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Development of the Rapid Immunoassay Methods for the Screening of Listerial Contamination

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Abstract

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Listeria is one of the most important food poisoning bacteria in the field of public health, primarily due to its pathogenic effects in humans. In severe cases, *Listeria* has been known to cause meningitis, septicemia, and abortions in pregnant women. The initial step in the development of a method for the rapid screening of *Listeria* food contamination involved the production of antibodies based on flagella antigen. *L. monocytogenes* 4b was cultured at 22℃ for 48 hours, and flagella were separated from the cultured cells via mechanical disruption with glass beads, followed by ultracentrifugation steps. The molecular weight of flagellin was approximately 33 kDa, according to the results of SDS-PAGE. The isolation of the flagella was also confirmed by electron microscopy. MAbs, IgY, and rabbit polyclonal antibodies against *Listeria monocytogenes* 4b were then developed via immunization with the purified listerial

flagella. Five monoclonal antibodies (2B1, 3B7, 4F12, 6F3, 7A3) were produced and characterized after a series of fusions. The epitopes of listerial flagellin for the five MAbs were found in three different sites, using sandwich ELISA.

In the case of IgY, 50 μg, 200 μg, and 400 μg of flagella were injected into the breasts of hens at two-week intervals. The 200 μg of flagella induced a high positive response in the immunized hens other than two groups. Anti-*Listeria* IgY was then purified via polyethylene glycol and affinity chromatography. Finally, we obtained and purified flagella-specific rabbit polyclonal antibody. All three-antibody variants exhibited specific reactions with the genus *Listeria,* but no cross-reactivity with other bacteria.

In order to detect *Listeria* spp. in food, a rapid immunochromatographic assay (ICA) and sandwich enzyme-linked immunosorbent assay (ELISA), based on the three antibody variants were developed. These antibodies were then compared to one another. In pursuit of this sandwich ELISA, the detection antibodies-MAb7A3, IgY, and rabbit polyclonal antibody-were conjugated with HRP, according to the periodate method. Sandwich ELISA trials were performed using Pair1, involving HRP-labeled MAb7A3, Pair2, involving HRP-labeled IgY, Pair3, involving HRP-labeled rabbit polyclonal antibody, all combined with MAb2B1, and coated onto microplate wells. In these tests, Pair2 was observed to exhibit a sensitivity 10 times lower than Pair1 and Pair3, in culture temperatures of both 22℃ and 30℃. Pair1 exhibited a sensitivity of 10^4 cells

per 0.1 ml at both temperatures. Pair3 exhibited sensitivities of 10^4 cells and 10^3 cells per 0.1 ml, at 22℃ and 30℃.

An ICA kit was then produced, based on the above results. A 40 nm gold colloid was constructed using 1% sodium citrate, and then confirmed by TEM. Detection antibodies, MAb, and rabbit polyclonal antibody were all labeled with this gold colloid, and then soaked in a glass membrane. The ICA kits were compared with pure cultures at temperatures of 22℃ and 30℃. *L. monocytogenes* 4b with Pair1 evidenced a detection limit of 10^5 cells/0.1 ml, and 10^6 cells/0.1 ml at 22℃ and 30℃. Pair3 evidenced a detection limit of 10^5 cells/0.1 ml at 22℃, but demonstrated a weak positive at 30℃. Pair1 exhibited a high degree of sensitivity, and an ICA was used with all 13 *Listeria* strains, and appeared on the positive line. These analyses were performed on both pork and pasteurized milk, both of which had been artificially contaminated with low levels of *L. monocytogenes* 4b culture. All 27 samples tested positive on both assays. Although all strains were not apparently detectable with equal sensitivity, an augmented culture duration of about 48 hours is sufficient to allow the appearance of detectable titers of *Listeria* strains. This result indicated that both assays were equally adequate for the detection of *Listeria* spp. in food. However, the ICA kit facilitated the more rapid detection of *Listeria* contamination, with an elapsed time of 15 minutes, as opposed to the 2 hours required for the sandwich ELISA assay.

As hybridomas exhibit chromosomal instability, a decrease in secreted antibodies tends

to result. In order to circumvent this limitation, DNA sequences of antibody variable regions from hybridomas cell were amplified and cloned. These products were then applied to immunochromatography, for use as a probe. Recombinant single-chain Fv antibody against flagella, representing a covalent linkage of the V_H domain and V_L domain through a peptide linker, were then constructed using the 7A3 and 2B1 hybridoma cell lines, and expressed in insect cells and *Escherichia coli*. Among the scFvs, only the eukaryotic fragment, Bac scFv 7A3 [V_H-$(Gly_4Ser)_3$-V_L], evidenced a specificity for flagella on immunoassay, FA, and Western blotting. The scFv 7A3 sequence exhibited 95% homology to germ line sequences, indicating that the V_H and V_L regions belonged to the J558-19 and 12-44 germ lines, respectively. The affinity of Bac scFv 7A3 for flagella was found to be 1/100 that of the intact monoclonal antibody. This Bac scFv 7A3 was tested for efficacy as a detecting probe in ICA. The coupling of the gold-conjugated Bac scFv 7A3 with MAb2B1 exhibited a detection limit of 30 μg /ml flagella. In this study, we demonstrated that scFv can be used as a detection antibody in the production of ICA.

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LIST OF ABBREVIATIONS

BHI	Brain heart infusion
CDR	Complementarity determining region
CNBr	Cyanogen bromide
ELISA	Enzyme-linked immunosorbent assay
FR	Framework region
ICA	Immunochromatographic assay
IgY	Yolk immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
LEB	*Listeria* enrichment broth
L. monocytogenes	*Listeria monocytogenes*
MAb	Monoclonal antibody
MOI	Multiplicity of infection
scFv	Single-chain Fv fragment
SEM	Scanning electron microscopy
Sf9	A cell line originated from *Spodoptera frugipderda*
TEM	Transmissible electron microscopy
V_H	Variable heavy chain
V_L	Variable light chain

General introduction

1. Characterization of the *Listeria* genus

The genus *Listeria* belongs to the *Clostridium* subbranch, and includes six species: *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi.* The *L. murrayi* species is now included in the species *L. grayi* (Rocourt *et al*, 1992). Members of the *Listeria* genus are gram-positive, facultative anaerobic bacilli. They multiply at a temperature range between 1 and 45℃. All *Listeria* species are immobile when grown at 37℃, but they exhibit mobility when cultured at a temperature range between 20 and 25℃. This phenomenon is attributed to a ciliature which is composed of several peritrichous flagella (Peel *et al*., 1988A). Furthermore, *Listeria* strains are classified according to serotype, based on their flagella and somatic antigens. Although there are 13 *L. monocytogenes* serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7, only *L. monocytogenes* (1/2a, 1/2b, 4b) is a pathogen in both humans and animals (Schuchat *et al*., 1991). *L. ivanovii* is primarily responsible for abortions in sheep. The clinical manifestations of *Listeriosis* include mild flu-like symptoms, gastroenteritis, septicemia, central nervous infections (meningitis), and abortions. The population at highest risk for this affliction includes persons in whom T-cell mediated immunity has been impaired, such as pregnant women, newborns, the elderly (over 65 years), and immunocompromised patients (Hof, 2003). In such

cases, listeriosis is associated with a 20-30% fatality rate (Allerberger, 2003).

2. Pathogenesis of *Listeria monocytogenes*

L. monocytogenes can infect both epithelial and macrophage-like cells (monocytes, macrophages, or polymorphonuclear leucocytes) (Mounier *et al*., 1990). The mechanism underlying the cellular attachment of *L. monocytogenes* to non-phagocytic cells has been attributed to internalin in *L. monocytogenes*, which is able to bind to E-cadherin, the eukaryotic receptor found in adherens-type junctions (Luo *et al.*, 2003). Subsequent to phagocytosis, the vacuoles in which the pathogens first appear are lysed, at which time *L. monocytogenes* escapes into the cytoplasm and replicates. A halo of host actin filaments surrounds the pathogen, later relocating to form a polar tail. These formations generate the propulsive force which pushes *Listeria* organisms through the cytoplasm of the host cells. When the bacterium reaches the surface of the infected host cell, it forms pseudopods, and is then taken up by neighboring cells (Kuhn and Gebel, 1999).

The products of virulence genes are the positive regulator (encoded by *prf*A), a phosphatidylinositol-specific phospholipase C (encoded by *plc*A), listeriolysin (encoded by *hly*), a metalloprotease (encoded by *mpl*), a protein associated with actin polymerization (encoded by *act*A), and a phosphatidylcholine-specific phospholipase C (encoded by *plc*B). Other genes include *inl*A, *inl*B, *inl*C and the *iap* gene, which code for internalin (InlA), InlB, InlC and protein 60, respectively.

*lma*A also encodes a protein capable of inducing delayed-type hypersensitivity reactions in *L. monocytogenes*-immune mice, *lmsod* coding for superoxide dismutase, and *cat* coding for catalase (Gohmann *et al*., 1990, Gedde *et al*., 2000, Kuhn and Goebel, 2001).

3. Epidemiology

Members of the genus *Listeria* can be isolated from samples of soil, sewage, water, silage, decaying vegetation, and animal fecal materials (Fenlon, 1999). It has been suggested that these bacteria are saprophytic, and can survive and thrive under a variety of adverse conditions, including a vast range of pH (4.3 to 9), temperatures (-0.4-50℃), and salt concentrations (10%) (Cole *et al*, 1990, Farber and Peterkin, 1991). Therefore, the bacteria maintain a series of options with which they can survive and contaminate food for long periods of time. Outbreaks of listeriosis are primarily reported in advanced countries, with different consumption patterns and food processing protocols than developing countries (Rocourt *et al*., 2003). Contaminated ready-to-eat (RTE) foods, including soft cheese, cooked and chilled poultry products, sausage, and seafoods carry a high measure of risk for the public transmission of *L. monocytogenes.* However, non-foodborne *Listeria* transmissions have also been reported, albeit rarely (McLauchlin,1996, Rocourt *et al*., 2000).

No outbreaks associated with *Listeria*-contaminated food have yet been reported in Korea. In the USA, however, during a 3-month period in 2002, 46

culture-confirmed cases, including seven deaths and three stillbirths or miscarriages caused by *L. monocytogenes* infections, have been linked to food consumption, and were associated with such products as sliceable turkey deli meat (Philadelphia Dept.of Public Health *et al*, 2002).

4. Control of *Listeria* in Food

Many countries have established different legal limits with regard to the amount of *Listeria* allowed in foods, as the infective dose has yet to be delineated. The United States, Denmark, Italy, Australia, and Korea all have imposed rigid legal policies. All of these countries recall products when *L. monocytogenes* exceeds one cell in 25 g. Moreover, the European Community Directive on milk and milk-based products establishes a zero-tolerance policy on the organism. As described above, as the minimum infective dose of *L. monocytogenes* has not yet been determined, France, Germany, and the Netherlands accept levels up to 100 cfu/g. In the United Kingdom, the guidelines for ready-to-eat foods differentiate between four different classifications based on the number of found *L. monocytogenes:* of the complete absence of *L. monocytogenes* in 25 g of material is satisfactory, <100 cfu/g is fairly satisfactory, 10^2-10^3 cfu/g is unsatisfactory, and >1000 cfu/g is unacceptable (Axelsson and Sorin, 1998).

5. Isolation and detection of *Listeria*

The most reliable method for the detection of *Listeria* is known as the cold

enrichment technique (Gray *et al*, 1948). However, this method is limited by virtue of its long incubation time (up to several months). More advanced techniques, which utilize a variety of selective agents in the enrichment broth and agar, have been developed. Widely-used detection methods based on enrichment/plating have been adopted and recommended 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 by the Association FranÇaise de Normalisation (AFNOR) in Europe. The USDA method is generally suitable for the detection of *Listeria* in meat products, and the FDA method is generally acceptable for other food products. However, there is no universally accepted protocol at this time (Beumer and Hazeleger, 2003). These regulatory bodies use selective agents, including acriflavine, nalidixic acid, and cycloheximide, to allow for the recovery of any injured cells, and to surpress other bacteria in enrichment and isolation media. However, these media were unable to distinguish *L. monocytogenes* from non-pathogenic *Listeria*. Therefore, traditional biochemical tests are required, including nitrate reduction, the fermentation of a range of sugars, the catalase test, the oxidase activity test, and CAMP (Christie, Atkins, Munch-Petersen) tests, as well as a series of other useful tests. Culturing on blood media tends to facilitate the separation of the haemolytic species from the non-haemolytic species. Commercially-available media, the Biosynth Chromogenic Medium (BCM) *L. monocytogenes* detection system, CHROM agar, and Agar Listera Ottavani & Agosti (ALOA) medium, all based on chromogenic

compounds, have also proven useful (Beumer and Hazeleger, 2003). ALOA medium, for example, which utilizes the chromogenic compound, X-glucoside, makes it possible to differentiate a colony of *L. monocytognes* (light blue color surrounded by an opaque halo) from a colony of other *Listeria* spp. (light blue or blue color). (Vlaemynck *et al*., 2000).

Although the time required for these methods is less than the time required for cold enrichment, all are too time-consuming and laborious for routine applications. Therefore, rapid detection methods have been developed, based on the polymerase chain reaction (PCR), and immunoassays. Various commercially-available methods for the identification of *Listeria* spp. exist, including the BAX system (Qualicon), the GENE-TRAK *Listeria monocytogenes* Assay (GENE-TRAK) based on DNA detection and VIDAS (bioMerieux Vitek), Listeria Tek (Organon Teknika), VIP for *Listeria* (BioControl), the Lister test (Vicam), and the Pathatrix (Matix Microscience Ltd) system based on immunoassay. Immunoassay-based methods include immunoprecipitation, ELISA, immunomagnetic separation, and enzyme-linked fluorescent assay (ELFA). Some of these have been adopted by the Association of Official Analytical Communities (AOAC) International.

The accurate identification of bacteria may also be achieved using polymerase chain reaction (PCR)-based methods. Researchers have also reported of the amplification of *L. monocytogenes* genes, including *iap*, *hly*A, *act*A, *plc*, *prf*A, *inl*A, *mpl*, and 16s rRNA (Cooray *et al*., 1994, Kang *et al*., 1997, Duffy *et al*., 1999, Scheu *et al*., 1999, Choi and Hong, 2002, Kaclikova *et al*., 2003).

Recently, real-time PCR using a non-specific fluorescent dye, SYBR Green Ⅰ, and TaqMan PCR using FAM and TAMRA, have been been added to the library of PCR-based methods. In real-time PCR protocol, additional agarose-gel electrophoresis to determine the molecular weight of the amplified products is normally not performed (Hein *et al*., 2001, Norton, 2002, Bhagwat, 2002).

Immunoassays, in large part, detect not only *Listeria* spp., but also *L. monocytogenes*. Many researchers have developed monoclonal antibodies exhibiting specificity for flagella or whole cells (Farber and Speris, 1987, Butman *et al*., 1988, Mattingly *et al*., 1988, Skjerve *et al*., 1990, Bhunia and Johnson, 1992, Kathariou *et al*., 1994, Loiseau *et al*., 1995). These developments revealed that MAbs would be good material for the development of an ELISA test.

Immunochromatography constitutes another rapid detection method, which is convenient and simple, and can achieve its results within 15 minutes of loading the sample into the device. This test is used extensively in the detection of a variety of antigens, including viruses (Sang *et al*., 1998, Esfandiari and Klingeborn, 2000), bacteria, parasites (Bhaskar *et al*, 1996), toxins (Shyu *et al*., 2002), and antibiotics (Watanabe *et al*., 2002). Commercial kits for *Listeria* detection include the Clearview kit (Oxoid) and Reveal for *Listeria* (Neogen). These methods involve the conjugation of latex with detection antibodies.

All of the previously described methods, both PCR and enzyme immunoassay-based, require prior cultural enrichment for the augmentation of the number of *Listeria* spp. in various foods. Therefore, investigators have largely

focused their efforts on the reduction of cultural enrichment time.

6. Aims of this study

Although PCR-based methods are quite accurate, these systems require a series of lengthy enrichment steps for the amplification of low pathogen numbers, as do all of the other detection methods. Thus, such methods are not suitable for large sample numbers, due to the time requirements associated with the additional steps, most notably for electrophoresis to determine the exact size of the amplified PCR product. Reagents for performing PCR are also relatively expensive.

The goal of this study, then, was to develop a specific, rapid, reliable, and convenient assay for the detection of *Listeria* spp. Although only *L. monocytogenes* is pathogenic in humans, the detection of *Listeria* spp. is generally indicative of poor hygiene, underlining the importance of broader-spectrum detection. Therefore, specific flagella antibodies, monoclonal antibodies (MAbs), yolk immunoglobulin (IgY), and rabbit polyclonal antibody, which reacts with genus-specific *Listeria*, were produced. Using these antibodies, a sandwich ELISA and immunochromatographic assay (ICA) were developed, for the screening of *Listeria* spp. in pure cultures and in foods. Furthermore, a single-chain Fv antibody was applied and used as a capture and detection antibody in the development of ICA protocol.

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Chapter I.

Production of flagella-specific antibodies

ABSTRACT

Expressed flagella were observed on cell surfaces via electron microscopy, when the bacteria were cultured at 22℃. The flagella of *L. monocytogenes* 4b were then purified from cultured cells by mechanical disruption with glass beads, followed by ultracentrifugation. The molecular weight of flagellin was measured to be approximately 33 kDa by SDS-PAGE. MAbs, IgY, and rabbit polyclonal antibodies evidenced responses to *Listeria monocytogenes* after immunization with purified listerial flagella. Five monoclonal antibodies (2B1, 3B7, 4F12, 6F3, 7A3) were characterized in these trials. Using these MAbs, sandwich ELISA revealed that the epitopes of listerial flagellin for the five MAbs were located in three different sites. Adequate immune response for IgY production was observed upon injection with 200 μg of *L. monocytogenes* flagella. Anti-*Listeria* IgY was purified via polyethylene glycol and affinity chromatography. Finally, the specific polyclonal antibody was obtained from immunized rabbits. All three antibody variants exhibited spcificity for the *Listeria* genus, and no evidenced no cross-reactivity with other bacteria tested in this experiment, including *E. coli* O157:H7 and *Salmonella enteritidis*. Therefore, those antibodies would appear to be serviceable probes for use in immunoassays to detect *Listeria* in food products.

INTRODUCTION

The genus *Listeria* is a rod-shaped, gram-positive bacterium, and the species *Listeria monocytogenes* has been associated with human listeriosis. Foods contaminated with *Listeria,* including fish, shellfish, vegetables, milk, dairy products, and meat can be a source of human listeriosis (Farber and Peterkin, 1999). Meat may be contaminated contact of the carcasses with feces containing *Listeria* spp. during carcass processing, or as the consequence of ineffective sanitization procedures (Chasseignaux *et al*, 2001, Beumer, 2003).

Kanuganti (2002) reported that the incidence of *Listeria* spp. contamination in ground pork was about 1.7%, among which *L. monocytogenes* comprised 50.2% in the meat. *L. monocytognes* 4b has most commonly been implicated in human listeriosis; however, *L. monocytognes* 1/2a, 1/2b, and 1/2c, as well as *L. innocua,* have also been isolated in meat. *L. welshimeri* is also present in meat, to a lesser degree, followed by *L seeligeri* and *L. ivanovii* (Jay, 1996).

According to the reports of Hood and Zottola (1997) and Vatanyoopaisarn *et al*. (2000), the attachment of *L. monocytogenes* to stainless steel and, presumably, meat is attributable to the flagella, which express only between 20 and 25℃. This temperature-dependent motility of *L. monocytogenes* is the result of its peritrichous flagella. The flagella filament in this species is composed of one or two repeating linear arrays of protein subunits (flagellin), with some carbohydrates and lipids (1-5%) (Nossal and Ada, 1971).

Listeria strains are subdivided according to serotype, 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 *Listeri*a spp. are classified into A, B, C, or D type flagella antigens; however, *L. grayi* exhibits only the E type of flagella antigen (Table 1) (Seeliger and Hohne, 1979).

There have been several studies which produced a variety of specific MAbs for whole cells (Butman *et al*., 1988, Torensma *et al*., 1993, Loiseau *et al*., 1995) and crude cell surface proteins (Bhunia and Johnson, 1992, Kathariou *et al*., 1994). The reactivity patterns exhibited by their MAbs were specific to the *Listeria* genus, or specific to *L. monocytogenes* and one or more other *Listeria* species. However, no available reports have focused on flagella-specific IgY. As compared to IgG, IgY does not bind to proteins A or G, rheumatoid factor, or mammalian Fc receptors, and has not been determined to interfere with mammalian IgG, and to activate the mammalian complement (Larsson and Sjoquist, 1988, Larsson and Lindahl, 1993, Carlander *et al*., 2000). Also, there are several adventages to use IgY technology. Only egg collection without bleeding, long storage at 4℃ for at least 1 year, and the simple isolation of IgY can be obtained (Gassmann *et al*., 1990). These advantages may make IgY ideal for application in immunoassays.

In this study, we generated MAbs, IgY, and rabbit polyclonal antibody, all of which reacted specifically with the flagella of *Listeria* spp. This constitutes an adequate tool for the development of the detection kit.

Table 1. *Listeria* spp. used in this study

Organism	Serotype	Strain	H antigen
Listeria monocytogenes	1/2a	HPB 410*	AB
Listeria monocytogenes	1/2b	HPB 503	ABC
Listeria monocytogenes	1/2c	HPB 12	BD
Listeria monocytogenes	3a	ATCC 19113**	AB
Listeria monocytogenes	4a	ATCC 19114	ABC
Listeria monocytogenes	4b	HPB 3	ABC
Listeria monocytogenes	4c	ATCC 19118	ABC
Listeria monocytogenes	4d	ATCC 19117	ABC
Listeria seeligeri		ATCC 35967	ABC
Listeria innocua		ATCC 33090	ABC
Listeria ivanovii		ATCC 19119	ABC
Listeria welshimeri		ATCC 35897	ABC
Listeria grayi		ATCC 19120	E

* HPB, Health Protection Branch, Health and Welfare Canada; **ATCC, American Type Culture Collection.

MATERIALS AND METHODS

1. Bacterial strains, media, and cultures

The 13 *Listeria* strains used in this study were listed in Table 1. These bacteria were grown in tryptic soy broth (TSB; Difco, MD, USA) supplemented with 0.2% glucose and 0.6% yeast extract, at 22℃ for 48 hours, and the concentration of each bacterium was determined by plating on tryptic soy agar supplemented with 0.2% glucose and 0.6% yeast extract, and 24-48 hours of incubation at 37℃.

The 11 non-*Listeria* organisms, *Salmonella enteritidis, Salmonella typhimurium, Escherichia coli* K88ab, *Pseudomonas aerugenosa, Escherichia coli* O157:H7, *Streptococcus mastits* strain 1, *Streptococcus mastits* strain 2, *Rhodococcus equi, Staphylococcus aureus* strain 1 and 2, and *Bacillus subtilis* were also used, all of which had been isolated from animals in Jeju by the Microbiology Laboratory at Cheju National University in Korea, after inoculation samples in 10 ml of Brain heart infusion (BHI; Difco, MD, USA) broth for 24-48 hours at 37℃. The bacterial populations in each broth culture were determined by plating on BHI agar at 37℃ for 24-48 hours.

2. Preparation of flagella

L. monocytogenes serotype 4b (ATCC19115) was grown in TSB at 22℃, for 24 hours, until the stationary growth phase was reached. Flagella were isolated

according to the procedures described by Peel *et al*., 1988 (A). In brief, this bacterium was harvested by centrifugation at 7,000 rpm for 20 minutes, and washed three times with phosphate-buffered saline, pH 7.2 (PBS). 24 glass beads (2 mm diameter) were used to detach the flagella by 30 minutes of vigorous shaking. This suspension was then centrifuged at 7,000 rpm for 30 minutes. After centrifugation of the supernatant from the previous centrifugation step at 14,000 g for 40 minutes, the flagella in the pellet were resuspended in PBS, and stored at -20℃. Protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, CA, USA).

3. Scanning electron microscopy (SEM) and transmissible electron microscopy (TEM)

The presence of flagella in the cell was confirmed via SEM. *L. monocytogenes* 4b, grown at 22℃ and 37℃ on BHI agar, were sliced and fixed in 2% glutaraldehyde at 4℃ overnight, and 1% osmium tetraoxide was added. After 2 hours of incubation at 4℃, the BHI agar was dehydrated by sequential ethanol concentrations, and dried with CO_2 gas for 20 minutes. The gold coating was performed at 16 mA for 2 minutes. These samples were examined by scanning electron microscopy (HITACHI 2460N, Japan). *Listeria* treated by glass beads was also observed by using SEM.

For TEM, purified flagella were fixed with 4% paraformaldehyde for 1 hour at 4℃. Then, the fixed flagella were negatively stained for 5 minutes with 1%

phosphotunstic acid (pH7.2, SPI-Chem, PA, USA) on a copper grid (400mesh, SPI-Chem, PA, USA) coated with formvar (TAAB, Berkshire, England) in 1% chloroform. The dried grid was then examined by transmission electron microscopy (JEM 1200EXⅡ, Japan).

4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Whole bacteria cell extracts or purified flagella were denatured at 100℃ for 3 minutes with a loading buffer. After boiling, the samples were loaded onto wells of 4% stacking gel with 12% separating gel, then electrophoresed. This gel was transferred to Immunobilon membranes (Pierce, IL, USA). The blotted membrane was stained with Ponceau's solution in order to confirm the transblot of proteins. The blot was blocked with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 3% BSA, and allowed to react with flagella-specific MAb and secondary anti-mouse IgG, conjugated with horseradish peroxidase (Sigma, MO, USA). The reaction was developed using a mixture of 4-chloro-1-naphthol (Sigma, MO, USA).

5. Production of monoclonal antibodies

Monoclonal antibodies to flagellin were produced by immunizing Balb/c mice against 50 ㎍/ml of flagella. Equal numbers of splenic lymphocytes and murine myeloma SP2/0 cells were fused, as described previously by Kohler and

Milstein (1975). MAbs-secreting hybridomas were screened by ELISA and cloned. For the production of ascite fluid, 6-week old Balb/c mice were injected with 500 ㎕ of pristane. After one week had passed, hybridoma cells (1×10^5 cells/ml) produced flagella-specific antibodies, which were injected intraperitoneally into pristine-primed mice. 7-10 days later, the ascite fluid containing the MAbs was collected, centrifuged (5,000 rpm, 10 minutes), and stored at -20℃ until use.

6. Production of anti-flagella rabbit antibodies

Antibodies against *L. monocytogenes* 4b flagella were generated by the immunization of 16-week-old New Zealand White rabbits. One hundred ㎍/ml of flagella in complete Freund's adjuvant was injected subcutaneously into different sites on the back for the first dose, and incomplete adjuvant for two weekly doses. Blood was collected at each immunization, and the presence of antibodies was verified by ELISA.

7. Production of IgY

Each group of three general laying hens (24 weeks old) was initially given an intramuscular injection into the breast region with 50 ㎍, 200 ㎍ and 400 ㎍ of purified flagella emulsified with an equal volume of complete Freund's adjuvant. After two weeks, the hens received biweekly boosters with an incomplete adjuvant. Seven days after the first injection, eggs were collected daily for 4 months. The eggs were stored at 4℃ until verification of the antibody titer by

ELISA.

8. The extraction of IgY

IgY was extracted as previously described (Hassl and Aspöck, 1988). Egg yolk was diluted by four-fold with 3.5% polyethylene glycol 6000 (PEG), and incubated at room temperature (RT) for 20 minutes. After centrifugation at 10,000 rpm for 20 minutes, PEG was added to a final concentration of 12% in the supernatant. This solution was incubated for 20 minutes at RT. The precipitate containing IgY was recovered by centrifugation at 10,000 g for 10 minutes, then resolubilized in PBS and stored at 4℃.

9. Purification of antibodies by affinity chromatography

Antibodies were purified by affinity chromatography using flagellin-sepharose 4B beads. In brief, three grams of CNBr-activated 4B (Pharmacia, Uppsala, Sweden) were conjugated with flagella (1.5 mg/ml). These gels were packed into columns (10 cm) and washed several times with PBS. Ascite fluid, rabbit antibodies, and crude IgY were introduced (1 ml/min) into the column, washed with PBS, and eluted with 3 M NaSCN. Purified antibodies were dialyzed by PBS twice.

10. Enzyme-linked immunosorbent assay (ELISA)

For ELISA, polystyrene plates (Costar, NY, USA) were coated with flagella

(1 ㎍/ml) by overnight incubation at 4℃, then blocked with PBS containing 3% BSA at 4℃ for 30 minutes. After three washes with PBS, either MAbs, rabbit polyclonal antibodies, or IgY were added to each well, and incubated at RT for 30 minutes. After three washes, the plates were incubated for 30 minutes at RT with either goat anti-mouse IgG, goat anti-rabbit IgG, or goat anti-chicken IgG conjugated with horseradish peroxidase (Sigma, MO, USA). After 30 minutes, the chromogenic reactions were developed at RT for 30 minutes with ABTS. Optical density (OD) was determined at 405 nm with a plate reader (SLT, Salzburg, Austria).

11. Preparation of horse-radish peroxidase (HRP)-labeled antibodies

The conjugation was performed according to the method described by Wilson and Nakane (1978). Four milligrams of HRP were dissolved in distilled water, after which 0.2 ml of 0.1 M $NaIO_4$ was added and incubated for 20 minutes with gentle mixing. At that time, the color turned from brown to dark green. This solution was subsequently dialyzed against 1 mM sodium acetate buffer (pH 4.4) for 20 hours at 4℃, and the pH was raised to 9.5 by the addition of 20 ㎕ of 0.2 M sodium carbonate-bicarbonate buffer (pH 9.5). This mixture was added to 8 mg of each antibody, and incubated for 2 hours at RT. Subsequently, 100 ㎕ of $NaBH_4$ was added. After 2 hours, the HRP-conjugated antibody was dialyzed against PBS.

12. Selection of an MAb pair for sandwich ELISA

Each MAb was diluted in carbonate buffer (pH 9.6) to a concentration of 10 ㎍/ml, and 100 ㎕ of diluted MAb was added to ELISA plates. The plates were incubated for 2 hours at 37℃, and overnight at 4℃. The wells were blocked with PBS containing 3% BSA for 30 minutes at 4℃. After three washes with PBS, 100 ㎕ of flagella solution (100 ㎍/ml) was added and incubated for 30 minutes at RT. After washing, 100 ㎕ of each HRP-conjugated MAb was added into each well, and incubated for 30 minutes at RT. After the addition of ABTS (KPL, MD, USA) and 30 minutes of incubation at RT, absorbance was measured at 405 nm.

RESULTS

1. Production and purification of flagella

L. monocytogenes 4b grown at 22℃ and 37℃ was confirmed by SEM. Many flagella were seen on the cells which were cultured at 22℃, but no flagella could be seen on the 37℃ culture sample, as described in the textbook (Fig. 1). In order to isolate the flagella, we used glass beads (2 mm diameter) and vigorous shaking. After 30 minutes, the flagella were cut away from the cell surfaces (Fig. 2). Then, the purified flagella were suspended with PBS, followed by ultracentrifigation, and confirmed by TEM (Fig. 3). The flagellin was shown at 33 kDa by 12% SDS-PAGE (Fig. 4).

2. MAbs against flagella antigen

In primary screening, using ELISA, several culture supernatants were found to specifically recognize flagella. Five MAbs: 2B1, 3B7, 4F12, 6F3, and 7A3, all of which exhibited a high degree of specificity for flagella, but evidenced no cross-reactivity to any other bacteria used in this study by ELISA, were produced after two fusions (Table 2). Each ascite sample was then purified with a CNBr-activated 4B-flagella column. Among these, when we performed SDS-PAGE and Western blotting with MAb2B1, this MAb reacted with a single protein band of flagellin (Fig. 5, 6).

Flagella epitopes were characterized according to ELISA results (Table 3). At

least three different epitopes of these flagella were detected, and classified the MAbs into three groups, according to their exhibited specificity against the epitopes [MAb2B1 (I), MAb 4F12 (II) and MAbs 3B7, 6F3, 7A3 (III)]. Three MAbs in the third group (3B7, 6F3 and 7A3) produced weak OD in ELISA when used as detection and capture antibodies, meaning that they recognized the same epitopes. When the MAbs in all three groups were used as capture or detection antibodies, high OD values in the ELISA tests were observed.

3. IgY against flagella antigen

ELISA was also used to monitor the development of antibody levels using egg yolk diluted by a factor of 1,000 with PBS-T (Fig. 7). The yolk antibody also evidenced no cross-reactions to any other bacteria, and the antibody levels of all groups increased 30 days after initial immunization. Group 2 (200 μg) exhibited antibody titers which were comparatively higher than those of the other two groups. All hens were boosted twice at two-week intervals, with 200 μg of flagella, in order to confirm the most appropriate antigen concentrations. Antibody levels are shown in Fig. 8. Only group 3 antibody levels were found to have decreased.

Crude IgY extract (60 mg/ml) was loaded onto the CNBr-activated 4B-flagella column at a flow rate of 1 ml/min. The bound IgY was eluted by chaotropic salts, and immediately dialyzed with PBS. The recovery of IgY, measured at 280 nm, was approximately 1 mg/ml. As shown in Fig. 9, the flagella

were found to be very pure, and the IgY did not react with any of the other bacteria used in this study, and exhibited high signals with the flagella in the ELISA tests (Table 2).

4. Production of rabbit polyclonal antibody

Immunization of rabbits with flagella produced an antiserum. When the optical density was more than 1.5, the rabbits were sacrificed, and separated the anti-flagella serum. Rabbit polyclonal antibody levels increased during the immunization process (Fig. 10), and were also purified with the CNBr-activated 4B column.

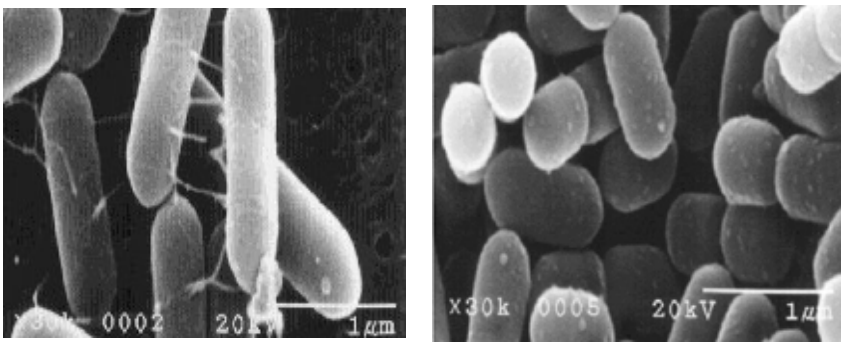

Fig. 1. Scanning electron micrograph of *Listeria monocytogenes* 4b (ATCC 19115). Flagella were seen on the cells grown at 22℃ (Left), whereas no flagella were expressed on the cells grown at 37℃ (right).

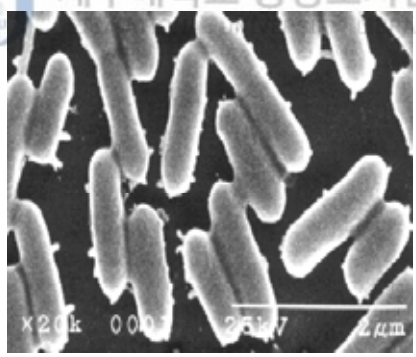

Fig. 2. Scanning electron micrograph of *Listeria monocytogenes* 4b flagella separated out by disruption with glass beads.

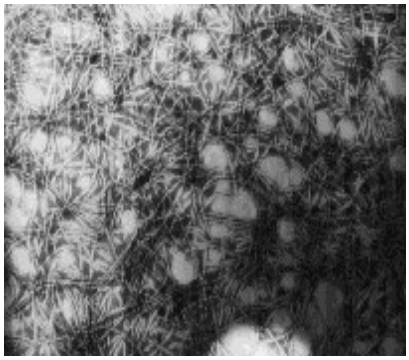

Fig. 3. Transmissible electron micrograph of flagella of *Listeria monocytogenes* 4b (ATCC 19115), which were purified via disruption with glass beads, followed by ultracentrifugation.

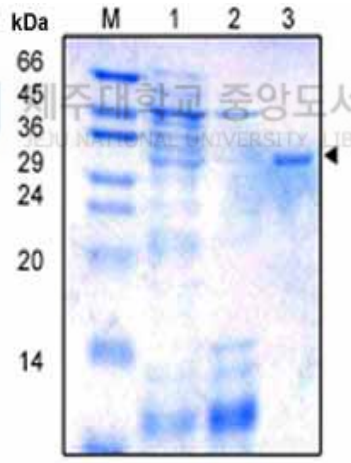

Fig. 4. SDS-PAGE of flagellin from *L. monocytogenes* 4b, according to the mentioned purification steps. The arrow denotes the position of the 33 kDa flagellin. Lane M, low molecular marker; lane 1, whole cell; lane 2, after glass bead treatment; lane 3, purified flagellin. About 10 μg of proteins were loaded into each lane.

Table 2. Specificity analysis of antibodies via ELISA with bacterial species

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

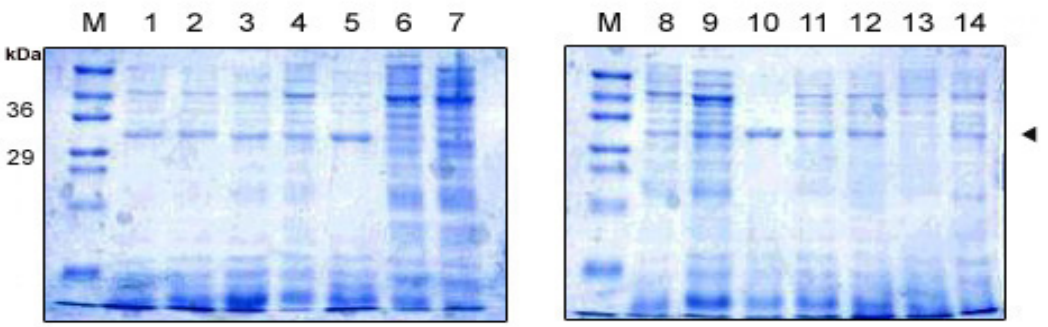

Fig. 5. SDS-PAGE of *Listeria* extracts from different *Listeria* spp. The arrow denotes the position of the 33 kDa flagellin of *Listeria* species. Lanes; 1, *L. grayi*; 2, *L. innocua*; 3, *L. ivanovii*; 4, *L. monocytogenes* 1/2a; 5, *L. monocytogenes* 1/2b; 6, *L. monocytogenes* 1/2c; 7, *L. monocytogenes* 3a; 8, *L. monocytogenes* 4a; 9, *L.*

monocytogenes 4b; 10, *L. monocytogenes* 4c; 11, *L. monocytogenes* 4d; 12, *L. murrayi* ; 13, *L. seeligeri*; 14, *L. welshimeri*.

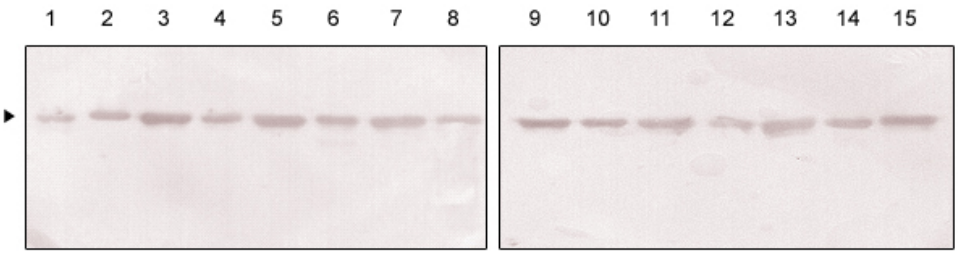

Fig. 6. Western-blot analysis of *Listeria* extracts from different *Listeria* spp. with MAb2B1. The arrow denotes the flagellin. Lanes; 1, *L. grayi*; 2, *L. innocua*; 3, *L. ivanovii*; 4, *L. monocytogenes* 1/2a; 5, *L. monocytogenes* 1/2b; 6, *L. monocytogenes* 1/2c; 7, *L. monocytogenes*; 8, *L. monocytogenes* 4a; 9, *L. monocytogenes* 4b; 10, *L. monocytogenes* 4c; 11, *L. monocytogenes* 4d; 12, *L. murrayi*; 13, *L. seeligeri*; 14, *L. welshimeri*; 15, flagellin.

Table 3. Sensitivity of the sandwich ELISA, with different pairs of monoclonal antibodies

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

-: negative (OD>0.1), +: positive (0.1≤OD≤1), ++: strong positive (1<OD).

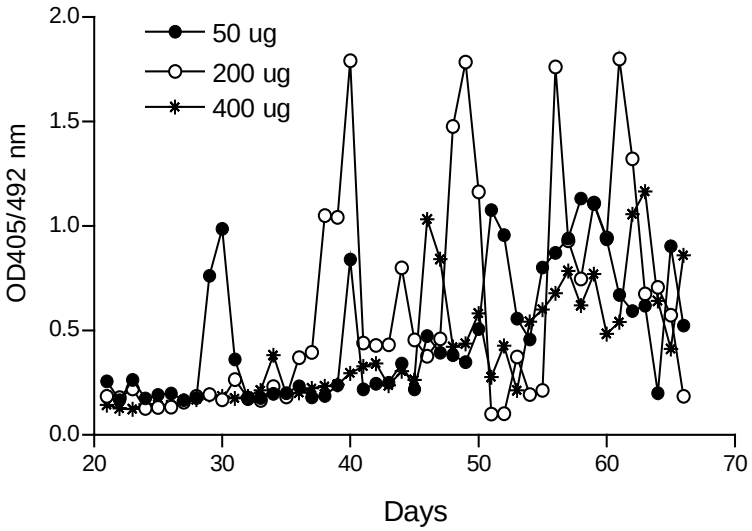

Fig. 7. Antibody levels in egg yolk from hens immunized against purified *Listeria* flagellin. Group 1, injected with 50 ㎍ (-●-); group 2, injected with 200 ㎍ (-○-); group 3, injected with 400 ㎍ (-*-). The number on the X-axis denotes the days after the first immunization.

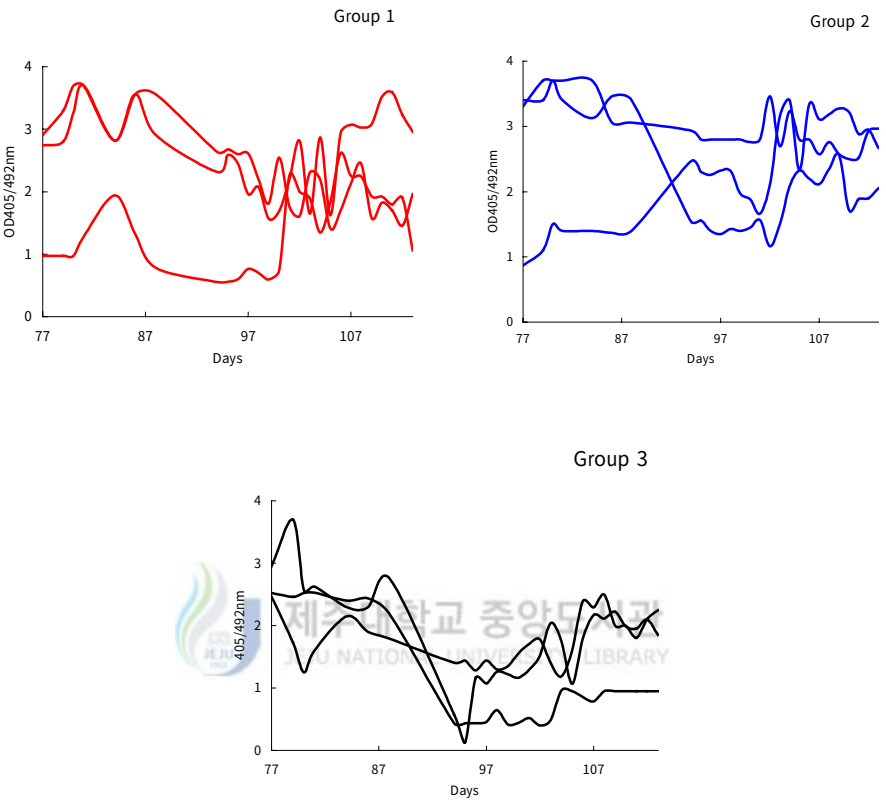

Fig. 8. Antibody levels in egg yolk. 200 μg of antigen was injected for all groups, from 70 to 113 days. Group 1, hens injected with 50 μg; group 2, hens injected with 200 μg; group 3, hens injected with 400 μg.

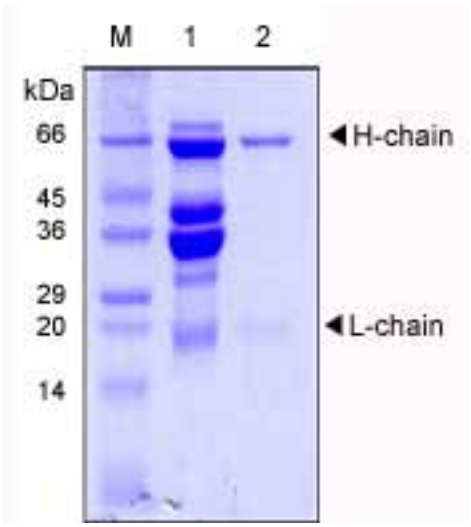

Fig. 9. SDS-PAGE analysis of IgY purified from immunized egg yolks. Lane M, low molecular marker; lane 1, crude yolk extract (25 μg of protein was loaded); lane 2, IgY fraction after affinity chromatography (15 μg of protein was loaded).

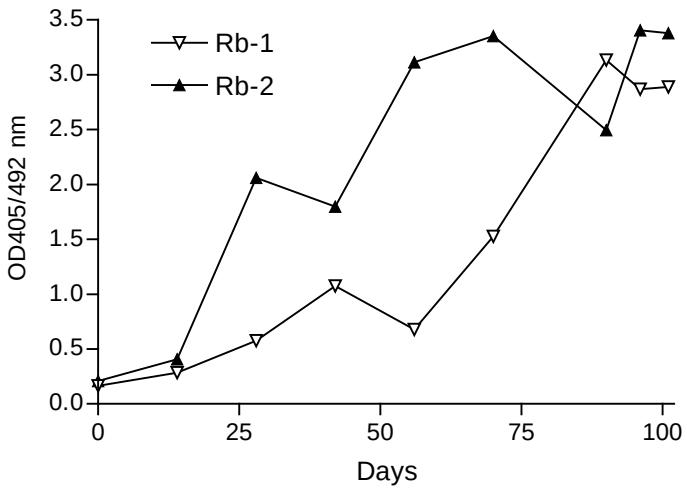

Fig. 10. Change in antibody levels in rabbit serum during the immunization period.

DISCUSSION

The filament of the flagellum is composed of one or two repeating linear arrays of protein subunits (flagellin), with some carbohydrates and lipids (1-5%) (Peel *et al.* 1988A). In order to construct flagella-specific antibodies, the flagella were purified using glass beads, followed by ultracentrifugation according to the methods described by Peel *et al.* (1988B). The molecular weight of flagellin is 33 kDa, according to the SDS-PAGE analysis done in this study, which is the same as was reported by Dons *et al*. (1992), but differs from the 29 kDa reported by Peel *et al*. (1988A). These different results with regard to the molecular weight of flagellin may be ascribable to differences in culture conditions and strains. Dones *et al.* (1992) pointed out that this may also be due to post-translational modifications, or to protein degradation.

Using these flagella, three kinds of antibodies--MAb, IgY and rabbit polyclonal antibody--were produced. Farber and Speirs (1987) and Skjerve *et al*. (1990) reported the use of flagella-specific MAbs in an immunomagnetic seperation technique and dot EIA for the detection of *Listeria* species in foods. They demonstrated that the flagella-specific MAbs could be used as detection probes.

Three groups of flagella-specific MAbs--MAb2B1 (I), MAb 4F12 (II) and MAbs 3B7, 6F3, 7A3 (III) were also generated. The third group of MAbs--3B7, 6F3 and 7A3--exhibited lower signals on sandwich ELISA when they were used

as capture and detection antibodies, which indicated that they competed for the same epitopes. When we used capture and detection antibodies from different groups, we were able to produce strong signals on ELISA, and it is obvious that different epitopes are recognized by these three groups of MAbs. Therefore, we selected detection and capture antibodies from different groups of Mabs, in order to establish the sandwich ELISA.

Immunoglobulins from all chickens' egg yolks increased 5 weeks after the initial injection, consistent with the results reported by Bollen *et al*. (1996). When we determined the IgY levels of the egg yolk of randomly chosen eggs by ELISA, the IgY levels were found to be unsteady during the entire monitoring period, consistent with the report of Leuw *et al*. (1997). This fluctuation in IgY levels is quite different from the steady-state level of immunoglobulins in the blood, and might represent the normal pattern in immunized eggs. Therefore, Leuw *et al.* (1997) suggested that IgY production should be performed with at least 5 chickens, checking the antibody titers and characteristics at different immunization times. Later, three-hen groups were injected with 200 μg of flagella. 50 microgram-injected and 200 μg-injected hen-groups maintained antibody levels, while a third group of hens (injected with 400 μg) exhibited a reduction in antibody levels. It can be concluded that 200 μg of antigen was sufficient to generate high yolk antibody titers.

IgY can be purified from egg yolk by using a combination of chromatography and precipitation, with materials and processes such as

polyethylene glycol, ammonium sulfate, chloroform, ultrafiltration, and gel filtration (Hassel and Aspöck, 1988, Akita and Akai, 1993, Svendsen *et al*., 1995, Bollen *et al*., 1996). In this study, we used PEG to precipitate lipids and proteins in egg yolk, and CNBr-activated Sepharose 4B-flagella column to purify the IgY. The IgY yield was low (1 mg/ml), possibly due to the low column capacity as compared to other types of affinity columns, including the TG19318/Emphaze column and the T-gel column (Hansen *et al*., 1998, Verdoliva *et al*., 2002). By analyzing purified IgY with SDS-PAGE, we determined that the band of the IgY light chain was weaker than that of the IgY heavy chain, a pattern which was previously reported by Hansen *et al.* (1998). The light chain of IgY appeared to stain poorly with Coomassie dye.

Polyclonal antibody from rabbits exhibited the same specificity for *Listeria* spp. as did IgY. However, a number of previous studies have reported that antibodies from hen egg yolk are superior in several key aspects to antibodies from serum (Tini *et al*., 2002). Immunized eggs can supply abundant amounts of antibody (IgY), and this may also substitute for IgG in mammals. This advantage, in particular, caused us to use IgY in the development of a novel ELISA technique in this study. This study demonstrated that IgY may represent a substitute for mammal IgG. In conclusion, all three antibody variants proved to be highly specific for *Listeria* flagella, and would constitute excellent probes for *Listeria* detection in food products.

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Chapter II.

Development and evaluation of immunochromatographic assays and sandwich enzyme-linked immunosorbent assays in the screening of listerial contamination in foods

ABSTRACT

A rapid immunochromatographic assay (ICA) and a sandwich enzyme-linked immunosorbent assay (ELISA) were developed in this study, using monoclonal antibodies, IgY and rabbit polyclonal antibody for the detection of *Listeria* spp. in foods. Sandwich ELISA tests were performed using Pair1, with HRP-labeled MAb7A3; Pair2, with HRP-labeled IgY; and Pair3, with HRP-labeled rabbit polyclonal antibody, all combined with MAb2B1 coated on microplate wells. Pair2 exhibited a sensitivity 10 times lower than Pair1 and Pair3, at both 22℃ and 30℃. In the development of this ICA method, a 40 nm gold colloid was constructed by using 1% sodium citrate, which was confirmed by TEM. 13 serotypes of *Listeria* pure culture were introduced to glass fiber membranes. Positive lines appeared for all of them. However, the other bacteria used in this study, including *Salmonella*, *E.coli* O157:H7, and *Staphylococcus aureus*, were not detected with detection antibodies. ICAs were compared on pure culture at 22℃ and 30℃. Pair1 exhibited an *L. monocytogenes* 4b detection limit of 10^5 cells/0.1 ml at 22℃, and a detection limit of 10^6 cells/0.1 ml at 30℃. These analyses were performed on pork and pasteurized milk, both of which had been artificially contaminated with low levels of *Listeria monocytogenes* 4b culture. All 27 samples were confirmed positive by both assays. This demonstrated that both assays had equal efficiency with regard to the detection of *Listeria* spp. in foods. However, the ICA could be completed more rapidly (15 minutes) than the sandwich ELISA (2 hours) on the enrichment culture.

INTRODUCTION

The minimum infective dose of *L. monocytogenes* has not yet been definitively determined. Therefore, the United States, Australia, and Korea have established legal limits with regard to the number of *L. monocytogenes* which can be present in meat products. In all three countries, a zero tolerance policy in 25 g of raw meat has been adopted. *Listeria* spp. from inoculated or naturally contaminated food material can be isolated by various methods, as well as enrichment broths and plating media, including McBride *Listeria* agar, LPM agar, Oxford agar (OXA), PALCAM agar, University of Vermont broth (UVM), and Fraser broth. The methods provided by the U.S. Food and Drug Administration (FDA) and the U. S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) were used to isolate *L. monocytogenes* from different foods.

The FDA method is normally used to detect *L. monocytogenes* in milk, milk products, seafood, and vegetables. A 25 ml or 25 g sample is introduced to 225 ml of *Listeria* Enrichment broth (LEB), mixed, and incubated at 30℃ for 4 hours. This sample is then mixed with selective agents (acriflavine HCl, nalidixic acid sodium salt, cycloheximide) and incubated for an additional 20 or 24 hours at 30℃, then streaked onto OXA and LPM agar, and incubated for 24-48 hours at 35℃ and 30℃, respectively. Another USDA-FSIS method is used to detect *L. monocytogenes* in meat and poultry products. A 25 g meat sample is introduced to 225 ml UVM broth, and stomached for 2 minutes. After 20 hours or 24 hours of

culturing at 30℃, 1ml of this culture solution is added to 10 ml of Fraser broth for 24-26 hours at 35℃. Fraser broth enrichment culture is then streaked to onto a modified Oxford agar (MOX) plate, and incubated at 35℃ for 24-48 hours. If *Listeria*-like colonies are not present, 24 additional hours of incubation in Fraser broth culture should be performed, at which time the broth culture is streaked to a second MOX plate. Ferron and Michard (1993) compared the two methods. They determined that the USDA method was superior, detecting 69% of all positive samples, as opposed to the FDA method, which detected 34% of all positive samples. However, both conventional methods require at least 7-10 days for the isolation and identification of *Listeria* spp.

PCR-based techniques are quite sensitive, and tend to be more rapid than conventional culture methods. The detection limits of these techniques are $\log_{10}$ 4.0-$\log_{10}$ 5.4 cfu ml^{-1} in enriched meat sample cultures. However, the key advantage of this technique involves the recovery of *L. monocytogenes* from the meat, without the PCR reaction inhibitors. In order to circumvent such drawbacks, Duffy *et al*. (1999) reported the use of a surface adhesion-based extraction procedure, which includes the immersion of a polycarbonate membrane into an enriched food sample. Sheridan *et al*. (1997) also recovered bacteria using polycarbonate membranes, and detected *Listeria* via immunofluorescent techniques. Although PCR-based methods have demonstrated improved sensitivity and specificity, they are not suitable for the screening of *Listeria* spp. in foods. As described above, the direct detection of *Listeria* spp. by PCR-based

methods remains limited, due to PCR reaction-inhibitory substances in food, nucleic acids from other bacteria, and the volumes of the sample sizes necessary (Cooray *et al*., 1994, Olsen, 2002). Furthermore, PCR methods are relatively expensive for screening, and are prohibitively so at large sample sizes.

Enzyme immunoassays constitute another method for the detection of *Listeria* spp. in foods. Various monoclonal antibodies which react with different *L. monocytogenes* antigens have been developed by many researchers (Farber and Speria, 1987, Butman *et al*., 1988, Bhunia and Johnson, 1992, Torensma *et al*., 1993, Loiseau *et al*., 1995). These MAbs have not proven specific for *L. monocytogenes*, and also reacted with other *Listeria* spp. Several enzyme-linked immunosorbent assays for the detection of *Listeria* are currently commercially available, including the *Listeria* Rapid Test (Oxoid), Listeria Tek^{TM} (Organon Teknika), and VIDAS (bioMerieux Vitek), among others.

Until now rapid methods, including immunoassays and polymerase chain reaction (PCR) assays, generally require 48 hours of sample enrichment in order to obtain detectable levels of bacteria (10^5-10^6 cfu ml^{-1}) (Dever *et al*., 1993), as the level of *L. monocytogenes* in meats is as low as 100/g (Jay, 1996). Following 48 hours of enrichment, the two methods mentioned above, although they are sensitive and specific, take at least 2 or 3 additional hours, respectively, for the completion of testing. Hence, the slow turnaround time for results becomes an issue. Therefore, the development of a rapid, inexpensive, and convenient method, which requires no instruments when used in mass-screening surveys, is a clear

necessity.

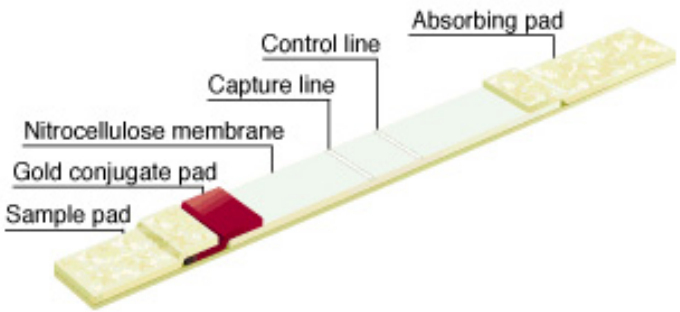

Fig. 1. Diagram of a rapid immunochromatographic assay.

Fig. 1. displays the principles of immunochromatography assay (ICA). When the liquid sample was applied to the sample pad, it migrated by capillary diffusion to the conjugate pad, which was composed of immobilized colloidal gold labeled antibodies. The antigen and gold conjugate then moved onto the nitrocellulose membrane (NC), and captured the antibody in capture lines by immobilization. The capture lines manifested as red lines. The control line also appeared red, indicating that the test is complete, and this requires a minimum of 10-15 minutes. The immunochromatographic assay (ICA) was developed using colloidal gold. Because colloidal gold, which is produced by the reduction of tetrachloric acid ($HAuCl_4$), exhibits high stability, as well as better mobility and sensitivity, it is more commonly used for ICA than is latex (Chandler *et al*., 2000, Shyu 2002). Furthermore, Paek *et al*. (2000) reported that trehalose was an effective stabilizer, with a shelf-life of about 2 years at room temperature. This assay also can be used for blood (Shin *et al*., 2001), serum (Reithinger *et al*., 2002), and enrichment culture samples (Gryko, 2002). In this study, we developed a rapid

immunochromatographic assay (ICA) for the screening of *Listeria* spp., using flagella-specific MAbs, IgY, and rabbit polyclonal antibodies,and also compared the reliability of this ICA with that of the sandwich enzyme-linked immunosorbent assay (ELISA). Although only *L. monocytogenes* is restricted by laws concerning food safety, the screening of all *Listeria* spp. can indicate levels of hygiene being observed during meat processing and distribution.

MATERIALS AND METHODS

1. Bacteria

The 13 *Listeria* strains, 11 non-*Listeria* organism strains, and cultural methods used in this study were identical to those used in Chapter One.

2. Isotyping of monoclonal antibodies

Immunoglobulin isotyping of MAbs was done with the Mouse-Hybridoma Subtyping Kit (Boehringer, Mannheim, Germany).

3. Titration of HRP-labeled antibodies by ELISA

The flagella were diluted in 10 ㎍/ml with 0.05 M carbonate buffer (pH 9.5), and added to each well of a 96-well microtiter plate. After 2 hours of incubation at 37℃ and overnight incubation at 4℃, the plates were blocked with PBS containing 0.2% BSA for 30 minutes at 4℃. After three washings with PBS, each HRP-labeled antibody sample was serially diluted with PBS containing 0.02% Tween. The plates were then incubated for an additional 30 min at RT. After washing, 100 ㎕ of ABTS was added and incubated for 30 minutes at RT. The absorbance was measured at 405 nm with an ELISA plate reader.

4. Sandwich ELISA

Sandwich ELISA was performed on microtiter plates (Costar, NY, USA).

Each well was coated with purified MAb2B1 in 0.05 M carbonate buffer (pH 9.5), via 1 hour of incubation at 37℃, and an overnight incubation at 4℃, after which the wells were washed three times with PBS. The heat-inactivated (100℃ for 20 minutes) 13 *Listeria* serotypes and 11 non-*Listeria* strains were tested after 48 hours of incubation in TSB at 22℃ and 30℃, respectively. A 100 ㎕ aliquot of each sample was then added into wells coated with MAb2B1. The plates were incubated for 30 minutes at RT, and then washed three times with PBS. 100 microliters of each optimally-diluted MAb7A3, IgY, and rabbit polyclonal antibody sample was labeled with HRP, which was added into each well of the plates. The plates were incubated for 30 minutes at RT, and washed three times with PBS. Antigen-antibody reactions were visualized by the addition of ABTS to each well, and the absorbance was measured at 405 nm, using an ELISA plate reader.

5. Immunochromatographic assay

5-1. Preparation of 40 nm gold : 100 ml of deionized water was added to 88 ml of 20 nm gold chloride while boiling, after which, 8 ml of 1% sodium citrate was added. Then, 0.8 ml of 1% gold chloride (Sigma, MO, USA), mixed with 80 ml of deionized water, was dropped slowly at a rate of 1 ml/min, and was boiled for another 20 minutes. The density and size of the gold in the preparations were checked both by spectrophotometry at 528 nm, and TEM.

5-2. Antibody-gold conjugates : 160 ㎍/ml of MAb7A3, IgY and rabbit polyclonal antibodies were added to 10 ml of a 40 nm gold particle suspension, which was then adjusted to a pH of 7.5, using 10% NaOH. The mixture was then allowed to stand for 30 minutes for conjugation. The reaction was then blocked by the addition of 500 ㎕ of 10% bovine serum albumin (BSA), and incubated for 10 minutes. The pH of the suspension was adjusted again to 7.5, using 0.1 N HCl. Conjugates were collected by centrifugation at 11,000 g for 1 hour at 20℃, and the supernatant was discarded. The conjugates were finally suspended in 100 mM phosphate buffer containing 6% sucrose, 2% Tween, 2% BSA, and 0.05% NaN_3.

5-3. Immunochromatographic test : The 1.5 mg/ml of goat anti-mouse IgG (Sigma, MO, USA) and 3 mg/ml of MAb2B1 were applied 2 cm and 1.6 cm from the bottom of the nitrocellulose membrane (Whatman, Ontario, Canada) respectively, and dried for 1 hour at RT. The MAb7A3-gold conjugate was soaked in a sheet of glass fiber pads (mdi, Ambala Cantt, India) for 1 hour at 37℃. This assay was carried out with the pre-enriched culture initially applied to the sample pad, followed by 100 ㎕ of dilution buffer (3% sucrose, 1% tween, 1% BSA, 0.05% NaN_3). After 15 minutes, samples with the appearance of red-colored bands at the Test line (T) and goat anti-mouse IgG at the Control line (C) were considered positive for *Listeria* spp.

6. 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℃ for 48 hours, was diluted to 9-10 cells, 5-6 cells, and 1-2 cells/ml with TSB, then inoculated into 25 g of pork. These samples were enriched using the USDA method, but the temperature of the second enrichment was 30℃. Pasteurized milk (25 ml) was also artificially contaminated, as previously described on pork, and the enrichment was performed using the FDA method. The assays were carried out on 27 samples, among which 3 and 6 were contaminated at each concentration in pork and milk, respectively. These culture fluids were then analyzed using both sandwich ELISA and ICA methods.

RESULTS

1. Development of sandwich ELISA

MAb7A3 and MAb2B1 were shown to be the best hybridoma for the recognition of distinct epitopes on flagella selected for the development of sandwich ELISA and ICA. The MAb2B1 and MAb7A3 subclasses were determined as IgG1 and IgG2a, respectively, with the kappa light chain.

The MAb7A3, IgY, and rabbit polyclonal antibody were used as detection antibodies. The detection antibodies were labeled with HRP by the periodate method. Serial dilutions of the detection antibodies were assayed for flagella-binding ability, as is shown in Fig. 2. The optimal dilutions of detection antibodies were determined using an approximate optical density of 2.5 on an ELISA reader. Optimal dilutions were 1000× for HRP-labeled MAb7A3, 50× for HRP-labeled IgY, and 250× for HRP-labeled rabbit polyclonal antibody.

The pairs consisted of combinations of Mab2B1 with HRP-labeled MAb7A3 (Pair1), HRP-labeled IgY (Pair2) and HRP-labeled rabbit polyclonal antibody (Pair3), all coated onto microtiter plate wells. The sandwich ELISA developed using the antibodies in this study could be used to detect all 13 *Listeria* serotypes, and exhibited no cross-reactivity with other bacteria (Data not shown). *L. monocytogenes* 4b was cultured at 22℃ and 30℃, respectively. The numbers of *L. monocytogenes* were calculated by the plate count method, and serially diluted with TSB. The detection limit of the assay using Pair1 was 1×10^4 cells per 0.1 ml,

and the same at 30℃ (Fig. 3). Using pair2, the detection limit was 1×10^5 cells per 0.1ml at 22℃, and 1×10^6 cells per 0.1 ml at 30℃. The detection limit of sandwich ELISA using Pair3 was 1×10^4 cells per 0.1 ml at 22℃, and 1×10^3 cells at 30℃. The sensitivity associated with this assay was higher than in any other two-pair systems. However, the optical density, between 10^5 cells and 10^3 cells per 0.1 ml for cultures at 30℃, did not increase appreciably. Two Pairs, 1 and 3, exhibited higher sensitivity than did Pair2. Therefore, we selected Pairs1 and 3, for use in our ICA protocol.

2. Development of ICA

In this ICA method, 1% sodium citrate was used for the reduction of tetrachloric acid, in order to induce the formation of gold atoms. The diameter of the colloid was confirmed to be 20-40 nm by TEM. On EM, some particles were observed to be aggregated, but most of them were individual and round with the expected size of 20-40 nm (Fig. 4). The materials for ICA included an absorption pad (cellulose membrane), a nitrocellulose membrane coated with goat-anti mouse IgG and MAb2B1 on the surface, 40 nm gold-labeled MAb7A3, and a rabbit polyclonal antibody conjugate pad and sample pad (glass fiber membrane).

Pure cultures of the 13 *Listeria* strains were dropped onto glass fiber membranes. 15 minutes later, positive lines appeared on all tests (Fig. 8). However, the other bacteria described above were not detected by the detection antibodies (Fig. 5). These ICAs were carried out on pure cultures, at 22℃ and

30℃. Pair1 exhibited an *L. monocytogenes* 4b detection limit of 10^4 cells per 0.1 ml and 10^6 cells per 0.1 ml, respectively (Fig. 6). Pair3 exhibited an *L. monocytogenes* 4b detection limit of 10^5 cells per 0.1 ml in a 22℃ culture. However, the ICA which used Pair3 exhibited no signals in the 30℃ culture (Fig. 7). These results indicated that the combined use of two MAbs was essential for the accurate detection of *Listeria*.

3. Detection of *L. monocytogenes* 4b in foods

Pork and milk samples which had been artificially contaminated with low numbers (9-10, 5-6, 1-2 and 0 cells/ml) of *L. monocytogenes* 4b cultures were detected by two methods: sandwich ELISA, and ICA using Pair1 antibodies. All samples yielded positive responses for *L. monocytogenes* 4b (Table 1, Fig. 9). We calculated the negative results for ELISA according to the following formula: negative $OD_{average}$+0.2. A culture sample was considered to be positive if its OD was higher than the negative index. Both the sensitivity and the specificity of ICA, as determined by ELISA, were 100%. The values were calculated according to the protocol discribed by Livingston *et al*. (2002).

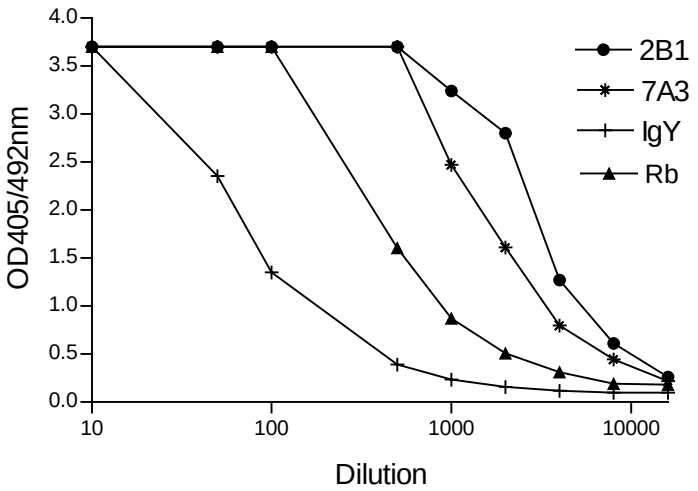

Fig. 2. Titration of detection antibodies.

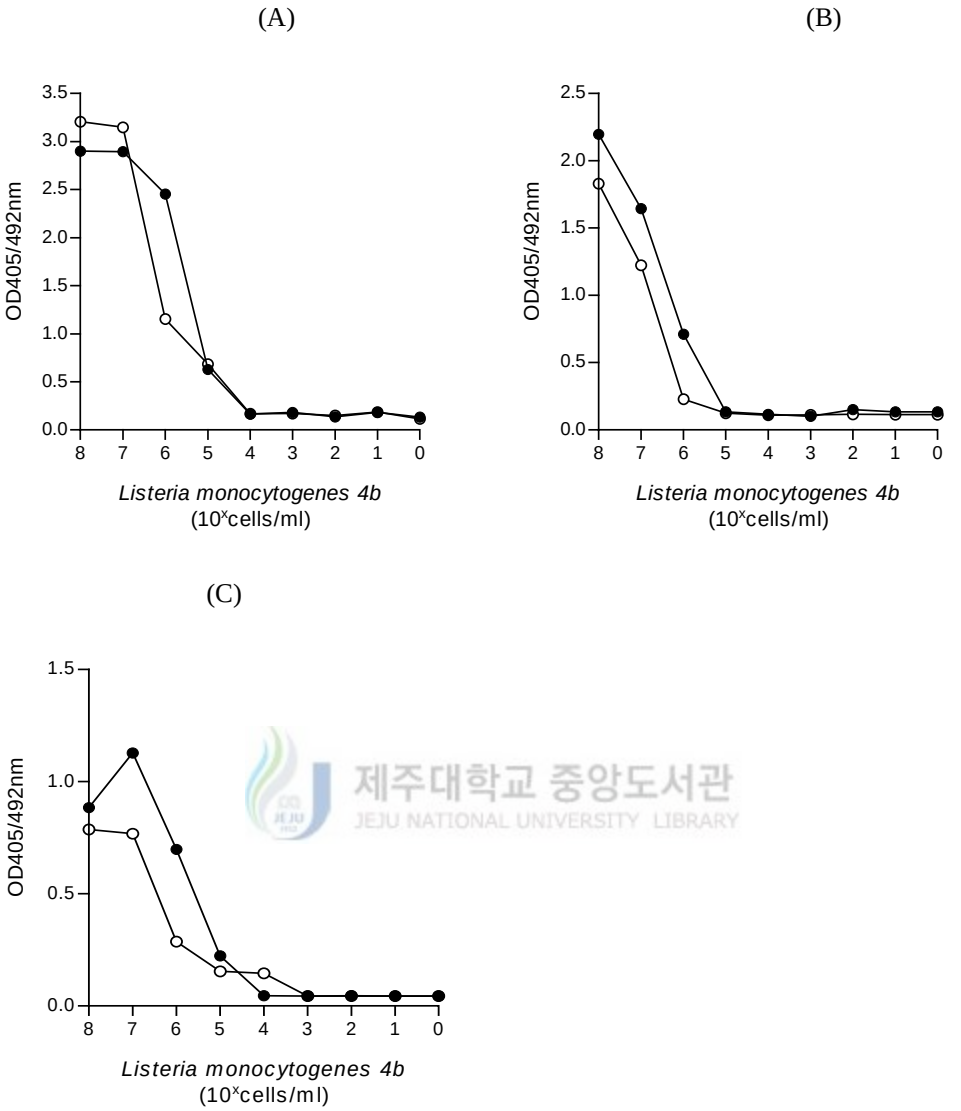

Fig. 3. The sensitivity of sandwich ELISA with regard to the detection of *Listeria monocytogenes* 4b (ATCC 19115). The cell was cultured in TSB at 22℃(●) and 30℃(○). A, Pair1, the combination of HRP-labeled MAb7A3 and MAb2B1 coated onto microplate wells; B, Pair2, the combination of HRP-labeled IgY and MAb2B1 coated onto microplate wells; C, Pair3, the combination of HRP-labeled rabbit polyclonal antibody and MAb2B1 coated onto microplate wells.

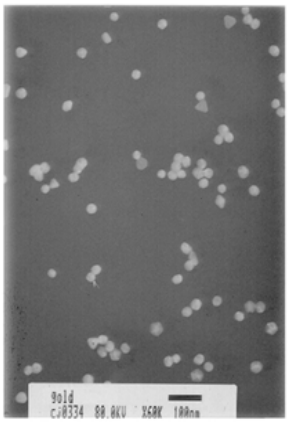

Fig. 4. Transmission electron micrograph of 40 nm gold colloid (×60 K).

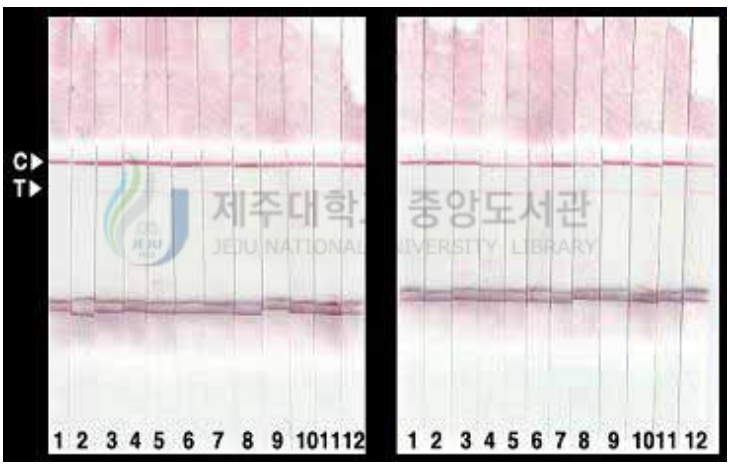

Fig. 5. Specificity analysis of immunochromatographic assay. One hundred microliters of non-*Listeria* culture were dropped. Left and right were used for Pairs 1 and 3. Lanes; 1, *Salmonella enteritidis*; 2, *S. typhimurium*; 3, *E.coli* K88ab; 4, *Pseudomonas aerugenosa*; 5, *E.coli* O157:H7; 6, *Streptococcus mastitis* strain 1; 7, *Streptococcus mastitis* strain 2; 8, *Rhodococcus equi*; 9, 10, *Staphylococcus aureus* strain 1 and 2; 11, *Bacillus subtilis*; 12, PBS.

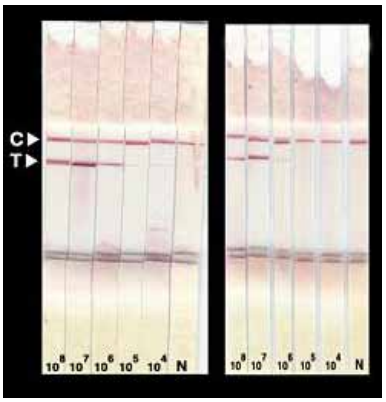

Fig. 6. Detection limit of immunochromatographic assay with *L. monocytogenes* 4b (ATCC 19115) using Pair1 in pure cultures at 22℃ (left) and 30℃ (right).

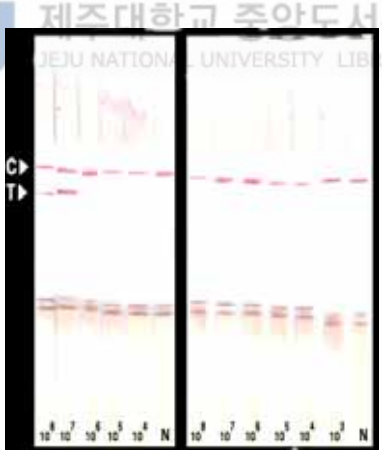

Fig. 7. Detection limit of immunochromatographic assay with *L. monocytogenes* 4b (ATCC 19115) using Pair3 in pure cultures at 22℃ (left) and 30℃ (right).

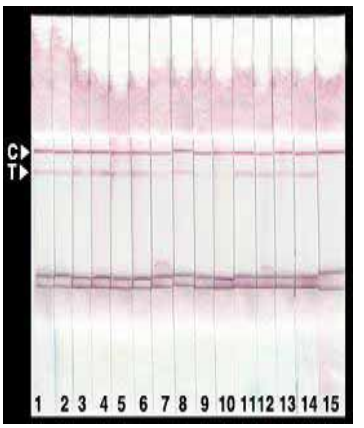

Fig. 8. Specificity of immunochromatographic assay. 10^6 cells/100 ㎕ of 13 *Listeria* strains were dropped. Lanes; 1, *L. grayi*; 2, *L. innocua*; 3, *L. ivanovii*; 4, *L. monocytogenes* 1/2a; 5, *L. monocytogenes* 1/2b; 6, *L. monocytogenes* 1/2c; 7, *L. monocytogenes* 3a; 8, *L. monocytogenes* 4a; 9, *L. monocytogenes* 4b; 10, *L. monocytogenes* 4c; 11, *L. monocytogenes* 4d; 12, *L. grayi*; 13, *L. seeligeri*; 14, *L. welshimeri*; 15, PBS.

Table 1. Detection of spiked *L. monocytogenes* 4b by sandwich ELISA and ICA

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

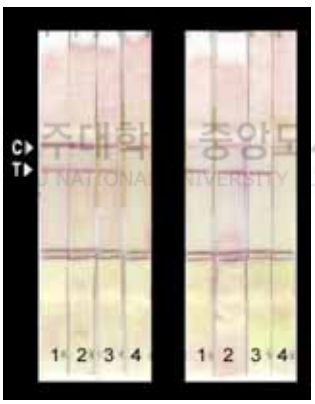

Fig. 9. Immunochromatographic assay for the detection of artificially contaminated 9-10 (lane 1), 5-6 (lane 2), 1-2 (lane 3), and 0 (lane 4) *L. monocytogenes* 4b (ATCC19115) cells in pork (Left) and milk (right) samples. The samples were enriched using the USDA method, but in the second enrichment, the temperature was changed from 35℃ to 30℃, and the FDA method was utilized.

DISCUSSION

As these two MAbs, IgY, and rabbit polyclonal antibody all exhibited positive reactivity for flagella, the expression of which is temperature-dependent, we compared the sensitivity of the two methods at culture temperatures of 22℃ and 30℃. Asturias *et al*. (1999) reported that a specific polyclonal serum would not incur conformational changes which could affect epitopes. Therefore, the polyclonal antibodies, IgY, and rabbit antibody were used as detection antibodies in sandwich ELISA and ICA.

The sensitivity of ELISA using IgY as a detection antibody (Pair2) was 10 times lower than that of the ELISA which used two MAbs at a culture of 22℃. In contrast to these results, Vejaratpimol *et al*. (1998) reported that they obtained better sensitivity results by using IgY as a detection antibody, than by using MAb as a detection antibody. This result may be due to the different specificities and affinities inherent in different antibodies. Compared to the commercially-available ELISA method, which exhibits a sensitivity of nearly 5×10^4 to 10^5 bacteria ml^{-1}(Capita *et al*. 2001), the detection limit of the sandwich ELISA which used the Pair1 antibodies (combination of HRP-labeled MAb7A3 and MAb2B1 coated onto microplate wells) was 10^4 cells/0.1ml at both 22℃ and 30℃. It can, then, be concluded that sensitivity is not affected by the culture temperature, as opposed to the results obtained when Pair2 antibodies were used. Pair3 exhibited a sensitivity of 1×10^4 cells per 0.1 ml at 22℃, and 1×10^3 cells at 30℃. Therefore, we chose

two pairs, 1 and 3, for the development of ICA.

For the development of ICA method, 40 nm gold was produced using 8 ml of trisodium citrate, and the gold size was confirmed via TEM. On EM, the colloidal gold exhibited variations in size, from 20 to 40 nm. However, Chaudhuri and Raychaudhuri (2001) previously reported that gold sizes of 20-40 nm were suitable for the development of diagnostic assays, and the result confirmed these, as the ICA using 20-40 nm gold in this study exhibited excellent sensitivity. When ICA was performed using Pair3 (combination of gold-labeled rabbit polyclonal antibody and MAb2B1 coated onto nitrocellulose membrane), a weak signal on the 30℃ culture was detected. Hence, Pair1 (combination of gold-labeled MAb7A3 and MAb2B1 coated onto nitrocellulose membrane) was selected for this ICA. The detection limit of this ICA protocol was 10^5 cells/0.1ml and 10^6 cells/0.1ml at 22℃ and 30℃. As described above, due to the fact that *Listeria* spp. are motile and express abundant flagella at 22-26℃ (Peel *et al*., 1988A), the detection limit of ICA for the sample cultured at 22℃ was 10 times higher than that associated with the sample at 30℃. The detection limit of the sandwich ELISA was the same at both temperatures (10^4 cell/0.1ml). This result suggests that the sandwich ELISA is inherently more sensitive than the ICA in pure cultures. Watanabe *et al*. (2002) also demonstrated that the sensitivity of ICA was 10 times lower than that of sandwich ELISA for the detection of dihydrostreptomycin in milk. 25 g or 25 ml portions of pork and milk were artificially contaminated. The second enrichment of the spiked pork samples was

performed at 30℃, due to our utilization of temperature-dependent flagella-specific MAb. As was described above, because *Listeria* can contaminate foods at very low levels, and can grow even at refrigeration temperatures, the detection of even low-levels of *Listeria* contamination in food is very important. Therefore, in this study, two foods were contaminated with 1-10 cells/ml of *L. monocytogenes* 4b. Both assays yielded positive results for all samples.

The two methods applied appear sufficiently sensitive for the detection of *Listeria* spp. Although all strains may not be detected with equal sensitivity, an additional 48 hours of culturing in enrichment medium should almost always be sufficient for the accumulation of detectable titers of *Listeria* strains. This ICA and sandwich ELISA were used as a screening test, and are potentially applicable to other foods, including vegetables, cheese, etc. However, the ICA can be used more conveniently, and it may provide a faster approach to diagnosis, with regard to the detection of initial *Listeria* contamination.

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Chapter III.

Application of the single-chain Fv antibody to immunochromatographic assays for the detection of *Listeria* spp.

ABSTRACT

Recombinant single-chain Fv antibodies were constructed from the hybridoma cell lines, 7A3 and 2B1, and were expressed in both insect cells and *Escherichia coli*. The eukaryotic fragment, Bac scFv 7A3, exhibited specificity for flagella, but this affinity was 100 times lower than that associated with the intact monoclonal antibody. Conversely, the prokaryotic fragments had no affinity for *Listeria* flagella. The Bac scFv 7A3 sequence was used to demonstrate that the V_H and V_L region belonged to the J558-19 and 12-44 germ lines, respectively. Two milligrams per liter of Bac scFv 7A3 were purified after Ni-NTA chromatography. The detection limit of gold-conjugated Bac scFv 7A3 coupled with MAb2B1, when used in the immunochromatographic assay, was 30 μg/ml of flagella.

INTRODUCTION

Many recombinant antibodies, such as Fv, scFv, dsFv (disulphide stabilized Fv) and chimeric antibody (Verma *et al*. 1998), are currently used. Chimeric antibodies are humanized antibodies in which the complementary determining regions (CDRs) are of rodent origin (Richmann *et al*. 1988). The Fv fragment, which retains only an intact antigen binding site, has been proven to be unstable, and easily dissociates from the V_H and V_L domains. In order to alleviate this deleterious tendency, a single-chain Fv (scFv) fragment was composed using the heavy chain variable region (V_H), linked covalently to the light chain variable region (V_L) by various flexible peptides, such as $(Gly_4Ser)_3$, which should not have any propensities for ordered secondary structures (Huston *et al*., 1988). Also, scFv is composed of V_H and V_L, linked by a disulfide bridge (Bird *et al*. 1988). scFv has been produced and used in several therapeutic applications, including cancer treatment and detoxication, as these molecules are possessed of several advantages: good penetration into solid tissue, rapid clearance from circulation, and low risk of immunogenicity (Haber, 1992).

The hybridomas, on the other hand, have their own drawback, namely the lack of antibody-generating ability. This is due to their inherent chromosomal instability (Choi *et al*., 1997). Therefore, scFv is also applied in the immunodiagnostic field. Navarro-Teulon *et al*. (1995) reported that anti-digoxin scFv expressed in both the *E.coli* expression system and the phage displaying

system, may constitute a useful probe for diagnosis. Kerschbaumer *et al*. (1997) reported that the fusion of scFvs, with a leucine zipper as a coating reagent and alkaline phosphatase as a detecting reagent, was also suitable for use in sandwich ELISA tests. Other approaches, including the use of bivalent bispecific antibodies, as well as the specific gp120 of HIV, were found to have some potential in both assays and therapeutic applications (Kontermann, 1997). Lemeulle *et al.* (1998) also expressed anti-digoxin scFv fragments in bacteria and insect cells. Emanuel and coworkers determined that recombinant antibody fragments (Fabs) captured by phage display technology also constituted excellent probes in the ELISA process, especially with regard to the detection of botulinum neurotoxin A/B. The use of scFv in this detection system may result in a greater variety of standardized tests, as well as a needed reduction in production costs (Navarro-Teulon *et al.*, 1995, Kerschbaumer *et al*., 1997).

Recently, recombinant antibodies have been used as sensor molecules in microarrays for clinical diagnoses, and also in therapeutic interventions, as recombinant antibodies exhibit well-characterized binding properties. Their specificity and large libraries can also be obtained (Holt *et al*., 2000, Pavlickova *et al*., 2004). When recombinant antibodies are applied to microarrays, a variety of different supports are available, including polystyrene or plastic, glass, porous gel pad slides, and filters. Using these supports and recombinant antibodies, several companies have been developing antibody microarray systems. Several case studies have reported that the detection limit of antibody microarrays may have to

be measured in the picogram range (Kusnezow and Hoheisel, 2002, Pavlickova *et al*., 2004). As described above, microarrays with recombinant antibodies appear to represent a very promising method for the standardization of diagnostic assays in proteome analysis. However, thus far, the development of single-chain Fv antibody array technology is necessary for the reduction of non-specific reactions, as well as to offer improved diverse detection methodologies, including the testing of proteolytical derivatives and antigen-associated membrane vesicles in serum samples (Pavlickova *et al*., 2004).

When the V_H and V_L genes are obtained by PCR, recombinant antibodies have been produced in various expression systems, including bacterial, insect, yeast, mammalian, and plant systems (McGregor *et al*., 1994, Casey *et al*., 1995, Poul *et al*., 1995, Lemeulle *et al*., 1998, Franconi *et al*., 1999, Reavy *et al*. 2000). Also, these molecules can be generated from phage display libraries (McCafferty *et al*., 1990, Chames and Baty, 2000). However, every scFv is composed of a different sequence of amino acids, and no unique expression system has yet been developed (Verma *et al*., 1998).

Common bacterial expression systems have been used, as they are relatively inexpensive, and they have the ability to produce proteins in large quantities. Verma *et al*. (1998) reviewed a detailed discussion of successful expression conditions for scFv in *E. coli* and refolding protocols. *E. coli* does not adhere to glycosylating proteins, nor to insoluble inclusion bodies in the cytoplasm, which contain unfolded proteins, and normally require a refolding step. Another

approach used in bacterial expression is to use leader sequences, which typically contain a *pel*B leader from the pectate lyase gene of *Erwinia carotovora*, and a leader sequence derived from alkaline phosphatase. This approach carries with it certain advantages, namely that its selection of antibodies may allow for direct purification and rapid screening from the supernatant.

Baculovirus expression systems are probably the most popular of the insect cell expression systems. As baculovirus can infect only insects, it is quite safe for use in the therapeutic field. Furthermore, insect cells are devoid of mammalian contaminants, due to their ability to grow in serum-free media. They produce a large amount of active proteins, as the insect cells are able to perform the majority of the required post-translational alterations. Some researchers have produced recombinant antibodies in the insect cell baculovirus system (Poul *et al*., 1995, Lemuelle *et al*., 1998). The systems associated with baculovirus are the Autographa California nuclear polyhedrosis (AcNPV), the Bombyxmori nuclear polyherosis (BmNPV), and the insect cell line Sf9, which derives from *Spodoptera frugipderda*. According to the report of Weiss *et al.* (1982), for successful foreign gene expression, a good quality growth media, careful culturing, and a viable and log growth phase of insect cells are necessary.

We previously produced a monoclonal antibody anti-flagella antibodies designated 7A3 and 2B1, which exhibit broad affinity for *Listeria* spp. Amplification of the V_H and V_L genes assembled via the flexible peptide linkers of the 7A3 and 2B1 hybridoma marked their production in insect cells and the *E.*

coli system. Until now, no immunochromatographic assays (ICA) predicated on an scFv approach have been designed. In this report, we demonstrated the feasibility of using scFv as a capture or detection agents in an ICA.

MATERIALS AND METHODS

1. Hybridoma

The 7A3 and 2B1 hybridomas have been previously described. In brief, these hybridoma were maintained in DMEM supplemented with 10% FBS, until cells were grown to a density of 1×10^7 cells/ml. They were then washed three times with PBS.

2. Bac scFv 7A3 expressed in insect cell system

2-1. Construction of the Bac scFv 7A3

Total RNA was extracted from 1×10^7 hybridoma cells, using the RNeasy Mini kit (Qiagen, MD, USA). Reverse transcription was also performed on the RNA, using reverse-transcription Superscript (Invitrogen, CA, USA) and Oligo (dT) primer. The variable regions of the heavy chain (V_H) and kappa light chain (V_L) were amplified from 2ul of first-strand cDNA, using *Taq* DNA polymerase with 30 PCR cycles (1 cycle is 1 minute at 94℃, 1 minute at 55℃, and 2 minutes at 72℃). The primers used in this PCR amplification were modified from those developed by Lemeulle *et al*. (1998) (Table 1). The FvHFBam and FvHR-Lin primers were used for the amplification of V_H; the FvLF-Lin and FvLRXho primers were used for the amplification of V_L. The FvHR-Lin and FvLF-Lin primers were contained in a sequence encoding for a short flexible peptide, $(Gly_4Ser)_3$. After gel purification with a QIAquick Gel Extraction kit (Qiagen,

Hilden, Germany), overlapping PCR was performed in order to construct the scFv gene, using the FvHFBam and FvLRXho primers for 30 cycles (1 minute at 94℃, 1 minute at 55℃, and 2 minutes at 72℃). Finally, the assembled scFv products were gel-purified.

Table 1. The oligonucleotide primers used for the amplification of the Bac scFv 7A3 fragment in the orientation V_H-$(Gly_4Ser)_3$-V_L

Primer	Sequence (5'-3')
FvHFBam	ATCGGATCCGCGGCCCAGCCGGCCATGGCCCAGGTCCAAGCCGCAGAGTCCGG
FvHR-Lin	*GCCAGAGCCACCTCCGCCTGAACCGCCTCCACC*TGCAGAGACAGTGACCAGAGTCC
FvLF-Lin	*TCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCG*GACATCCAGATGACCCAGACTCCATCCTCCTTATCT
FvLRXho	ACTCTCGAGTCATGCGGCCGCCCGTTTGATTTCCAGCTTGGTGCCTCC

The underlined sequences are the restriction enzyme sites (*Bam*HI and *Xho* Ⅰ) and the linker sequence, $(Gly_4Ser)_3$ is in italics.

2-2. Cloning and sequencing of the Bac 7A3 scFv gene

Gel-purified scFv was cloned into pGEM-T vector (Promega, WI, USA). The ligation mixture contained 5 ㎕ of 2 × Rapid Ligation buffer, 1ng of pGEM-T Easy vector, 37.5 ng of PCR product, and 1 ㎕ (3 Weiss units) of T4 DNA ligase. This mixture was incubated for 3 hours at RT. The 2 ㎕ of ligation mixture was transfected with *E.coli* JM109 for 45 seconds at 42℃, then treated

with 950 ㎕ of SOC medium. After 1 hour at 37℃ with shaking, the transformation culture was plated onto a LB plate containing ampicillin, IPTG, and X-Gal. The white colonies were confirmed and digested with *Bam*HI/*Xho*I, and the plasmid DNA was sequenced using the dideoxy chain termination method, with an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) and an ABI 377 DNA sequencer. The sequence data was analyzed using the DNASIS v2.5 program (Hitachi Software Engineering Co., Ltd for network).

2-3. Expression and purification of the scFv 7A3

As described in Fig 1, pBacPAK (Neurogenex) contains the hexahistidine peptide (His_6) tag sequence, previously digested *Bam*HI, and *Xho*I. A *Bam*HI/*Xho*I oligonucloetide of scFv 7A3 was cloned into the pBacPAK. Then, the expression of recombinant scFv was performed in insect cells following the manufacturer's instructions. In brief, Sf9 cells were cotransfected with 100 ng of pBacPak-His-scFv transfer plasmids, BacPAK6 viral DNA, and 4 ㎕ of Bacfectin reagent (Clontech, NJ, USA). This transfection mixture was then incubated at RT for 15 minutes, and infected with Sf9 cells. After a 5 hour incubation at 27℃, 1.5 ml of Grace's medium containing 10% FBS was added. Five days later, the supernatant was utilized in the isolation of viral DNA, and in the carrying out of PCR analysis. This baculovirus DNA was amplified using the primer sets: F (5'-AAATGATAACCATCTCGC-3'), and R (5'-

ACCGAGGAGAGGGTTAGGGAT-3'). Viral DNA was obtained after 30 cycles of PCR (94℃ 1 minute, 55℃ 2 minutes, 72℃ 3 minutes). In order to confirm the recombinant protein in the insect cells, infected Sf9 cells, with recombinant baculovirus in 60 mm petri dishes, were fixed with cold acetone at -20℃ for 30 minutes. After 1 hour of incubation at 37℃ with the monoclonal anti-poly histidine clone His-1 (Qiagen, Hilden, Germany), the plate was washed three times with PBS. FITC-conjugated anti-mouse IgG was added and incubated for 1 hour at 37℃. The stained Sf9 cells were examined under a fluorescence microscope (Olympus IX70, Tokyo, Japan). High Five cells, grown in TC-100 (Sigma, MO, USA), and containing 5% FBS were then infected with the recombinant baculovirus at a multiplicity of infection of 1 in spinner culture (10^6 cells/ml). After 7 days, the pellet was harvested and purified with Bac scFv7A3, according to the instructions provided in the Ni-NTA purification kit (Invitrogen, CA, USA).

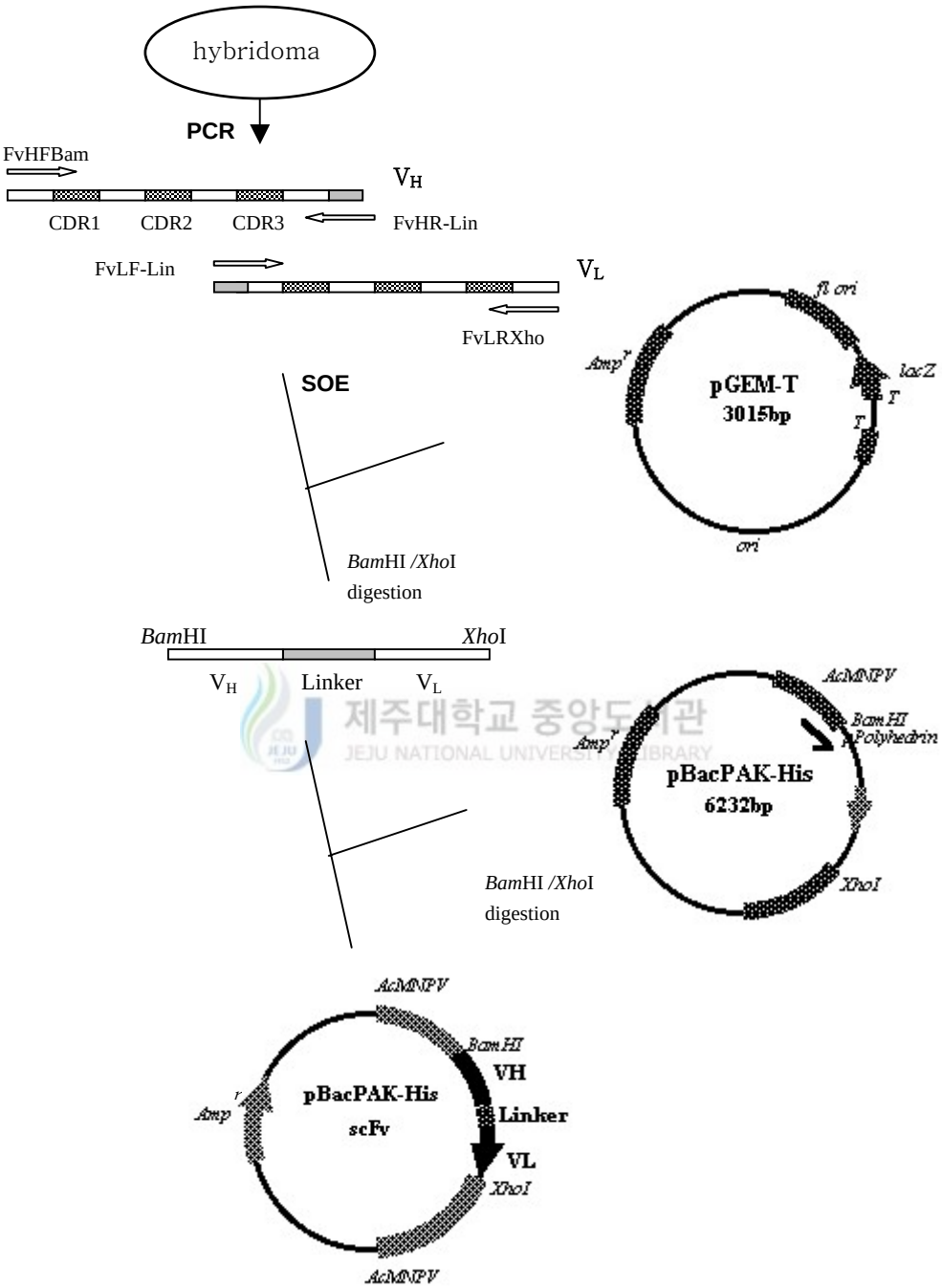

Fig. 1. Flow diagram for the cloning of a single-chain Fv antibody gene and the generation of its pBacPAK-His-scFv expression construct.

3. LH scFvs expressed in a bacterial cell system

3-1. Construction of LH scFvs

cDNAs were synthesized and described previously, except for specific primer sets containing GS $(G_4S)_3$ and the EK(GSTSGSGKPGSGEGSTKG) linker (Table 2), which were used to produce V_L-GS-V_H and V_L-EK-V_H, respectively. The primers used in the PCR amplification were modified from those developed by Min *et al*. (2001). The PCR conditions used were: 30 cycles of 30 seconds at 94℃, 30 seconds at 50℃, and 1 minute at 72℃. The PCR products were gel-purified and cleaved with *Sfi*I and *Not*I. A modified PUC19 plasmid, SNBN119, contained the *pel B* signal sequence for secretion, $(His)_6$ tags, and the unique restriction sites *Sfi*I and *Not*I were used in the cloning and expression of LH scFv 7A3 and 2B1.

Table 2. The oligonucleotide primers used for the amplification of LH scFvs in the orientation of V_L-GS-V_H and V_L-EK-V_H

Primer	Sequence (5'-3')
Mouse L Sfi F	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCGACATTSAGCTSACCCAGTCT
Mouse H Not R	GAGTCATTCTGCGGCCGCTGAGGAGACGGTGACCGTGGT
GS-L-R	*AGAGCCACCTCCGCCTGAACCGCCTCCACC*-CCGTTTBAKYTCCARCTTKGT
GS-H-F	*GGCGGAGGTGGCTCTGGCGGTGGCGGATCG*-AGGTSMARCTGCAGSAGTCWG
EK-L-R	*TTCACCACTCCCGGGTTTGCCGCTACCGGAAGTAGAGCC*CCGTTTBAKYTCCARCTTKGTSCC

EK-H-F	*AGCGGCAAACCCGGGAGTGGTGAAGGTAGCACTAAAGGT*CAGGTSMARCTGCAGSAGTCWGGG

The underlined sequences show the restriction enzyme sites (*Sfi*I and *Not*I) and linker sequences, $(Gly_4Ser)_3$ and EK, in italics.

3-2. Expression and purification of LH scFvs

SNBN119-ligated LH scFvs were transformed into *E coli* BMH cells. After confirming the formation of these plasmids via their cleavage with *Sfi*I and *Not*I, single colonies were grown in 50 ml of 2 × YT medium, supplemented with 20% glucose and 100 μg/ml of ampicillin, at 30℃ overnight. After the centrifugation of the above culture, the pellets were resuspended in 50 ml of 2 × YT medium, and incubated for 5 hours at 25℃, along with the addition of 0.25 mM IPTG. In order to isolate LH scFvs from the periplasm, the cells were harvested by 5 minutes of centrifugation at 10,000 rpm. The pellets were then resuspended and purified under native conditions, according to the instructions provided by Invitrogen.

The cell lysates containing LH scFv under denaturing conditions were resuspended in 20 ml of 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Trizma, 20 mM imidazole and pH 8.0, then incubated at 4℃ overnight. After 30 minutes of centrifugation at 10,000 rpm, the supernatant was incubated for 1 hour at RT on 0.5 ml of nickel- NTA agarose (Qiagen, Hilden, Germany) equilibrated with PBS. After centrifugation at 1,300 rpm and two washes in 25 ml with PBS, the column

was washed with 10 ml of 0.1 M Trizma, 0.5 M NaCl, and pH 7.4 buffer containing 40 mM of imidazole. The column was washed one more time with the 0.1 M Trizma, 0.5 M NaCl, 40 mM imidazole and pH 8.0 buffer. The scFv was eluted with 250 mM of imidazole, dialyzed in PBS, and checked for activity via ELISA. Finally, electrophoresis was performed for the determination of purity. The protein content was determined by a BCA Protein assay (Pierce, IL, USA), using bovine serum albumin as a standard, and by measuring the absorbance at 595nm.

4. Sodium dodecyl sulfate-polyacrlyamide gel electrophoresis (SDS-PAGE) and Western blotting

The fractions containing scFv were analyzed by electrophoresis on a 15% polyacrylamide gel. After separation, the protein bands in the gel were stained with Coomassie brilliant blue G-250, according to Laemmli's method (1970). The flagella were electrophoresed on 12% polyacrylamide gel, and transferred to nitrocellulose membranes (Pierce, IL, USA). The transblotted membranes were then blocked in TBS (25 mM Tris, 150 mM NaCl) containing 3% bovine serum albumin for 1 hour at RT, and were washed three times with a TBS containing 0.05% Tween 20 (TBS-T). ScFvs (about 20 μg/ml) was incubated for 1 hour in TBS-T, and then the monoclonal anti-poly histidine clone His-1 (about 10 μg/ml in TBS-T) was incubated for 1 hour at RT. After three washings in TBS-T, the immunoblot was incubated for 1 hour with an alkaline phosphatase-conjugated

goat anti-mouse IgG in TBS-T. ScFv fragments were visualized with the phosphatase substrate, NBT/BCIP (KPL, MD, USA).

5. Detection of antigen-binding activity by ELISA

ELISA was performed nearly the same way as described previously in Part1. Each well was coated with 10 ㎍/ml of flagella in a 0.05 M carbonate buffer (pH9.5), incubated at 37℃ for 1 hour, and then overnight at 4℃. 100 microliters of each two-fold diluted scFv 7A3 was added to each well on the plates. After 2 hours at 37℃, the goat anti-histidine conjugated HRP was added and incubated for 1 hour at 37℃. The antigen-antibody reaction was visualized when ABTS was added to each well for 30 minutes. The absorbency was then measured at 405 nm with an ELISA plate reader. Furthermore, the sensitivity of scFv was determined by the ELISA involving the coating of the flagella at a 10-fold serial dilution.

6. Immunochromatographic assay

This method was the same as the method described in Cahpter Two. ScFv was used as the capture and detection antibody. Therefore, two different approaches were explored for the construction of an ICA protocol. The pairs consisted of combinations of gold-labeled MAb and scFv coated onto the membrane (Pair1), or the combination of gold-labeled Bac scFv 7A3 and MAb coated onto a membrane (Pair2). The comparative sensitivity of *L. monocyotogenes* 4b in food was performed as was described in Chapter Two.

RESULTS

1. Cloning and expression of scFvs in insect cells and *E. coli*

ScFv 7A3, a covalent linkage of the carboxyl terminus through the V_H domain, and the amino end of the V_L domain through a $(Gly_4Ser)_3$ peptide linker, was cloned in the pBacPAK baculovector. The expected nucleotide size of scFv 7A3 when digested with the restriction enzymes *Bam*HI and *Hind*III was 771 bp (Fig. 2). The recombination of pBacPAK-His-scFv with the baculovirus DNA was transfected into Sf9 cells. In order to ascertain whether the protein was expressed in the insect cells, the recombinant baculovirus was identified via IFA (Fig. 3). The Bac scFv 7A3 was then purified under native conditions. After the purification of a density of 10^6 cells/ml in 800 ml High Five insect cells on a nickel column, we had obtained 200 μg/ml of pure scFv.

Other scFvs, such as LH scFv 7A3, LH scFv 7A3 EK, and LH scFv 2B1 EK were cloned in a prokaryotic vector, SNBN119, containing the *pel B* signal sequence for secretion, $(His)_6$ tags for purification, and the unique restriction sites *Sfi*I and *Not*I (Fig. 2). All of the recombinant proteins exhibited V_L-linker-V_H orientation, and among them, LH scFv 7A3 EK and LH scFv 2B1 EK joined with the EK linker. In order to determine whether the protein presented itself in an insoluble or soluble fraction, cells were sonicated or treated in urea, and the supernatants were then purified. The analysis of purified scFvs revealed that LH scFv 7A3 and LH scFv 7A3 EK were presented in soluble pellets. However, LH

scFv 2B1 EK was probably located within the inclusion bodies. After purification, the four scFvs were subjected to SDS-PAGE, then detected by Coomassie's brillant blue (Fig. 4). The results revealed that the scFv proteins, LH scFvs and Bac scFv 7A3, could be detected at sizes of about 32 and 41 kDa, respectively. These scFvs exhibited higher molecular weights than the expected size, 28.527 kDa for the scFv GS linker, 28.860 kDa for the scFv EK linker, and 29.304 kDa for Bac scFv.

2. Detection of flagella using scFvs by ELISA

ScFvs samples purified from *E. coli* and insect cells using the polyhistidine tail were applied to flagella-coated wells in order to determine its activity. Fig. 5 demonstrates that the scFvs from *E. coli* were unable to detect flagella, with the exception of Bac scFv 7A3 (20 μg/ml), which was from insect cells. Furthermore, the antigen specificity of the baculovirus-derived single-chain Fv antibody, Bac scFv 7A3, was demonstrated by Western blotting (Fig. 6). However, in the case of Bac scFv 7A3, the serial dilution of flagella was tested, and was found to be at least 100 times lower than MAb7A3 with regard to sensitivity. Bac scFv 7A3 proved able to detect approximately 10 μg/ml of flagella (Fig. 7).

3. Sequence of the Bac scFv 7A3 gene

The nucleotide sequences of Bac scFv 7A3 were cloned in pGEM-T easy vector (Fig. 8). A sequence comparison using the data provided by the reports of

Schäble *et al*. (1999), Thiebe *et al*., (1999) and Haines *et al*. (2001) revealed that the V_H region of the 7A3 antibody belonged to the J558 family, exhibiting 95% homology with the J558-19 germline gene. The V_L region exhibited 95% homology with the 12-44 genes of the V_K 12/13 family.

4. Immunochromatographic assay using Bac scFv 7A3

In order to develop the scFv-based ICA protocol, gold was conjugated with 200 μg/ml Bac scFv 7A3 for Pair1. Pair1 consisted of gold-labeled Bac scFv 7A3 and MAb2B1 coated onto membranes. Pair2 consisted of gold-labeled MAb2B1 and 200 μg/ml Bac scFv 7A3 coated on a membrane. Assembled ICAs were performed with serial dilution of *L. monocytogenes* 4b, in order to evaluate the sensitivity afforded by different approaches. However, no positive reactions ensued with any of the pairs. When the serial diluted suspension of purified flagella was dropped into the ICA, the sensitivity of Pair1 exhibited about 30 μg/ml of flagella (Fig. 9).

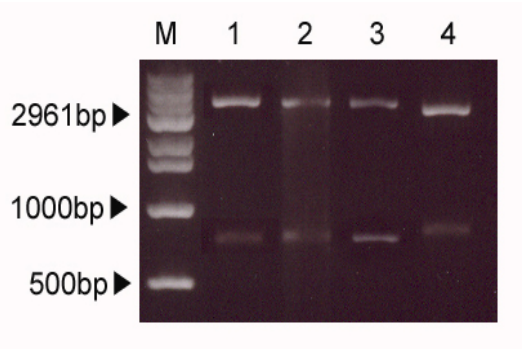

Fig 2. PCR analysis reveals scFvs inserted into different vectors by restriction enzymes. Lanes; M, molecular marker; 1, LH scFv 7A3; 2, LH scFv 7A3 EK; 3, LH scFv 2B1 EK; 4, Bac scFv 7A3. LH scFv 7A3, LH scFv 7A3 EK, and LH scFv 2B1 EK were used in SNBN119 vector containing *Sfi*I and *Not*I. Bac scFv 7A3 was used in pBacPAK containing *Bam*HI and *Xho*I.

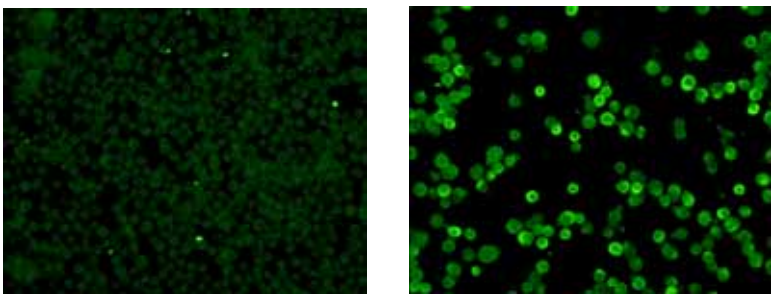

Fig. 3. Detection of recombinant single-chain Fv antibody (Bac scFv 7A3) in pBacPAK-His-scFv transfected into Sf9 cells by immunofluorescent assay with monoclonal anti-poly histidine clone His-1 (about 10 μg/ml). The left indicates a control, and the right shows pBacPAK-His-scFv transfected into Sf9 cells.

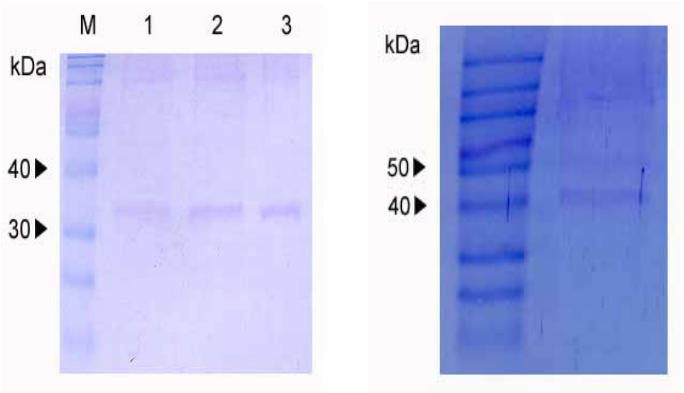

Fig. 4. SDS-PAGE analysis of scFvs purified with nickel-NTA agarose. Samples were electrophoresed onto 15% SDS-polyacrylamide gel. Lane M, low molecular marker; lane 1, LH scFv 7A3; lane 2, LH scFv 7A3; lane 3, LH scFv 2B1 (left); Bac scFv 7A3 (right).

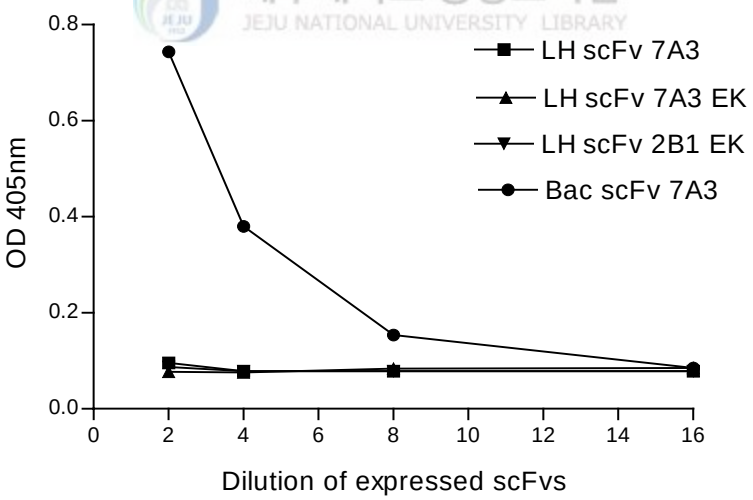

Fig. 5. Binding activity of the purified baculovirus expressed Bac scFv 7A3, and *E. coli* expressed scFvs, LH scFv 7A3, LH scFv 7A3 EK, and LH scFv 2B1 EK for flagella.

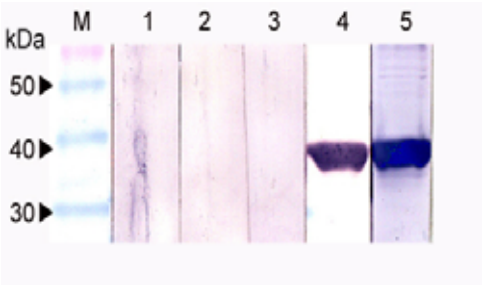

Fig. 6. Reactivity of the Bac scFv 7A3 on the Western blot analysis of flagella from *L. monocytogenes* 4b. The flagella were loaded on 12% SDS-PAGE gel. Western blotting was performed, using scFvs as a primary antibody. Lane M, 1ow molecular marker; lane 1, LH scFv 7A3; lane 2, LH scFv 7A3 EK; lane 3, LH scFv 2B1 EK; lane 4, Bac scFv 7A3; lane 5, MAb7A3.

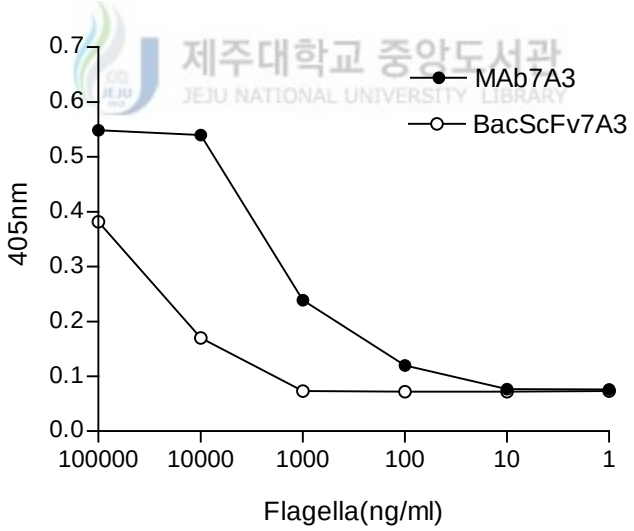

Fig. 7. Determination of the ELISA detection limit for flagella. Wells of a microtiter plate were coated with flagella at concentrations ranging from 100 ㎍/ml to 1 ng/ml. 20 ㎍/ml Bac scFv 7A3 and MAb7A3 were incubated as primary antibodies.

CAG GTC CAA GCC GCA GAG TCC GGG GCT GAG CTG GCA AGA CCT GGG GCT TCA GTG

Gln Val Gln Ala Ala Glu Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val

AAG TTG TCC TGC AAG ACT TCT GGC TAC ACC TTT ACT AGG TAC TGG ATG CAG TGG

Lys Leu Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr Arg Tyr Trp Met Gln Trp

VH CDR1

GTA AAA CAG AGG CCT GGA CAG GGT CTG GAA TGG ATT GGG ACT ATT CAT CCT GGA

Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Thr Ile His Pro Gly

GAT AAT AAT ACT AAG TAC ACT CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GCA

Asp Asn Asn Thr Lys Tyr Thr Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala

VH CDR2

GAT AAA TCC TCC ACC ACA GCC TAC ATG CAA CTC AGC AGC TTG ACA TCT GAG GAC

Asp Lys Ser Ser Thr Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp

TCT GCG GTC TAT TAT TGT GCA AAT GAC TAC TAT AAT GAC GAC GGG TTT GCT TAC

Ser Ala Val Tyr Tyr Cys Ala Asn Asp Tyr Tyr Asn Asp Asp Gly Phe Ala Tyr

VH CDR3

TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA GGT GGA GGC GGT TCA GGC GGA

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala Gly Gly Gly Gly Ser Gly Gly

Linker peptide $(Gly_4Ser)_3$

GGT GGC TCT GGC GGT GGC GGA TCG GAC ATC CAG ATG ACC CAG ACT CCA TCC TCC

Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met Thr Gln Thr Pro Ser Ser

TTA TCT GCA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT CGA ACA AGT GAA AAT

Leu Ser Ala Ser Val Gly Glu Thr Val Thr Ile Thr Cys Arg Thr Ser Glu Asn

ATT TAC AGT TAT TTA GTA TGG TAT CAG CAG AAA CAG GGA AAA TCT CCT CAG CTC

Ile Tyr Ser Tyr Leu Val Trp Tyr Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu

VL CDR1

CTG GTC TAT AAT GCA GAA ACC TTA GCA GAA GGT GTG CCA TTA AGG TTC AGT GGC

Leu Val Tyr Asn Ala Glu Thr Leu Ala Glu Gly Val Pro Leu Arg Phe Ser Gly

VL CDR2

AGT GGA TCA GGC ACA CAA TTT TCT CTG AAG ATC AGC AGC CTG CAG CCT GAA GAT

Ser Gly Ser Gly Thr Gln Phe Ser Leu Lys Ile Ser Ser Leu Gln Pro Glu Asp

TTT GGG AGT TAT TAC TGT CAA CAT TAT TAT GGT TCT CGC CGG ACG TTC GGT GGA

Phe Gly Ser Tyr Tyr Cys Gln His Tyr Tyr Gly Ser Arg Arg Thr Phe Gly Gly

VL CDR3

GGC ACC AAG CTG GAA ATC AAA CGG GCG GCC GCA TGA

Gly Thr Lys Leu Glu Ile Lys Arg Ala Ala Ala ***

Fig. 8. The nucleotide and amino acid sequences of the baculovirus expressing Bac scFv 7A3. Residues corresponding to the complementarity-determining (CDRs) regions were boxed and shaded.

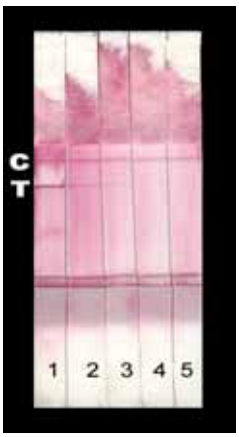

Fig. 9. Detection limit of the immunochromatographic assay using the purified flagella. Lanes, 1, 3 mg/ml; 2, 300 μg/ml; 3, 30 μg/ml; 4, 3 μg/ml; 5, PBS.

DISCUSSION

Antibody engineering has developed with the use of a variety of expression systems and applications in biology and biotechnology. In this study, we cloned a highly specific anti-flagella scFvs with a series of primer sets. The cloned scFvs were expressed in *E. coli* and insect cells, and then compared with regard to their activities. Finally, scFvs were used in the development of a novel immunochromatographic assay, which allowed for a cheap and reliable approach to *Listeria* detection.

Specific scFvs for flagella were produced under two different expression systems, namely, *E. coli* and insect cells. First, the pBacPAK vector containing the polyhedrin promoter and polyhistidine tail was used for expression in insect cells. A few reports have dealt with scFv expression. These authors exploited the secretory pathway, using an antibody k chain secretion signal peptide, the baculovirus GP67 signal sequence. These studies also utilized a mouse V47 VH signal sequence for the secretion of scFvs into the medium (Laroche *et al*., 1991, Kretzschmar *et al*., 1996, Lemeulle *et al*., 1998). They were able to purify 4.8 mg-32 mg of monomeric scFvs per liter of insect culture. However, the secretory pathway used in other reports differs from that used in this study, as the produced protein was almost completely cell-associated. The yield of Bac scFv 7A3 in this system was 2 mg per liter in the spinner culture. The detected amount of protein was lower than the amount obtained by other recombinant antibodies in the

secretion sequence containing the baculovirus vector.

Second, the *E. coli* vector, SNBN119, which contained the *pel* B signal sequence and a unique restriction enzyme site, was used in the cloning and expression of scFvs. Using this vector, we expressed LH scFv 7A3, LH scFv 7A3 EK, and LH scFv 2B1 EK. These recombinant single-chain Fv antibody constructs exhibited the V_L-linker-V_H domain order, but the Bac scFv 7A3 was oriented in reverse, i.e. V_H-linker-V_L. Anand *et al*. (1991) reported that orientation was enhanced in the levels of secreted functional scFv. However, Huston *et al*. (1988) showed that V_H-V_L orientation led to the formation of a higher affinity binding site for scFv than did reverse configuration. LH scFv 2B1 EK accumulated and formed insoluble cytoplasmic inclusion bodies, even though the *pel B* signal sequence was contained in the expression vector, consistent with the report of Jonge *et al*. (1995).

Functional ELISA tests revealed that scFvs, like LH scFv 7A3, LH scFv 7A3 EK and LH scFv 2B1 EK, from the *E. coli* system were unable to detect flagella, with the exception of Bac scFv 7A3. Lemeulle *et al*. (1998) discussed the different character of the scFvs produced in *E. coli* and baculovirus expression systems. However, this study indicated that this difference may have resulted from the reverse orientation of scFvs. The Bac scFv 7A3 from the insect cells exhibited an affinity of 1/100 of that of the native monoclonal antibody. The causes of this lower affinity may have included the absence of constant domains, or the possibility that the linker peptide may not have been sufficient to stabilize

the conformation of the antigen-binding domain. Therefore, some recombinant scFv or Fab fragments may have exhibited lower affinities (He *et al*., 1995, Huston *et al*., 1988, Koo *et al*., 1998). However, the ability of scFv varies in this regard. Byrne *et al*. (1996) demonstrated that scFv which could recognize atrazine exhibited better sensitivity than did the intact antibody.

The V_H sequence of Bac scFv 7A3 exhibited some sequence differences from sequenced members in the antibody database. The Bac scFv 7A3 V_H gene mutated, evidencing 13 amino acid changes as compared to the germ line J558 family. These changes were clustered in the CDRs, particularly in CDR2. This implies that the CDR2 in the V_H of Bac scFv 7A3 may play an important role in the determination of the specificity of flagella binding. Furthermore, framework 1 of V_H exhibits 4 amino acid changes. Many authors have reported that the CDRs and framework1 of mutations were affected by the fine specificity properties exhibited by the various antibodies. In contrast, the V_K in Bac scFv 7A3 had only 7 amino acid changes when compared with its possible germ line gene, 12-44. This property may contribute to the lesser degree of specificity evidenced by this variant (Jahn *et al*., 1995, Li *et al*., 1996, Lemeulle *et al*. 1998).

Some reports have involved the use of recombinant antibodies in the development of an immunoassay. Kerschbaumer *et al*. (1997) reported on the utility of scFv in sandwich ELISA as a coating and detecting reagent. They generated a coating reagent by the fusion of a leucine zipper protein. Subsequently, a detecting reagent was fused with scFv to enzymatically-active

alkaline phosphatase for the reduction step. This product, when developed by ELISA, evidenced a higher sensitivity than did the ELISA which utilized polyclonal antibodies.

Thus far, none of the previous reports of anti-flagella recombinant single-chain antibodies have been described in terms of immunochromatographic assay applications. In this study, however, Bac scFv 7A3 was tested as a capture antibody, as well as a gold-labeled detection antibody with two different sets of antibodies in ICA. Pair1, consisting of the combination of gold-labeled detection antibody Bac scFv 7A3 and MAb2B1 stripped on a nitrocellulose membrane, evidenced no signal when exposed to an *L. monocytogenes* culture and subjected to ICA. A similar result was seen for the opposition pair, Pair2. However, when the serial diluted flagella were dropped into the gold-labeled Bac scFv 7A3 soaked glass membrane, Pair1 exhibited a sensitivity of 30 μg/ml. This result might be attributable to a lower avidity than that of MAb7A3, as well as to the generally low levels of gene expression observed in the baculovirus expression system. Also, this particular scFv was relatively poor in the lysine, tryptophan, and cysteine residues that attach to gold particles, which may have impaired the sensitivity (Chandler *et al*., 2000).

Emanuel *et al*. (2000) reported on a recombinant Fab which exhibited 2-4 fold higher sensitivity than the MAb which had been successfully integrated into the ICA. However, this report did not generate detailed data. If the drawbacks inherent in this approach could be improved, the combination of recombinant

antibodies with capture and detection antibody in an ICA would carry with it many advantages. Such an ICA protocol could result in the development of a standardized diagnostic system, and would also be less expensive. As described elsewhere in Chapter Two, ICA has become one of the most rapid screening methods for the detection of microorganisms. Due to its low cost and high affinity for recombinant antibodies, these methods produce efficient and effective probes. In this study, a single-chain Fv antibody which is specific for flagella was produced, and demonstrated the possibility that scFv could be used as a detection antibody in the production of diagnostic kits.

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GENERAL CONCLUSIONS

Listeria outbreaks are primarily reported in advanced countries. For instance, during a 3-month period in 2002, the US logged seven deaths and three stillbirths, or miscarriages, resulting from *L. monocytogenes* infections, all of which were linked to the consumption of foodstuffs. In Korea, changing consumption patterns and methods of food processing have increased the potential risk of a *Listeria* outbreak. Even though only the strain *L. monocytogenes* is pathogenic in humans, the detection of *Listeria* spp. overall can be used as an indicator for poor hygiene. The purpose of this study, then, was to develop a specific, rapid, reliable, and convenient assay for the detection of *Listeria* spp.

Chapter Ⅰ focused on the production of specific flagella antibodies, monoclonal antibodies (MAbs), yolk immunoglobulin (IgY), and rabbit polyclonal antibodies, all of which were designed to react with genus-specific *Listeria*. For this, the flagella of *L. monocytogenes* 4b, as confirmed by EM, were purified from cultured cells. The molecular weight of the flagellin was approximately 33 kDa according to SDS-PAGE. Each antibody against *Listeria monocytogenes* was developed by immunization with purified listerial flagella. Five monoclonal antibodies were characterized, and all indicated that the epitopes of listerial flagellin were located in three different sites. Other antibodies, IgY and rabbit polyclonal antibody, also exhibited good responses to the flagella. All three antibody variants evidenced specific reactions to the *Listeria* genus, and showed

no cross-reactivity with the other bacteria tested in this experiment, including *E. coli* O157:H7 and *Salmonella enteritidis*. The antibodies produced in this study could all be used as serviceable probes for developmental immunoassays.

Chapter Ⅱ focused on the development and evaluation of novel sandwich ELISA and immunochromatographic assay (ICA) protocols. The three tested pairs: Pair1, the combination of HRP-labeled MAb7A3 and MAb2B1, Pair2, the combination of HRP-labeled IgY and MAb2B1, and Pair3, the combination of HRP-labeled rabbit polyclonal antibody and MAb2B1, were coated onto microplate wells and subjected to sandwich ELISA. As the flagella were expressed in a temperature-dependent manner, the sensitivity of both assays was compared using 22℃ and 30℃ culture samples. Pair2 exhibited a sensitivity 10 times lower than that associated with Pairs 1 and 3, at both temperatures. Therefore, the development of the ICA was predicated on the results of the sensitivity of the respective sandwich ELISA tests. When Pair1 and Pair3 were used in the ICA, Pair1 exhibited a detection limit of 10^5 cells/0.1ml and 10^6 cells/0.1ml at 22℃ and 30℃, respectively. However, the detection limit of the ICA used for Pair3 at a culture temperature of 30℃ was only very weakly positive. Finally, the ICA which used a monoclonal antibody pair was further evaluated, in order to determine its sensitivity in spiked food samples. When the 27 samples, artificially contaminated with low levels of *Listeria monocytogenes* 4b culture, were dropped into a glass fiber membrane, all samples were confirmed positive by both assays. This result demonstrated that both assays had the same

efficiency with regard to the detection of *Listeria* spp. in food. However, the ICA could be completed more rapidly (15 minutes) than could the sandwitch ELISA (2 hours) after the processing of the enrichment culture.

Chapter Ⅲ evaluated the application of a single-chain Fv antibody as a capture and detection antibody in an ICA. The single-chain Fv antibody was formed in heavy- and light-variable regions (V_H and V_L), connected by a linker peptide. We expressed scFvs in both insect cells and *E. coli*, and compared them in terms of their activity. Among these, only Bac scFv 7A3, expressed in baculovirus, demonstrated any activity for flagella when tested by ELISA, FA, or immunoblotting. A 723-base pair DNA of Bac ScFv 7A3 exhibited sequence differences from other sequenced members in the antibody database. When the Bac ScFv 7A3 was tested in the ICA as a capture and detection antibody with MAb2B1, there were no positive results for *Listeria monocytogenes* 4b. However, 30 μg/ml of flagella could be detected by the ICA with the gold-conjugated Bac scFv 7A3 and MAb2B1 pair.

This study was undertaken in order to develop a method for the rapid testing and screening of *Listeria* spp. in foods. In conclusion, flagella were proven to be a viable antigen for the induction of antibodies, MAbs, IgY, and rabbit polyclonal antibody, specific to *Listeria* spp. ICA and sandwich ELISA based on the MAbs pair were developed and evaluated. As described above, ICA appears to be a more practical screening protocol than sandwich ELISA, due to its shorter detection time, and improved simplicity. In addition, the last part demonstrated the

possibility of using scFv as a gold-conjugated detection antibody in the ICA. scFv expressed in insect cells may constitute a new reagent in the field of rapid testing.

ABSTRACT in KOREAN [국문초록]

리스테리아 오염 스크리닝을 위한 신속 진단법 개발

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리스테리아는 사람에 감염시 심각할 경우 뇌막염 및 패혈증, 임신부에게는 유산을 일으킬 수 있는 공중보건학 상 중요한 식중독균 중의 하나이다. 이를 검출하기 위한 진단법 개발을 위해 첫번째로 리스테리아 편모항원를 생산하였다. 22℃에서 48시간 배양한 *Listeria monocytogenes* 4b를 glass bead를 이용하여 편모를 떨어뜨린 후 초원심분리를 이용하여 정제하였다. 정제된 편모를 전기영동 한 결과 33 kDa의 크기를 나타내었으며 정제과정의 편모는 SEM 및 TEM으로 확인하였다. 편모 특이 항체로는 단클론항체, 난황항체 및 토끼에서 다클론항체를 생산하였다. 단클론항체는 융합을 통해 5개의 편모 특이 단클론항체를 생산하였으며, 효소면역법으로 5개의 단클론항체는 크게 3개의 그룹으로 나눌 수 있었으며 이를 통해 편모에 적어도 3개의 에피토프가 존재함을 알 수 있었다. 난황항체인 경우 고역가의 항체 생산을 위해 50 ㎍/ml, 200 ㎍/ml, 400 ㎍/ml의 편모를 닭의 가슴부위에 접종한 결과 다른 그룹에 비해 200 ㎍/ml의 항원농도가 가장 높은 항체수준을 유지하고 있음을 알 수 있었으며 난황에서의 항체는

polyethylene glycol과 친화성컬럼을 통과하여 정제하였다. 마지막으로 토끼에서도 편모특이 다클론항체를 생산한 후 정제하였다. 생산된 3 종류의 항체는 본 실험에서 사용한 리스테리아와 특이반응을 보였으며, 여타의 다른 세균과는 반응하지 않음을 효소면역법을 통해 확인하였다.

Sandwich ELISA 개발을 위해 단클론항체 7A3, 난황항체, 토끼 다클론항체를 periodate법으로 HRP와 접합시켜 detection항체로 사용하였다. 각 well에 단클론항체 2B1을 코팅한 후 *Listeria monocytogenes* 4b를 반응시킨 후 위의 detection항체들을 각각 반응시킨 결과 난황항체를 이용한 경우의 민감도가 제일 낮은 것으로 나타났다. 단클론항체인 경우 22℃ 및 30℃ 모두에서 1×10^4 cells/0.1ml의 민감도를 보였으며 토끼 다클론항체인 경우 1×10^4 cells 와 1×10^3 cells/0.1ml 의 민감도를 나타내었다. 이의 결과를 바탕으로 면역크로마토그래피법 (ICA)을 개발하였다.

먼저 40nm골드를 만들기 위해 reducer로 sodium citrate를 사용하여 작성하였다. 작성된 골드는 TEM으로 확인하였다. 40nm골드는 단클론항체 및 토끼 다클론항체에 각각 접합시켜 glass membrane에 적신 다음 37℃에서 1시간 말린 후 ICA에 적용하였다. 이 또한 비리스테리아균과 반응을 보이지 않았으며 본 실험에서 사용된 리스테리아와는 특이반응을 보였다. 개발된 ICA의 민감도를 알아 보기 위해 22℃와 30℃에서 각각 키운 *Listeria monocytogenes* 4b를 10배 계단희석하여 점적한 결과 22℃ 와 30℃ 에서 각각 10^5 cells/0.1ml 와 10^6 cells/0.1ml 보였다. 그러나 토끼 다클론항체인 경우 22℃ 에서 10^5 cells/0.1ml 을 보였으나 30℃에서는 약한 반응을 보였다. 따라서, 이후의 ICA는 단클론항체 조합으로 실시하였다. Spike실험은 25g의

돈육 및 25ml의 우유에 *Listeria monocytogenes* 4b를 적은 수로 희석 후 접종하였다. 모든 27개의 spiked 시료는 ICA 및 sandwich ELISA에서 양성을 나타내었다. 따라서, 리스테리아종 사이의 민감도가 동일하지 않더라도, 48시간의 배양은 본 실험에서 개발된 진단방법으로 검출할 수 있을 만큼의 세포수로 증가할 수 있기 때문에 두 방법 모두 리스테리아를 충분히 스크리닝 할 수 있을 것으로 여겨지며 특히 ICA는 15분이면 양성 및 음성을 판단할 수 있기 때문에 sandwich ELISA 보다 더 효율적으로 사용이 가능하다고 판단 되었다.

진단킷트에 사용되는 단세포군 세포주는 염색체의 불안정성으로 인해 항체생성이 감소되는 단점을 가지고 있어 본 연구에서는 단세포군 세포주의 유전자를 클로닝 한 후 재조합항체를 발현하여 진단킷트에 적용하고자 하였다. 편모항체를 생산하는 단세포군 세포주에서 mRNA를 추출한 후 V_H와 V_L을 Linker peptide로 연결한 유전자를 클로닝하고 곤충세포와 대장균에 각각 발현하였다. 발현된 재조합항체 (scFv) 중 baculovirus system에서 유래된 Bac scFv 7A3만 효소면역법, FA 및 Western blotting assay에서 biological activity를 나타내었다. 염기서열 분석 시 V_H는 germ line J558 family의 J558-19와 V_L인 경우 V_K 12/13 family의 12-44와 각각 95%의 상동성을 보였다. MAb7A3과 Bac scFv 7A3의 편모에 대한 검출한계 비교시 각각 0.1 ㎍/ml과 10 ㎍/ml을 보여 주어 MAb7A3이 100배 높은 민감도를 나타내었다. 발현된 재조합항체를 이용하여 ICA에 detection 항체로 적용하여 보았다. 40nm gold에 접합된 재조합항체를 glass membrane에 적신 후 리스테리아를 점적한 결과 양성 반응을 보이지 않았으나 편모항원인 경우 30 ㎍/ml까지

검출이 가능하였다. 따라서, 발현시킨 재조합항체는 ICA에서 detection 항체로서의 응용이 가능함을 보여주었다.

감사의 글

저의 소박한 노력의 결과가 끝을 맺었습니다. 공허함과 부족함을 뒤로하고 우연히 읽었던 책에서 '우리는 닻을 올렸으며 수평선은 희망으로 가득 차 있다'라는 구절이 떠오릅니다. 이 논문이 앞으로 저의 삶에 조그마한 원동력이 되었으면 합니다.

많이 부족한 저를 받아주시고 지속적인 관심과 가르침으로 채워주셨던 임윤규 교수님께 무한한 존경과 감사를 드립니다.

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또한 언제나 기쁨과 슬픔을 같이 나눌 수 있는 친구들, 선배, 후배들 그리고 저에게 용기를 주었던 모든 이들에게도 감사드립니다.

진심어린 애정과 인내심으로 곁에서 한결같이 지켜봐 주는 식구들,,,, 끝없는 믿음을 보여주시는 엄마, 지금 계셨더라면 누구보다도 기뻐해 주셨을 아빠에게 무한한 사랑과 고마움을 전합니다.