Application of loop mediated isothermal amplification (LAMP) method for detection of *Edwarsiella ictaluri* on patin (*Pangasius* sp.) fish

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Abstract. *Edwarsiella ictaluri* is a potential bacteria as a pathogen causing Enteric Septicemia of Catfish (ESC) and also still a big problem in catfish because it can cause death to 100%. This study aims to develop diagnosis DNA based on molecular using Loop Mediated Isothermal Amplification. Method (LAMP). Detection DNA of *E. ictaluri* bacteria from 9 isolates was extracted at Fish Health Laboratory, in Depok. used four pieces of specific primers from Eip 18 gene of *E. ictaluri*. DNA amplification, for LAMP reactions without a thermocycler machine, using only an incubator / water bath / oven, at 65 ºC for 1 hour. The result of detection with LAMP, showed that 8 isolates were positive of *E. ictaluri* marked by SYBR green I color change from orange to green. While 1 isolate was negatively marked with SYBR Green remain orange. The LAMP results are also verified with PCR using outer primer F3 and B3 which showed positive *E. ictaluri*. LAMP can detect ESC disease simple, quickly, easily, economically in 1 hour compared by PCR and LAMP method can be used as an alternative diagnostic test for fish diseases, especially in areas with limited laboratory infrastructure.

Keywords: *Edwarsiella ictaluri*; eip 18 gene; LAMP; PCR; SYBR green

1. Introduction

*Edwarsiella ictaluri* is a Gram-negative bacteria that causes Enteric Septicemia of catfish (ESC), which is a potential pathogen and is still a big problem in catfish cultivation because it can cause up to 100% mortality. Outbreaks of ESC disease by *E. ictaluri* bacteria caused a failure in catfish to cultivate in Indonesia, especially in Jambi in 2002 [1]. Species other than catfish reported can be infected with *E. ictaluri*, were *Oreochromis niloticus* [2], *Plecoglossus altivelis* [3, 4], *Morone americana*, *Oncorhynchus tshawytscha*, O. Mykiss [5] and Danio rerio [6] can also be infected by these bacteria experimentally. Early detection of ESC disease can help reduce the infection by *E. ictaluri* in catfish. Biochemical identification by isolating bacteria from internal organs such as the kidneys, liver or brain, this diagnostic method takes time, especially for *E. ictaluri* because of its slow growth. [7]. One of the rapid diagnostic methods used to detect disease is the LAMP [8].

The Loop Mediated Isothermal Amplification (LAMP) method is the mechanism of DNA synthesis and enzymatic separation of DNA chains with DNA polymerase Bst and four primers designed to recognize six target DNA sequences. DNA synthesis by transfer is a strand initiated by the inner and outer primers that are hybridized to the ends of the target and resulting in a stem-loop DNA structure. LAMP has high selectivity because it recognizes 6 different target sequences at the beginning of the
reaction and 3 regions at each end of the DNA fragment four primers [9]. Compared to PCR, LAMP has several advantages because only requires fairly simple equipment, fast procedures, and easy reading of the results [10]. LAMP amplifies DNA through DNA polymerase Bst by eliminating the denaturation stage and the reaction is carried out without using PCR because it is very specific and has high sensitivity, is fast, and economical so that it can be used with limited equipment and makes it easier for fish farmers to monitor diseases caused by bacteria. This study aimed to develop detection in application of the LAMP method to diagnose ESC disease due to *E. ictaluri* infection in catfish.

2. Materials and methods

2.1. *E. ictaluri* bacteria isolate

*E. ictaluri* bacteria were obtained from the culture collection of the Fish Health Laboratory, Depok. This bacterial was isolated from catfish infected with *E. ictaluri* at the catfish culture center in West Java and has been carried out by characterization and biochemical testing. The bacteria were cultured in TSA at 28 °C for 24-48 hours and then prepared for DNA extraction.

2.2. *E. ictaluri* DNA extraction

Bacterial DNA was extracted using by boiling method, in which the colonies were taken with a sterile loopful inserted into a sterile microtube and added with TE buffer (500µL) and heated for 10 minutes at 95°C. After heating, 5 µl of triton-X was added and heated again at 95°C for 5 minutes. Then the sample was centrifuged at 4000 rpm (5 minutes) and then supernatant obtained was transferred to a new microtube and used as a DNA template and quantified using a nanodrop at a wavelength of 260/280 nm with a purity of 1-2, then after which it was used as a template for LAMP and PCR reactions µl or stored at 20°C until use.

### Table 1. LAMP reaction mixture for DNA amplification of *E. ictaluri*.

| Component          | Reaction of LAMP | concentration stock | final of reaction | reaction volume (µl) |
|--------------------|------------------|---------------------|-------------------|----------------------|
| ThermoPol buffer   | 10x              | 1x                  | 2.5               |
| Primer FIP         | 100 µM           | 20 µM               | 2                 |
| Primer BIP         | 100 µM           | 20 µM               | 2                 |
| Primer F3          | 100 µM           | 10 µM               | 0.5               |
| Primer B3          | 100 µM           | 10 µM               | 0.5               |
| Betaine            | 5M               | 400M                | 4                 |
| dNTP               | 25 mM            | 1mM                 | 0.5               |
| BST DNA polymerase | 8,000 U/ml       | 8U                  | 1                 |
| NFW                | -                | -                   | 9.5               |
| Template DNA       | 100 ng/ µl       | 100ng/ µl           | 2                 |
| Total              |                  |                     | 25                |

2.3. Detection by Loop Mediated Isothermal Amplification (LAMP)

LAMP was performed in a 25-µl total reaction mixture containing four primers each, F3 primer (forward outer primer), B3 (Backward outer primer), FIP (Forward inner primer), and BIP (Backward inner primer) as shown in table 2. The mixture was incubated at 65 °C for 60 min. Visual detection based on the turbidity of the by-product of DNA amplification was performed in real-time. In addition, a positive amplification was indicated by a color change after adding 0.5 µl of SYBR Green I to the reaction mixture and observing under UV light. The components of the reaction mixture for DNA amplification by the LAMP method are presented in table 1.

2.4. PCR of *E. ictaluri* with external primers F3 and B3

The PCR amplification was performed with two external LAMP primers (F3 and B3) to detect the results of LAMP and to ensure that the LAMP correctly amplified the target. The PCR
reaction was carried out in a 25 μl reaction mixture consisting of 12.5 μl of go green taq master mix promega, 1 μl of F3 and B3 primers respectively, 8.5 μl of free water nucleases and 2 μl of template DNA with 30 PCR cycles at denaturation temperature 92°C for 2 minutes, annealing at 64°C for 1 minute, elongation at 72°C for 1 minute and final elongation at 72°C for 5 minutes. The target PCR band generated from external primers F3 and B3 is 234 bp. [8]. The amplified products were analyzed by gel electrophoresis in 2% agarose gels, stained with ethidium bromide, and then observed under gelog.

### Table 2. Primer sequence was used for E.ictaluri detection (Eip 18 gene) by LAMP.

| Primer name | Primer sequence for E.ictaluri detection |
|-------------|------------------------------------------|
| F3          | 5’-TAA GAC TCC AGC CCT CGG-3’             |
| B3          | 5’- TTC CCT CGC TGG AAG TGG-3’           |
| FIP         | 5’- GCC CGC AGG AAA CCA TTG ATT TTTT CCG CCT TAC CGC TCT GAT-3’ |
| BIP         | 5’-GAG GCC CCG GAG CAG TCA TA TTTT GCG ATA AGT TCG CCT TCT GT-3’ |

2.5. Sensitivity of LAMP DNA E. ictaluri

The sensitivity test of the LAMP E. ictaluri reaction was carried out on positive samples using 10 fold dilution of E. ictaluri DNA as templates with LAMP and PCR assays, as described above and observed with a UV light.

3. Results and discussion

E. ictaluri bacterial infection in catfish shows symptoms, anemia in the gills, white spots on the liver, spleen and kidneys, pimples, body full of wounds which can be seen in figure 1. E. ictaluri comes from the family Enterobacteriaceae, is facultative anaerobic, rods, Gram negative, oxidation negative, glucose negative and nitrate negative [11,12]. his study has succeeded in detecting E. ictaluri using the LAMP method and can be seen in figure 2, the sample shows positive E. ictaluri, it can be seen that under UV light and could be observed by the naked eye cause amplified LAMP reaction mixtures contained magnesium pyrophosphate with turbidity in a positive sample and no turbidity in negative sample. Naked eye detection by a change of color of SBYR Green I from orange to green is due to the presence of fluorescence bonds in a positive sample under UV light. In the negative sample, the SBYR Green I color was orange (see figure 2).

The use of SYBR Green I for visual observation of LAMP amplification products is simple without the use of electrophoresis and does not require staining with ethidium bromide. Only 0.5 μl of SYBR Green I was added to the LAMP reaction mixture and it was enough to see the color change from orange to green indicating a positive sample [13]. The addition of SYBR green I DNA fluorescence dye will bind to double-stranded DNA and glow when exposed to UV light. Before fluorescent with UV can see the difference in color between positive and negative results, but with UV light, the difference will be seen more clearly [13].

The LAMP product can quickly become visible visually because LAMP amplifies the target DNA in very large quantities and produces magnesium pyrophosphate as a by-product. Magnesium pyrophosphate is formed as a result of the reaction between magnesium ions and pyrophosphate ions released by dNTP during nucleic acid polymerization [14]. E. ictaluri biochemical test for identification takes at least 48 hours with a long time, maybe the disease has spread to all fish populations [15]. Likewise, detection with PCR takes approximately 3 to 4 hours, while detection with LAMP only takes 1 hour.
LAMP detection is a method that has high sensitivity and specificity besides being simple and fast, both amplification and detection reactions in target genes can be carried out completely in one tube under isothermal conditions without using a thermal cycler machine for DNA amplification and does not require electrophoresis in agarose [16]. This isothermal condition is possible because, at a temperature of about 65°C, the double-stranded DNA is in dynamic equilibrium, allowing one of the primers to hybridize with its complementary sequence, thereby initiating DNA synthesis. The LAMP method is a direct method of molecular diagnostic testing based on the bacterial nucleic acid identification test. The LAMP method has the potential to be developed for the detection of pathogens in various infectious diseases in fish and is very efficient in rapid diagnosis for surveillance of these bacterial diseases.

**Figure 1.** Catfish infected with *E. ictaluri*, found white spots on the kidneys and liver.

**Figure 2.** The result of *E. ictaluri* was amplified with LAMP method, positive samples showed that SBYR green I green change of color to green and negative samples showed SBYR Green I were orange. Samples 1 to 8 the positive, sample 9 were negative samples and 10 were Negative control and 11 were Positive control.
The results of the verification of *E. ictaluri* by PCR also showed the same results as LAMP for eight positive sample isolates of *E. ictaluri* into a target of band PCR were 234 Bp and 1 negative sample which can be seen in figure 3. DNA amplification by PCR took 3 hours to obtain PCR bands that were electrophoretic in agarose. LAMP detection for bacterial diseases in fish has been carried out on *E. tarda* [17], *Flavobacterium columnare* [8] as well as for the detection of viruses such as WSSV in shrimp [18], KHV [19], IHNV in Rainbow trout [20], iridovirus [21].

Results of the post-sample sensitivity test no. 1 detection of LAMP *E. ictaluri* test on catfish with 10 fold dilutions from $10^{-3}$ (100 ng/µl) - $10^{-10}$ (100 pg/µl) in figure 4 and table 3. shows that LAMP can detect up to a level of dilution 1.000.000 times with limit of detection of 1fg/µl compared with the PCR method in 100.000 times. Similar results were also described who reported detection limits of the LAMP test of $0.3 \times 10^{-3}$ ng for detecting *Trichinella spiralis* and $3.6 \times 10^{-3}$ ng by PCR using external primers [22]. However, [23, 24] showed that the sensitivity for detection of infection by *H. contortus* in stool samples from sheep was $10^{-5}$ ng/µl and in *fasciolosis* $10^{-4}$ ng. conducted the LAMP test to differentiate *F. hepatica* and *F. gigantica* showing a detection limit of $10^{-5}$ ng [25].

![Figure 3. Results of PCR amplicon of E. ictaluri with an outer primer of Lamp F3 and B3, running on agarose gel, M = Marker 100 Bp, no. 1 to no. 8 were positive E. ictaluri samples, and no. 9 was a negative sample, (-) negative control and (+) positive control.](image)

![Figure 4. The results of the LAMP and PCR sensitivity assays and the limit detection for sample no. 1, with 10 fold dilutions the DNA from E. Ictaluri.](image)
Table 3. Serial dilution results from LAMP reactions.

| Dilution | The limit of detection |
|----------|-----------------------|
| $10^{-1}$ | 100 ng                |
| $10^{-2}$ | 10 ng                 |
| $10^{-3}$ | 1 ng                  |
| $10^{-4}$ | 100 pg                |
| $10^{-5}$ | 10 pg                 |
| $10^{-6}$ | 1 pg                  |
| $10^{-7}$ | 100 fg                |
| $10^{-8}$ | 10 fg                 |
| $10^{-9}$ | 1 fg                  |
| $10^{-10}$| 100 aG                |

The detection limit of the LAMP test indicates the high sensitivity is derived from its ability to detect at least six copies of DNA in a reaction mixture. With the success of the LAMP method in amplifying DNA and the results of calculating the sensitivity value, it can be said that the LAMP method has validity after confirmation between the detection results of *E. ictaluri* bacteria with the LAMP method and the PCR method.

4. Conclusion

The results of detection of *E. ictaluri* bacteria using LAMP showed positive results for the eight isolates with visual observations seen from the color change of SYBR Green I from orange to green. Besides that, the application of the LAMP method is simple, fast, easy, and economical. LAMP can be used as an alternative method besides the PCR method for molecular diagnosis in small laboratories with limited infrastructure areas because it only requires an incubator/water bath.

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