control. The amplification curves shifted to the right (the thin line in Fig. 1). This shift is probably due to the influence of unknown natural inhibitors present in the river water. Nonetheless, the shift of the standard curve exhibited a strong correlation between the amount of DNA template and threshold time (Fig. 1). The lower limit of parE detection was 2.0 \times 10^2 \text{ copies per reaction in river water samples from upstream and midstream sites (Fig. 1). However, in the water samples from the downstream site, the } 2.0 \times 10^2 \text{ copy internal standard was not amplified. The inhibitors in the downstream sample seemed to be much stronger than those detected in the up- and midstream samples. Thus, the lower limit of parE detection probably increases with the sample quality.}

![Fig. 1. The influence of unknown contaminants from natural river water samples on parE-LAMP](image-url)
The amount of indigenous \textit{parE} in the model river (downstream; June and July 2004) was quantified by performing \textit{parE} qLAMP (Fig. 2). BCWD infection in the downstream ayu was observed in June and July 2004, which is the migration season for this species. When the concentrations of the purified \textit{parE} internal standards were higher than the concentration of extracted natural \textit{parE} DNA, a significant correlation was observed between the \textit{Tt} values and the copy numbers of the template DNA (thick solid line in Fig. 2). However, in the lower concentration range of the internal standard (i.e., standards $\leq$ extracted \textit{parE} DNA), DNA amplification occurred simultaneously in all the tubes (broken vertical line in Fig. 2). The log copy numbers of the template DNA in the samples of 1 June and 1 July (2004) were estimated from the linear points of intersection (dotted line in Fig. 2), which were $1.5 \times 10^4$ and $6.1 \times 10^2$ copies per reaction, respectively.

The LAMP method has been used to detect several viruses (Ihira et al. 2004; Sugiyama et al. 2005; Torinowa and Komiya 2006; Hagiwara et al. 2007; Parida et al. 2007), bacteria (Enosawa et al. 2003; Iwamoto et al. 2003; Torigoe et al. 2007; Miyagawa et al. 2008; Gahlawat et al. 2009; Yamazaki et al. 2009), and parasites (El-Matbouli and Soliman 2005; Thekisoe et al. 2009). Some authors compared LAMP with conventional PCR and real-time PCR and reported that LAMP was more sensitive than PCR, and was similar to nested and real-time PCR (Enosawa et al. 2003; Kuboki et al. 2003; Hagiwara et al. 2007; Torigoe et al. 2007). Gahlawat et al. (2009) reported that LAMP

![Fig. 2. Quantification of \textit{parE} DNA from \textit{F. psychrophilum} under inhibition by unknown contaminants in the natural river water samples. All the natural river water samples were collected from downstream sites of the model river when bacterial cold-water disease was detected in the ayu living there. Symbols indicate copy numbers of internal standard DNA. Intersections of each solid and broken line indicate indigenous \textit{parE} gene copy number in the downstream water. In this method, the standard curve for DNA quantification was specific for each natural sample. Each $R^2$ value for the standard curves (solid line) were as follows: June 1, 2004: 0.986; July 1, 2004: 0.993.](image-url)
was 10-fold more sensitive than real-time PCR. However, Sugiyama et al. (2005) observed that the sensitivity of their modified LAMP (60 min reaction) was slightly lower than that of real-time PCR. Real-time PCR is as rapid as LAMP, and neither method requires electrophoresis. The advantage is that LAMP is easier to perform than PCR or real-time PCR, because LAMP does not require expensive apparatus such as thermal cyclers (Enosawa et al. 2003; El-Matbouli and Soliman 2005; Sugiyama et al. 2005; Hagiwara et al. 2007; Torigoe et al. 2007; Miyagawa et al. 2008; Gahlawat et al. 2009). LAMP is suitable for processing multiple samples at the same time.

In this study, we identified a solution to the problem of unknown inhibitors in natural freshwater samples. In our method, a single primer set is used to detect the target gene and the internal standard. The amount of target gene in the river water samples was extrapolated from the standard curve, which was traced by adding serial dilutions of DNA standard to the each sample. This novel method attenuates the influence of unknown inhibitors in natural freshwater samples. The other obstacle to precise estimation of natural DNA is the loss of DNA during extraction. We did not focus on this aspect in this study. However, we believe a possible solution is recombinant bacteria-mediated transfer of recombinant DNA different from that of the target field.

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DNA增幅阻害物質が含まれている河川水試料中の

**Flavobacterium psychrophilum** の定量 LAMP 法

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天然河川水試料に存在する *Flavobacterium psychrophilum* の DNAを定量的 loop-mediated isothermal amplification（LAMP）法を用いて調べた。段階制御した DNA標準物質を天然河川水由来試料に直接添加することにより、DNA增幅阻害物質の存在に関わらず、*F. psychrophilum* の DNA量を正確に見積もることが可能になった。この方法は、天然河川水に存在する未知の物質の阻害効果を減少させる新規な方法である。