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Ultra highly sensitive method for detecting *Edwardsiella ictaluri* using high-gradient immunomagnetic separation with polymerase chain reaction

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ABSTRACT

Edwardsiella ictaluri causes an economically important bacterial disease in farm-raised catfish in the USA and abroad. To elucidate the route of infection for fish bacterial disease, it is important to monitor both fish health and trace bacteria in the water environment. In this study, we applied an ultra highly sensitive method to detect *E. ictaluri* using high-gradient immunomagnetic separation (HGIMS) with polymerase chain reaction (PCR). HGIMS is a magnetic separation method in which the magnetic force is strengthened by integrating a magnetic gradient between the magnetic filter and nearby column. Immunomagnetic beads were specifically designed to react with the target bacteria, allowing for more efficient collection. The accumulated beads were released from the filter by releasing the magnetic force. After the process, DNA was extracted from the concentrated cells, and PCR was applied to detect *E. ictaluri*. The HGIMS system had higher detection sensitivity using than the conventional method, and the total assay time, including sample preparation, was about 3.5 h. The optimal reaction time of immunomagnetic beads was 15 min and the flow rate of the HGIMS system was 10 ml min^{-1}. PCR products of the expected size were obtained from samples concentrated up to 10^2 cfu ml^{-1}. The proposed system appears to be suitable for highly sensitive detection of *E. ictaluri*. Improvements in the bacteria recovery ability of the immunomagnetic beads will further increase the detection limits.

1. Introduction

Aquatic products are precious sources of good quality protein and have excellent nutritional characteristics. They are indispensable foods, especially in traditionally healthy food diets. Production of offshore fisheries, however, tends to decrease due to overfishing without proper management of the marine environment and fishery resources [1,2]. As a result, in addition to the fishing industry, aquaculture has become indispensable for securing a stable supply of marine products for current needs [3]. Deterioration of the aquatic environment or disease brought in with other species imported from other regions or countries may negatively affect fish health [4,5]. The survival rate of fish in aquaculture farms greatly affects management and profits. Thus, appropriate methods for dealing with fish disease in aquaculture are required.

In the 1980s in Japan, bacterial cold water disease in cultivated Ayu (*Plecoglossus altivelis*) spread throughout the country [6,7]. Since then, several anti-epidemic measures such as colonization monitoring or the development of an appropriate breeding environment have been implemented. As a result, outbreaks of cold water disease have significantly decreased over the last several years. A new Ayu bacterial fish disease, *Edwardsiella ictaluri* infection, however, has recently attracted attention. *E. ictaluri* infection is a bacterial fish disease detected in dead Ayu (*Plecoglossus altivelis*) found in a river in 2007 in Japan [8,9]. Ayu is very popular in Japan as a food and as fishing game. A large number of juvenile Ayu reared in hatcheries or collected from local rivers and seacoasts are released annually to enhance Ayu stocks [10]. Because Ayu may carry the pathogen to rivers, care must be taken in treating the breeding water of the released Ayu. Live Ayu are also used as decoys in a Japanese cultural angling method called “Tomozuri” [11,12]. These activities are also thought to be another route of the spread of bacterial fish disease in Japan caused by delivery of the pathogen to rivers. Although *E. ictaluri* is ubiquitous, early infection does not always visibly harm fish and thus it is very difficult to find and distinguish this bacterial fish disease until the fish have been infected for a certain period of time and began to exhibit symptoms [13–15]. *E. ictaluri* has not yet resulted in a drastic loss and death of fish like Ayu, but it is now widely recognized as the main source of fish disease that could result in a huge

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economic loss. The course of invasion and infection of *E. ictaluri* in fish, however, is not clear. Efficient methods to regularly estimate the existence of the pathogen and changes in its levels in the habitat are useful and desirable for studying the pathogenic mechanisms and preventing the spread of infection.

In general, traditional agar plate cultivation methods are used to detect bacteria like *E. ictaluri* [16]. This method, however, is time-consuming and laborious, and depending on the type of bacteria, may take up to several days. Moreover, *E. ictaluri* is difficult to distinguish from other bacterial colonies because the colony shape varies [17,18]. These disadvantages can be overcome using a serologic method, e.g., immunoassay techniques and molecular biologic methods, e.g., polymerase chain reaction (PCR) assays. The immunoassay technique is based on the interaction between an antigen and antibody, and requires technology for detecting or quantifying the interaction. Several different immunoassays are available, and these techniques are used broadly in many fields. Enzyme-linked immunosorbent assay (ELISA) is an immunologic technique that can be adapted to detect antigens originating specifically from a targeted bacterial pathogen. This technique is useful as a screening or identification tool for detecting pathogens [19,20]. Immunofluorescence antibody techniques detect cells using fluorescence-labeled antibodies. Flow cytometry (FCM) has been extensively used in recent years to measure fluorescence-labeled cells [21–23]. FCM is based on the optical detection of particles passing through a tube that is irradiated using a laser source. Particles exhibit different light-scattering characteristics due to their size, shape, internal complexity, and granularity. Fluorescence-labeled particles can be detected using a fluorescence detector. PCR, a molecular technique for in vitro amplification of a DNA fragment via enzymatic replication, is also widely used. Products of PCR amplification are separated on an agarose gel, stained, and the resulting fluorescent DNA bands are detected. Although viable bacteria are not detected using PCR, the PCR technique is widely used in various fields because of its high sensitivity [24,25].

These methods can detect target bacterial cells rapidly, specifically, and sensitively compared with traditional agar plate cultivation methods, which are very commonly used for detecting bacteria. When using environmental samples or small volume samples, however, the traditional methods are problematic due to the low concentration of the target sample and time required for cultivation or sample concentration. Furthermore, sample preparation is required because impurities contained within the sample may inhibit detection.

Immunomagnetic separation (IMS) is an ideal tool for selectively isolating several bacterial species. IMS utilizes immunoreactions and magnetic force to isolate target bacteria from samples. In IMS, paramagnetic beads immobilize cells using a specific antibody to bind to the target cells, and the resulting magnetic complex can be easily separated by applying a magnetic field. Magnetic beads are now commercially available and are used in analytical chemistry and separation techniques because analytes can be rapidly purified by trapping the magnetic beads with a magnetic field [26,27].

Magnetic separation methods can be divided into two main classes: open-gradient magnetic separation (OGMS) and high-gradient magnetic separation (HGMS) [28,29]. In this study, we focused on HGMS, which can collect and separate magnetic beads by strengthening the magnetic force by increasing the magnetic field gradient. To increase the magnetic force, it is necessary to either increase the volume of the magnetic beads or strengthen the magnetic gradient. When magnetic materials are placed near the magnet, the magnetic flux density on the magnetic materials increases. Therefore, a magnetic gradient arises between the magnetic materials and surrounding area.

In the HGMS system, magnetic beads are deposited on a magnetic filter magnetized by an external magnet. Accumulated beads are released from the filter by removing the external magnetic force. The magnetic filter does not clog easily because the pore size of the magnetic filter is large compared with that of a membrane filter used for trapping bacteria. Therefore, it is not necessary to use high pressure to maintain sample flow. Furthermore, the filter can be washed and reused. HGMS can be used to separate weakly magnetized materials and fine particles because it generates a higher magnetic force than OGMS. This method improves the detection sensitivity 100 to 1000-fold compared with the conventional FCM method [30,31]. It is necessary, however, to collect cells effectively to obtain the higher sensitivity.

Here, we evaluated the use of high-gradient immunomagnetic separation (HGIMS) system to produce concentrated samples, and performed highly sensitive detection. For the application of magnetic separation methods, we studied the detection of *E. ictaluri* using an HGIMS under optimal experimental conditions measured by FCM. On the other hand, because the PCR method is widely used for detecting *E. ictaluri*, it can be used in combination with HGIMS to develop a highly sensitive detection method. We describe the following procedures relevant to the HGIMS and PCR techniques: (1) establishment of the optimum conditions for HGIMS, (2) detection of *E. ictaluri* cells using HGIMS and PCR, and (3) application of our proposed method for monitoring environmental water samples. The goal of this method was to enhance the sensitivity of bacterial detection using the combination of HGIMS and PCR compared with conventional methods like FCM or PCR.

2. Material and methods

2.1. Reagents

Anhydrous ferrous chloride and anhydrous ferric chloride were obtained from Strem Chemicals (Newburyport, MA, USA) and Wako Pure Chemical Industries Ltd. (Osaka, Japan) respectively. Rabbit anti-*Edwardsiella ictaluri Y1112* polyclonal serum was obtained from the Kitayama Labs Co., Ltd. (Nagano, Japan). Dextran from *Leuconostoc* spp. (Mr ~40,000) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA). Other chemicals were obtained commercially and were of analytical reagent grade.

2.2. Bacteria and growth conditions

In this study, we used the following strains: *Edwardshiella ictaluri* Y1112 was grown at 25 °C for 2 days in a modified tryptic soy culture medium; *E. hoshinae* NBRC 105699 was grown at 25 °C for 1 day in a modified nutrient broth with 0.5% NaCl culture medium; *Flavobacterium psychrophilum* SG 011227 was grown at 25 °C for 3 days in a modified altered *cytophaga* culture medium; *F. branchiophilum* FPC 520 was grown at 25 °C for 3 days in a modified altered *cytophaga* culture medium; *Lactococcus garvieae* H 9507was grown at 25 °C for 1 day in a modified BHI culture medium; *Escherichia coli* ATCC 25922 was grown at 25 °C for 2 days in a modified LB culture medium; and *Vibrio* sp. was grown at 25 °C for 1 day in a modified BPG culture medium. After the incubation, all the bacteria were killed by 0.3% formalin at 4 °C for 24 h. Before the experiment, all the bacteria were centrifuged (10,000 rpm, 6000 × *g*, 3 min) twice, washed with washing buffer (pH 7.2, 3.0% bovine serum albumin,0.5% Triton® X-100 containing phosphate buffer), and stored in phosphate buffer (pH 7.2, 3.0% bovine serum albumin).

2.3. Primer design

The *E. ictaluri*-specific primers: Edi-F (5′-CAGATGAGCGGATTTCA CAG-3′) and Edi-R (5′-CGCGCAATTAACATAGAGCC-3′) described by Sakai et al. [32]. were used for specific amplification of the gene.

2.4. Preparation of the magnetic antibody beads

Ferromagnetic iron beads were prepared by reacting a mixture of