Development of a sandwich ELISA for the detection of *Listeria* spp. using specific flagella antibodies

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Five monoclonal antibodies (MAbs) and chicken immunoglobulin (IgY) were developed by immunizing with flagella purified from *Listeria monocytogenes* 4b and the five MAbs have been confirmed to be specific against three different epitopes of flagellin. The antibodies showed specific reaction to *Listeria* genus and no cross-reactivity with other bacteria tested in this experiment including *E.coli* O157:H7 and *Salmonella enteritidis*. Sandwich enzyme-linked immunosorbent assays (ELISA) using the MAbs and IgY were developed to detect *Listeria* species and the sensitivity and specificity of the developed ELISA have been analyzed. The detection limit of ELISA using MAb 2B1 and HRP labeled IgY was $1 \times 10^5$ cells/0.1 ml at 22°C and $1 \times 10^6$ cells/0.1 ml at 30°C. ELISA using the pair of MAbs (MAbs 2B1 and HRP labeled MAbs 7A3) detected up to 10⁶ cells/0.1 ml at 22°C and 30°C. Detection limit of sandwich ELISA using IgY was 10 times lower than MAb pair. Using the developed ELISA, we could detect several *Listeria* contaminated in food samples after 48 h-culturing. In conclusion, both MAbs and IgY have been proved to be highly specific to detect *Listeria* flagella and the developed sandwich ELISA using these antibodies would be useful tool for screening *Listeria* spp. in food.

Key words: *Listeria* spp., sandwich ELISA, flagella, monoclonal antibody, IgY

Introduction

The genus *Listeria* is a rod shaped gram-positive bacterium and the species *Listeria monocytogenes* has been associated with human listeriosis. Contaminated products with *Listeria*, such as fish, shellfish, vegetables, milk, dairy products and meat can be the source for human listeriosis [7]. In Korea, *L. monocytogenes* has been isolated from a variety of foods. No outbreak associated with these contaminated foods has been reported yet. Contaminated ready-to-eat foods, however, have public risk in the transmission of *L. monocytogenes*, because *L. monocytogenes* could grow at refrigeration temperature [14].

Methods to detect *Listeria* based on the enrichment/plating approach have been described by the Food and Drug Administration (FDA), U. S. Department of Agriculture (USDA) and the International Organization for Standardization (ISO) in the United States, and the Association Française de Normalisation (AFNOR) in Europe [5,11]. Although the analysis time required for these methods is shorter than that of cold enrichment, performing these methods is still time consuming and laborious for routine applications. Therefore, rapid detection methods have been developed based on immunoassay. Commercially available methods are varied for identification of *Listeria* including, VIDAS (BioMérieux, France), Listeria Tek (Organon Teknika, USA), Listeria VIP (BioControl, USA), Listeretest (Vicam, USA), Pathatrix (Matix Microscience, UK) etc. Some of them have been adopted by the Official Method of Analysis (AOAC).

The enzyme-linked immunosorbent assay (ELISA) is more reliable mass-screening methods to detect *Listeria* in food. Only presumptive positive results by ELISA need to perform PCR based method or culture confirmation. In this study, we generated flagella specific antibodies and developed the sandwich ELISA for the screening of *Listeria* spp.

Strains of *Listeria* are subdivided by serotyping based on flagella and somatic antigens. Five flagella antigens (A, B, C, D and E) combined into four flagella antigen types (AB, ABC, BD, E) have been identified. *L. monocytogenes* and
other Listeria spp. are classified to A, B, C, or D type flagella antigen; but L. grayi only have the E type flagella antigen [1]. According to the report by Vatanyoopaisarn et al. [22], attachment of L. monocytogenes to stainless steel and presumably meat surfaces is related to flagella which could expressed only between 20°C and 25°C. This temperature-dependent motility of L. monocytogenes is attributable to the possession of peritrichous flagella, which may indicate flagella specific antibodies are good probe for detecting Listeria. The objective in this study is to generate the monoclonal antibodies (MAbs) and IgY against flagella of Listeria monocytogenes 4b, and to compare the sensitivity and specificity of the sandwich ELISA for screening Listeria spp. using different sets of flagella specific antibody combinations.

Materials and Methods

Bacteria
The 13 species of Listeria and the 10 strains of non-Listeria organism were used in this study. The 13 species of Listeria included L. monocytogenes 1/2a (HPB 410), L. monocytogenes 1/2b (HPB 503), L. monocytogenes 1/2c (HPB 12), L. monocytogenes 3a (ATCC 19113), L. monocytogenes 4a (ATCC 19114), L. monocytogenes 4b (ATCC 19115), L. monocytogenes 4c (ATCC 19118), L. monocytogenes 4d (ATCC 19117), L. grayi (ATCC 19120), L. innocua (ATCC 33090), L. ivanovii (ATCC 19119), L. seeligeri (ATCC 35967), L. welshimeri (ATCC 35897). These bacteria were grown in tryptic soy broth supplemented with 0.2% glucose and 0.6% yeast extract (TSB, Difco, USA) at 22°C for 48 h, and the concentration of each cultured bacterium was determined by plating culture sample on tryptic soy agar supplemented with 0.2% glucose and 0.6% yeast extract and incubating at 37°C for 24-48 h.

As non-Listeria organism, Salmonella enteritidis, S. typhimurium, Escherichia coli K88ab, E.coli O157:H7, Pseudomonas aeruginosa, Streptococcus mastitis strain 1, Streptococcus mastitis strain 2, Rhodococcus spp., Staphylococcus aureus 1 and 2, Bacillus subtilis, all of which were isolated from animals in Jeju island, were inoculated into 10ml of Brain heart infusion (BHI, Difco, USA) broth for 24-48 h at 37°C.

Preparation of flagella
L. monocytogenes 4b (ATCC19115) was grown in TSB at 22°C for 24 h to the stationary phase of growth [16]. Flagella were isolated by following procedures described by Peel et al. [16]. Briefly, the bacteria were harvested by centrifugation at 7,000 rpm for 20 min and washed three times with phosphate-buffered saline, pH 7.2 (PBS). Glass beads (2 mm diameter) were used for detaching flagella by vigorous shaking for 30 min. Then, this suspension was centrifuged at 7,000 rpm for 30 min. After centrifugation at 14,000 g for 40 min of the supernatant from previous centrifugation, the flagella of pellet were resuspended in PBS and stored at -20°C. The protein concentrations were determined using protein assay kit (Bio-Rad, USA). Purified flagella analyzed by electrophoresis in 12% polyacrylamide gel.

Production of monoclonal antibodies
Monoclonal antibodies to flagellin were produced by immunizing BALB/c mice against 50 µg/ml of flagella and fusing equal numbers of splenic lymphocytes and murine myeloma SP2/0 cells as previously described by Kohler and Milstein [12]. MAbs-secreting hybridomas were screened by ELISA and cloned. The ascites containing MABs were collected, centrifuged (5,000 rpm, 10 min) and stored at −20°C until use.

Production of IgY
Each three-group of three general laying hens (24 weeks old) was initially injected intramuscularly in the breast region with 400 µg, 200 µg and 50 µg of purified flagella emulsified with an equal volume of complete Freund’s adjuvant. After two weeks, the hens were boosted using an incomplete adjuvant at biweekly intervals. Seven days after the first injection, eggs were collected daily for 4 months. The eggs stored at 4°C until checking the titer of antibody by ELISA. IgY was extracted using polyethylene glycol (PEG) 6000 as previously described [10].

Purification of antibodies by affinity chromatography and preparation of horse-radish peroxidase (HRP)-labelled antibodies
Antibodies were purified by an affinity chromatography using flagellin-sepharose 4B beads. Briefly, three grams of CNBr-activated sepharose 4B (Pharmacia, Sweden) were conjugated with flagella (1.5 mg/mL). These gels were packed into column (10 cm) and washed with PBS several times. Ascites and crude IgY were flowed (1 ml/min) into column, washed with PBS and eluted by 3 M NaSCN. Purified antibodies were dialysed two times with PBS. The detector antibodies were labeled with HRP (Sigma, USA) by periodate method described by Wilson and Nakane [25].

Selection for MAB pair for a sandwich ELISA
Each monoclonal antibody (MAB) was diluted in carbonate buffer (pH 9.6) to the concentration of 10 µg/ml and 100 µg of diluted MAB was added into ELISA plate (Costar, Netherlands). The plate was incubated for 2 h at 37°C and overnight at 4°C. The wells were blocked with PBS containing 3% Bovine serum albumin (BSA; Sigma, USA) for 30 min at 4°C. After washing three times with PBS, 100 µl of flagella (100 µg/ml) diluted with PBS containing 0.05% Tween-20 (PBS-T) was added and incubated for 30 min at room temperature (RT). After