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

Seong-Hee Kim[1], Min-Keun Park[2], Jin-Young Kim[2], Pham Duc Chuong[3], Yong-Soon Lee[4], Byoung-Su Yoon[5], Kyu-Kye Hwang[2], Yoon- Kyu Lim[2,*]

[1]*National Veterinary Research and Quarantine Service, Ministry of Agriculture and Forestry, Anyang 430-824, Korea*
[2]*Department of Veterinary Medicine, Cheju National University, Jeju 690-756, Korea*
[3]*Faculty of Animal Science and Veterinary Medicine, Thainguyen University of Agriculture and Forestry, Thainguyen, Vietnam*
[4]*Department of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[5]*Department of Biology, College of Natural Science, Kyonggi University, Suwon 442-760, Korea*

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 1×10^5 cells/0.1 ml at 22ºC and 1×10^6 cells/0.1 ml at 30ºC. ELISA using the pair of MAbs (MAbs 2B1 and HRP labeled MAbs 7A3) detected up to 10^4 cells/0.1 ml at 22ºC and 30ºC. 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), Listertest (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

*Corresponding author
Tel: 82-64-754-3367; Fax: 82-64-756-3354
E-mail: yklim@cheju.ac.kr

other *Listeri*a 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 aerugenosa*, *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 shacking 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 by 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

washing, 100 µl of each HRP conjugated MAb was added into each well and incubation for 30 min at RT. After adding ABTS (KPL, USA) and 30 min's incubation at RT, absorbance was measured at 405 nm.

Sandwich ELISA

The sandwich ELISA was performed on microtiter plates. Each well was coated with purified MAb 2B1 in 0.05 M carbonate buffer (pH 9.5) by incubating at 37°C for 1 h and at 4°C for overnight and the wells were washed three times with PBS. Heat-inactivated (100v for 20 min) 13 species of *Listeria* and 10 non-*Listeria* strains were tested after 48 h incubation in TSB at 22°C and 30°C, respectively. A 100 µl aliquot of each sample was added into wells coated with MAb 2B1. The plates were incubated for 30 min at RT and washed three times with PBS. One hundred ml of each optimally diluted MAb 7A3 and IgY labeled HRP was added into each well of the plates. The plates were incubated for 30 min at RT and washed three times with PBS. Antigen-antibody reaction was visualized by adding ABTS to each well and the absorbance was checked at 405 nm using ELISA plate reader (STL, Austria).

Detection of *L. monocyotogenes* 4b in foods

Pork was purchased from a local supermarket and cut into several pieces of 25 g each. *L. monocytogenes* 4b grown at 22°C for 48 h was diluted to 9-10 cells, 5-6 cells, and 1-2 cells/ml with TSB and inoculated into 25 g pork. These samples were enriched following the USDA method but second enrichment temperature changed 35°C to 30°C. Pasteurized milk (25 ml) was also artificially contaminated same as previously described with pork and enrichment was performed following the FDA method. The assays were carried out 27 samples among which 3 and 6 were contaminated at each concentration to pork and milk, respectively. These culture fluids were then analyzed using sandwich ELISA.

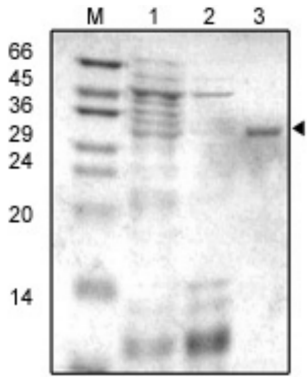

Fig. 1. SDS-PAGE analysis of *L. monocytogenes* 4b flagellin. The arrow indicates the position of 33 kDa flagellin. Lane 1, whole cell; Lane 2, after glass beads treatmant; Lane 3, purified flagellin. About 10 µg of proteins were loaded in each lane.

Results

Preparation of flagella antigen

L. monocytogenes 4b grown at 22°C and 37°C was observed by scanning electron microscopy (Hitachi 2460N, Japan). Many flagella were seen on cells cultured at 22°C but no flagella could be seen on 37°C culture sample as described in text book (data not shown). The flagellin was shown at 33 kDa by 12% SDS-PAGE (Fig. 1).

MAbs against flagella antigen

Using this flagella, MAb 2B1, 3B7, 4F12, 6F3 and 7A3 were produced after two fusions. They reacted with flagella without cross-reaction to any other bacteria used in this study by ELISA (Table 1). Flagella epitopes were characterized based upon ELISA (Table 2). We could detect at least three different epitopes of the flagella and classify MAbs into three groups by specificity against epitopes [MAb 2B1 (I), MAb 4F12 (II) and MAbs 3B7, 6F3, 7A3 (III)]. Three MAbs in third group (3B7, 6F3 and 7A3)

Table 1. Specificity analysis of antibodies (Abs) by ELISA with bacterial species

Bacterial strains	Monoclonal Abs					Polyclonal Abs	
	2B1	3B7	4F12	6F3	7A3	IgY	Rb
Listeria spp.	+	+	+	+	+	+	+
Salmonella enteritidis	-	-	-	-	-	-	-
Salmonella typhimurium	-	-	-	-	-	-	-
E. coli K88ab	-	-	-	-	-	-	-
Pseudomonas aerugenosa	-	-	-	-	-	-	-
E. coli O157:H7	-	-	-	-	-	-	-
Streptococcus mastitis strain 1	-	-	-	-	-	-	-
Streptococcus mastitis strain 2	-	-	-	-	-	-	-
Rhodococcus spp.	-	-	-	-	-	-	-
Staphylcoccus aureus 1	-	-	-	-	-	-	-
Staphylcoccus aureus 2	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-

Table 2. Sensitivity of the sandwich ELISA using different pairs of monoclonal antibodies

Capture Ab.	Detector Ab. (MAbs-HRP)				
	2B1	3B7	6F3	7A3	4F3
2B1	-	++	++	++	++
3B7	+	-	-	-	++
6F3	+	-	-	-	++
7A3	+	-	-	-	++
4F12	+	++	+	++	-

-: negative (OD<0.1), +: positive (0.1<OD<1.0), ++: strong positive (1.0<OD).

produced weak OD (< 0.1) in ELISA when they were used detector and capture antibodies, which means they recognize same epitope. When MAbs in three groups were used for capture or detector antibodies, we could get high OD in ELISA.

IgY against flagella antigen

ELISA was used to monitor the development of the antibody level using the yolk diluted 1,000 times with PBS-T. The yolk antibody also showed no cross reaction to any other bacteria (Table 1), and the antibody levels of all groups increased on 30 days after the first immunization. Group 2 (200 μg) showed the antibody titers comparatively higher than those of the other two groups (data not shown).

The preliminary extraction of immunoglobulin Y from egg yolk was performed to remove the large amount of lipoprotein and phosvitin granules [24]. To obtain the first crude extract, PEG 6000 was used. Then, crude extract of IgY (60 mg/ml) was loaded onto the CNBr-activated 4B-flagella column at a flow rate of 1 ml/min. Bound IgY was eluted by chaotropic salt and immediately dialyzed with PBS. The recovered IgY checked at 280 nm was close to 1 mg/ml. As shown in Fig. 2, IgY showed a very high purity and did not react with other bacteria with high specificity to flagella by ELISA (Table 1).

Development of sandwich ELISA

Among MAbs, MAb 7A3 and MAb 2B1 were shown to be good for not only recognizing distinct epitopes on flagella, but also producing enough quantity in ascites, and selected for developing a sandwich ELISA. The subclass of the MAb 2B1 and MAb 7A3 were identified as IgG_1 and IgG_{2a}, respectively, with kappa light chain.

The MAb 7A3 and IgY were used for detector antibodies. The pairs consisted of the combination of MAb 2B1 coated on microtiter plated wells and HRP labeled MAb 7A3 (Pair 1) or HRP labeled IgY (Pair 2). *L. monocytogenes* 4b was cultured at 22°C or 30°C for 24h and these cultures were diluted from 10^8 to 1 cells/ml in TSB. From these tubes 0.1-1ml were calculated the number of *L. monocytogenes* by plate count method and applied to sandwich ELISA. The

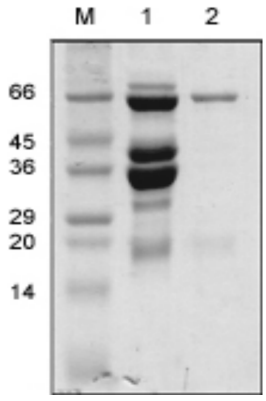

Fig. 2. SDS-PAGE analysis of IgY purified from immunized egg yolk. M: Low molecular marker, Lane 1, Crude yolk extract (25 μg of protein was loaded); Lane 2, IgY fraction of affinity chromatography (15 μg of protein was loaded).

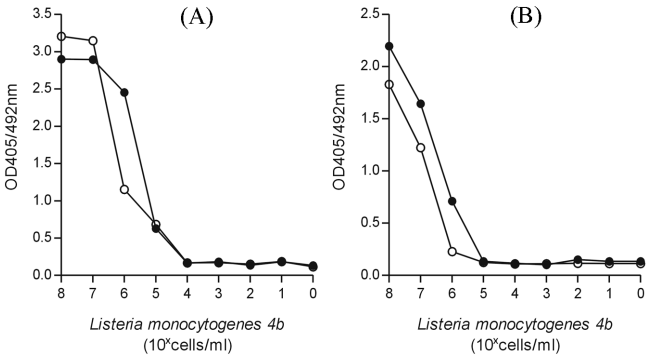

Fig. 3. Detection of *Listeria monocytogenes* 4b (ATCC 19115) with sandwich ELISA. The cell was cultured in TSB at 22°C (●) and 30°C (○). A, Pair1, the combination of HRP labeled MAb7A3 and MAb 2B1 coated on microplate wells; B, Pair2, the combination of HRP labeled IgY and MAb 2B1 coated on microplate wells.

detection limit of the assay using Pair 1 was $1°ø10^4$ cells/0.1ml for both samples from cultures at 22°C and 30°C (Fig. 3). Using Pair 2, the detection limit was 1×10^5 cells/0.1 ml at 22°C and 1×10^6 cells/0.1 ml at 30°C. Pair 1 was more sensitive than Pair 2 in detecting *L. monocytogenes*.

Detection of *L. monocytogenes* 4b in foods

Pork and milk artificially contaminated low number 9-10, 5-6, 1-2 and 0 cells/ml of *L. monocytogenes* 4b culture were detected by the sandwich ELISA using Pair1 (Table 3). We calculated negative results according to the following formula, negative $OD_{average} + 0.2$. Indices of ≤ 0.4 were considered negative. The culture samples were considered positive if its OD was higher than negative index.

Discussion

The flagella filament is composed of one or two repeating

Table 3. Detection of spiked *L. monocytogenes* 4b by sandwich ELISA

Type of Product	Contamination levels of *L. monocytogenes* 4b (CFU/ml)							
	0		1-2		5-6		9-10	
Pork	0/3*	0/3	3/3	3/3	3/3	3/3	3/3	3/3
Milk	0/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6

* : No. of positive samples/ No. of tested samples.

linear arrays of protein subunits (flagellin) with carbohydrate and lipid (1-5%) [15]. In order to make specific flagella antibodies, flagella were purified using by glass beads followed by ultracentrifugation according to Peel *et al.* [17]. The molecular weight of flagellin is 33 kDa with SDS-PAGE anlaysis in this study which is same as reported by Dons *et al*. [6], but differ from 29 kDa determined by Peel *et al*. [16]. Dones *et al.* [6] pointed that it may be caused by post-translation modification, or by degradation of protein.

Using these flagella, two kinds of antibodies, MAb and IgY, were produced. Farber and Speirs [8] and Skjerve *et al*. [18] reported the use of flagella specific MAbs in an immunomagnetic separation technique and dot EIA to detect *Listeria* species in foods. They showed the flagella specific MAbs could be used as detection probe.

Three groups of flagella specific MAbs, MAb 2B1 (I), MAb 4F12 (II) and MAbs 3B7, 6F3, 7A3 (III) were generated. The third group of MAbs, 3B7, 6F3 and 7A3 showed lower signal in sandwich ELISA when they were used as capture and detector antibodies, which indicated they competed for the same epitope. When we used capture and detector antibodies from different group, we could get strong signal in ELISA, and it is obvious that the epitopes recognized by these three groups of MAbs are different. Therefore, we selected detector and capture antibodies from different group of MAbs to establish sandwich ELISA.

The levels of IgY in egg yolk were not steady during whole period of monitoring, which was also reported by Leuw *et al*. [13]. The fluctuation of IgY level is quite different from the steady state level of immunoglobulins in blood, and might be normal pattern in immunized egg. 200 μg of antigen was enough to generate high titer of yolk antibody compared with 50 μg and 400 μg of antigen.

IgY can be purified from egg yolk with a combination of chromatography and precipitation, such as polyethylene glycol, ammonium sulfate, chloroform, ultrafiltration, and gel filtration [2,3,10,19]. In this study, we used PEG for precipitation of lipids and proteins in egg yolk, and CNBr-activated Sepharose 4B-flagella column for purification of IgY. The yield of IgY was low (1 mg/ml) that could be due to low column capacity compare to other kinds of affinity columns, TG19318/Emphaze column and T-gel column [9, 24]. By analyzing purified IgY with SDS-PAGE, we found that the band of IgY light chain was weaker than that of IgY heavy chain, which pattern was previously reported by Hansen et al [9]. The light chain of IgY seemed to be stained poorly by Coomassie dye. A number of previous studies have been reported that hen egg yolk antibody has many advantages over antibodies from serum [20]. Immunized egg can supply abundant antibody (IgY) that may substitute for IgG from mammals, which led us to use IgY in establishing ELISA in this study.

Because the expression of flagella used in immunization in this study is dependent on temperature, we compared sensitivity of sandwich ELISA on culture at 22°C and 30°C. The sensitivity of ELISA using IgY as detector antibody was 10 times lower than that of ELISA using two MAbs at 22°C culture. In contrast to this result, Vejaratpimol *et al*. [23] reported that they got better sensitivity by using of IgY as detecting antibody than MAb. This result may due to different specificities and affinities of antibodies.

Compared to commercially available ELISA method that has nearly same sensitivity as 5×10^4 to 10^5 bacteria ml^{-1} [4], the detection limit of the sandwich ELISA using Pair1 antibodies, the combination of HRP labeled MAb7A3 and MAb 2B1 coated on microplate wells, was 10^4 cells/0.1 ml at both 22°C and 30°C. It can be concluded that the sensitivity is not affected by culture temperature compare to Pair 2 antibodies. Twenty-five-gram or ml portions of pork and milk were artificially contaminated. Second enrichment of spiked pork samples were performed at 30°C because of flagella specific MAb. As described above, because *Listeria* contaminate in foods very low levels and can grow refrigerate temperature, 1-10 cells/ml of *L. monocytogenes* 4b were contaminated to two foods. This developed sandwich ELISA showed positive all spiked samples.

Considering the specific reaction of MAbs and IgY with flagella, both antibodies were useful probe for detecting *Listeria* spp. Although all strains of *Listeria* may not be detected with equal sensitivity [21], 48h of culture was sufficient to obtain *Listeria* for the detection by ELISA developed by us, and this sandwich ELISA can be used to screen *L. monocytogenes* as well as other *Listeria* spp. contaminated in foods. Therefore, enzyme-immunoassay described in this paper would be quick, specific and simple enumeration procedure over traditional culture method. Furthermore, although only *L. monocytogenes* is pathogen for human, the screening *Listeria* spp. will be indicated poor hygiene.

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