Production of antibodies against *Salmonella* Enteritidis

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Abstract

Salmonella enterica serovar Enteritidis (SE) is a common pathogen of all mammal species and fowls. The increase of food-borne salmonellosis due to SE outbreaks in human causes pandemic disease. Our research aim is to produce and develop antibodies specific to SE which can identify and differentiate SE from other *Salmonella* spp. Monoclonal antibodies were produced by BALB/c which are immunized with whole cell of heat-killed plus sonicated and heat-killed plus SDS-mercaptoethanol treated SE. Monoclonal antibodies specificity against SE were determined by dot blotting and the antigen recognition was detected by Western blotting. And only twelve monoclonal antibodies against SE ATCC 13076 were selected from six immunized mice. Determination of isotypes, sensitivity, specificity and antigen recognition of twelve monoclonal antibodies were described in this report.

Keywords: *Salmonella* Enteritidis, monoclonal antibodies, dot blot

Introduction

Salmonellosis is one of the most common and widely pandemic food-borne diseases and caused by *Salmonella*. *Salmonella* group is the large variant group of bacteria which can be differentiated by using serotyping. The serotyping method by the Kauffman-White scheme based on O (somatic) and H (flagellar) surface antigens has been used to subtype *Salmonella enterica*. Today, this species consisted of >2,500 different serotypes (Braden 2006). Nevertheless, only 4 serotypes are reported as a mainly associated with human pathogens which include *Salmonella* Typhimurium (ST), SE, *Salmonella* Newport and *Salmonella* Javiana (Velge et al. 2005).

The human illness caused by SE infection has been increased through worldwide. Since 1990, this serovar has been displaced ST as the primary cause of salmonellosis in the world. SE is a human pathogen which regularly contaminate on eggs and poultry products. Although, the environment of chicken farm is a source of a rich number of *Salmonella* serotypes, SE is the main serotype found in birds, chickens and suspected eggs (Guard-Petter 2001).

The SE pandemic involves in many interactions of the pathogen with multiple factors. The contamination in hen farm transmission to poultry products and eggs is the vehicle for transferring to human food chain (Guard-Petter 2001). Identification of bacterial characteristics unique to SE should help to separate this serovar from other *Salmonella* serotypes in environment and food that will be useful for pandemic control of this pathogen. In addition, determination of SE in poultry products is required in order to ensure the safety of export and import foods (Braden 2006).

The several methods for *Salmonella* spp. detection in food have been developed such as culturing techniques, bacteriophage specificity, DNA hybridization, polymerase chain reaction (PCR) and immunoassay. The standard culturing techniques for *Salmonella* detection has multi-steps with taking 4-5 days for detection and confirmation. The PCR, DNA hybridization, and bacteriophage methods were developed to reduce time for detection in 2-3 days, but none of them has been identified as fast, specific, and user-friendly for screening food samples routinely. Immunoassay for bacterial detection has been shown to be a stable and reliable method with easy to use, gives specific for target host (Yan et al. 2003). Therefore, mice antibodies specific to SE for use as a material to identify and differentiate SE precisely from other *Salmonella* spp. and other bacteria will be produced in this study.

Methodology

Bacteria strain and antigen preparation

SE ATCC 13076, *Salmonella* spp. and other bacteria were cultured in tryptic soy broth (TSB) (Difco Laboratories, USA) with agitation at 37 °C for 24 h. All bacteria were harvested and resuspended in phosphate buffer saline (PBS), adjusted OD_{660} ~1. Antigens for immunization, SE ATCC 13076 was prepared in two forms; heat-killed plus sonicated and heat-killed plus Sodium dodecyl sulfate (SDS)-mercaptoethanol (Sigma-Aldrich, USA) treated forms. Antigen in heat-killed form was prepared by using bacterial suspension with heat-killed at 60 °C for 60 min. Another sonicated form was prepared by using heat-killed bacteria for sonicated 4 times for 15 min at 80 Hz. SDS-mercaptoethanol form was prepared by mixing 4% (w/v) SDS and 10% mercaptoethanol at ratio 1:1 (v/v) with heat-killed bacteria, and then this suspension was boiled for 90 sec and dialyzed against 3x changes of PBS 12 h.

Production of monoclonal antibodies

Immunization of mice with SE: six BALB/c mice of 8 weeks old were immunized by intraperitoneal injection with antigen. A mouse serum before immunization was collected for use as a negative control. Three mice were immunized by intraperitoneal injection with heat-killed forms mixed with sonicated forms and Freund's complete adjuvant (Sigma-Aldrich, USA). And other three mice were immunized by intraperitoneal injection with heat-killed forms mixed with SDS-mercaptoethanol treated forms. After that, all of mice were boosted with the same antigen with Freund's incomplete adjuvant (Sigma-Aldrich, USA) at two week intervals. A week after third boost, the mouse antisera were collected by tail breeding for checking the serum titer against SE by dot blotting. Three days before heart breeding, each of mice was final boosted by intraperitoneal injection with the same antigen without adjuvant. The antiserum of each mouse was collected and used for test it's characteristic and specificity against SE by dot blotting. Generation of hybridoma, the spleen cells of immunized mice were fused with P3X myeloma cells with 50% polyethylene glycol. Hybridomas against SE were screened and characterized by dot blotting and Western blotting.

Characterization of monoclonal antibodies

Specificity test by dot blotting: SE ATCC 13076, *Salmonella* spp. and other bacteria were used in heat-killed forms. All of bacteria were spotted on each block of 4x4 mm nitrocellulose membrane (Santa Cruz, USA) as 1 µl/spot and dried at 37 °C for 30 min. The dot blot membranes were blocked with skim milk in 0.05% Tween 20 (PBST). Then, the

membrane was incubated with antiserum or hybridomas supernatant in skim milk for 3 h and washed with PBST. Horseradish peroxidase-conjugates goat anti mouse IgG (GAM-HRP) (1:3,000) (Jackson Immuno, USA) in PBS was added onto the membrane and incubated at room temperature for 3 h and washed. After that, Diaminobensidine tetrahydrochloride (DAB) substrate solution (Sigma-Aldrich, USA) was added and reacted at room temperature for 5 min. Then, the reaction was stopped by using distilled water (Khamjing et al. 2001).

Sensitivity test: five-fold serial dilutions of heat-killed SE ATCC 13076 were diluted in PBS. One microlitre of each dilution was spotted onto a nitrocellulose membrane at the range of 1.14×10^4 – 8.90×10^8 CFU/ml and detected by using various monoclonal antibodies at dilution 1:10. The process for detection used dot blotting as described above.

Antigen recognition by Western blotting: sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method that used in this work was modified from Laemmli (1970). The method used 10% of separating gel for separating the samples. Protein marker PS10 (GeneOn, Germany) with a molecular weight ranging from 11 to 180 kDa was used as a standard molecular weight. Total protein of each antigen was assayed by using protein assay kit (Bio-Rad, USA). About thirty micrograms of proteins in each antigen were used to run gel electrophoresis (Mini-Protein® Tetra System for SDS-PAGE, Bio-Rad, USA) at 100 V for 90 min and gels were stained with Coomassie brilliant blue solution (USB Corporation, USA). After running completely, protein bands in gels were transferred to nitrocellulose membrane by using the semi-dye transblot apparatus (Bio-Rad, USA) with operated at 15V 30 min. The membrane was blocked in 5% skim milk in PBS incubated 30 min and washed three times with PBST. After that, the membrane was incubated with antiserum or hybridoma supernatant in 5% skim milk for 2 h and washed with PBST. GAM-HRP was incubated at room temperature for 3 h, washed and developed with DAB substrate solution.

Isotype determination: isotype of mouse immunoglobulins from hybridomas were determined for class and subclass based on sandwich ELISA using Sigma's Mouse Monoclonal Antibody Isotyping Kit (Sigma-Aldrich, USA).

Results

Production of monoclonal antibodies

Six BALB/c mice of 8 weeks were immunized by heat-killed plus cell sonicated form and heat-killed plus SDS-mercaptoethanol treated forms. Antiserum from six BALB/c mice was determined for the antiserum titer by dot blotting technique and specificity with various bacterial strains. The titers of all antisera were in the range approximately 3.2×10^4 to 1.02×10^6 CFU/ml. The negative control was non-immunized mouse serum (data not shown). The antiserum titers of six immunized mice were high enough to enable their use as spleen donors for the process of hybridoma production.

Each fused cells of the immunized mice were distributed into 96-wells plates. The fused cells secreting the antibodies against SE ATCC 13076 were selected. The total of 12 monoclonal antibodies were produced from six mice fusions (Table1). Six clones (SCR23, SCR26, SCR50, SCL18, SCL28 and SCN35) from mice immunized with heat-killed plus cell sonicated forms, whereas 6 clones (SDSR3, SDSR66, SDSL29, SDSL64, SDSL75 and SDSN36) from heat-killed plus SDS-mercaptoethanol treated forms were obtained.

Table 1 Summary of hybridoma production

Mouse no.	Number of well for culture	Percent of hybridoma	1st screen (well)	2nd screen (well)	Monoclone number
1	672	46.43	74	19	SCR23, SCR26, SCR50
2	672	40.92	46	8	SCL18, SCL28
3	672	40.48	67	10	SCN35
4	576	88.72	142	18	SDSR3, SDSR66
5	576	82.64	100	20	SDSL29, SDSL64, SDSL75
6	576	81.60	52	12	SDSN36

Characterization of monoclonal antibodies

All of monoclonal antibodies from Table 1 were tested for the specificity, sensitivity, isotype determination and antigen recognition by dot blotting, isotype test kit and Western blotting technique, respectively. The 12 monoclonal antibodies showed cross-reactivity to SE ATCC 13076, *S.* Typhi and showed weakly with *S.* Paratyphi A. (Figure 1). Moreover, SCR50 and SDSL75 reacted strongly with ST DMST 34042. SDSN36 showed weak cross-reactivity to *E. coli* ATCC 25922. SCL18 reacted weakly with *Salmonella* spp. Our monoclonal antibodies was specific only to *Salmonella* group and no cross reaction with other group of Gram-negative bacteria tested (except SDSN36).

Monoclonal antibodies could be divided into 3 isotype groups-; IgG2b (SCR23), IgG1 (SCR26, SCL18, SCL28, SCN35, SDSR3, SDSL29 and SDSL75) and IgM (SCR50, SDSR66 and SDSL64) (Table 2). The antigen recognition of individual monoclone was in the range of 35.6 to 155.0 kDa. SCR23, SCR26, SCR50 and SDSL29 recognized a smear ranging from 63.5 to 155.0 kDa. SCL18 recognized protein bands at molecular weights from 63.5 to 104.5 kDa and 45.1 kDa. The four monoclones, SCL28, SDSR3, SDSL64 and SDSL75 only reacted with 58.7 kDa protein strongly. And other three clones SCN35, SDSR66 and SDSN36 recognized 45.1; 39.5 & 58.7 and 35.6 & 39.5 kDa proteins, respectively. In addition, the sensitivity of all monoclonal antibodies was in the range of 7.12×10^{6} to 3.56×10^{7} CFU/ml (data not shown). Moreover, SCR23, SCL18, SCN35, SDSL29 and SDSN36 showed the highest sensitivity of 7.12×10^{6} CFU/ml by dot blot assay. Characteristics of monoclonal antibodies against SE were summarized in Table 2.

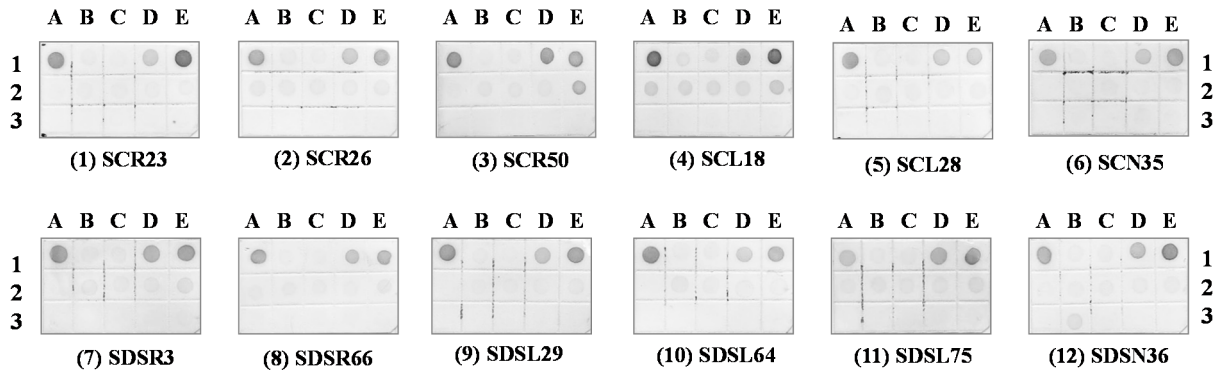

Figure 1 Specificity of monoclonal antibodies assayed by dot blotting. Heat-killed bacteria were spotted onto nitrocellulose membrane 1 µl/spot and treated with monoclonal antibodies which were screened. List of bacteria were spotted onto each block of nitrocellulose membrane as follows: Row 1: (A) SE ATCC 13076, (B) SE, (C) SE 1773-72; (D) S. Paratyphi A, (E) *S.* Typhi, Row 2: (A) ST ATCC 13311, (B) ST ATCC 14028, (C) ST (Copenhagen), (D) ST DMST 34038, (E) ST DMST 34042, Row 3: (A) *Citrobacter freundii*, (B) *Escherichia coli* ATCC 25922, (C) *Enterobacter cloacae*, (D) *Proteus mirabilis*, (E) *Serratia marcescens.*

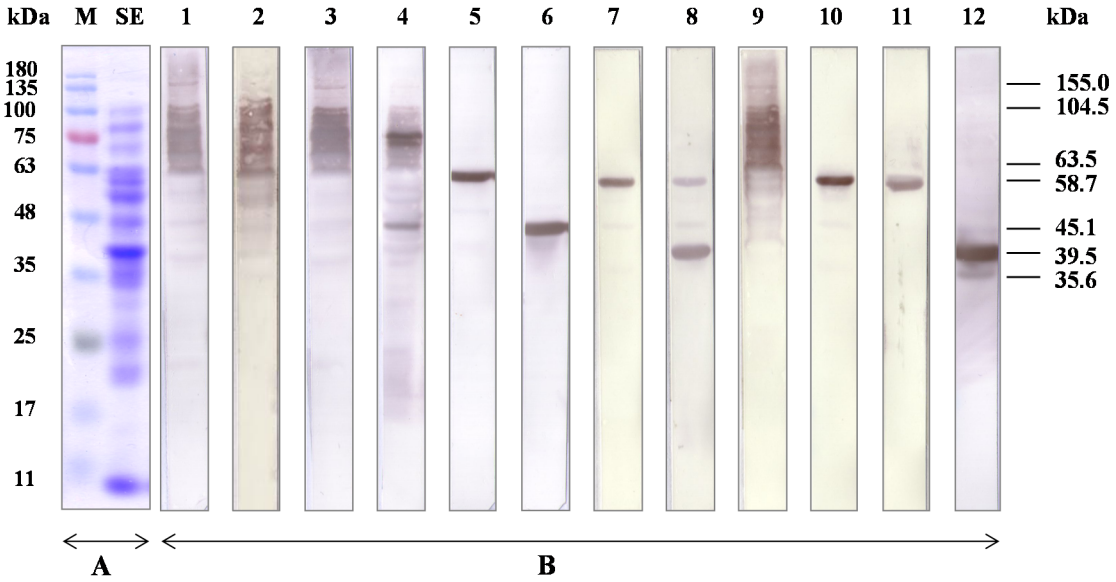

Figure 2 SDS-PAGE (A) and Western blot (B) of proteins from SE ATCC 13076. Cell lysate of SE ATCC 13076 (SE) was electrophoresed and stained with Coomassie blue (A), and transferred to membrane (B), then treated with various monoclonal antibodies at the 1/10 dilution. SE was treated with SCR23 (lane1), SCR26 (lane2), SCR50 (lane3), SCL18 (lane4), SCL28 (lane5), SCN35 (lane6), SDSR3 (lane7), SDSR66 (lane8), SDSL29 (lane9), SDSL64 (lane10), SDSL75 (lane11) and SDSN36 (lane12). M, molecular weight maker in kDa is indicated on the left side.

Table 2 Characteristics of monoclonal antibodies against *Salmonella* Enteritidis.

Clone	Isotype	Sensitivity (CFU/ml)	Antigen recognition size (kDa)	Specificity
SCR23	IgG2b	7.12×10^6	63.5-104.5, 155.0	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SCR26	IgG1	3.56×10^7	63.5-104.5	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SCR50	IgM	3.56×10^7	63.5-104.5, 155.0	SE ATCC13076, *S.* Paratyphi A, *S.* Typhi and ST DMST34042
SCL18	IgG1	7.12×10^6	45.1, 63.5-104.5	*Salmonella* spp.
SCL28	IgG1	3.56×10^7	58.7	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SCN35	IgG1	7.12×10^6	45.1	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SDSR3	IgG1	3.56×10^7	58.7	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SDSR66	IgM	3.56×10^7	39.5, 58.7	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SDSL29	IgG1	7.12×10^6	63.5-104.5, 155.0	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SDSL64	IgM	3.56×10^7	58.7	SE ATCC13076, *S.* Paratyphi A and *S.* Typhi
SDSL75	IgG1	3.56×10^7	58.7	SE ATCC13076, *S.* Paratyphi A, *S.* Typhi and ST DMST34042
SDSN36	IgM	7.12×10^6	35.6, 39.5	SE ATCC13076, *S.* Paratyphi A, *S.* Typhi and *E. coli* ATCC 25922

Discussion

Two forms of heat-killed plus sonicated and heat-killed plus SDS-mercaptoethanol antigens prepared from SE ATCC13076 in this research could be used to immunize BALB/c mice to produce the antibodies. A total of 12 monoclonal antibodies could react with SE but gave cross-reactivity with *S.* Typhi and *S.* Paratyphi A. In Western blot, monoclonal antibodies from our study recognize various epitopes of *Salmonella* antigens. Therefore cocktail mixture of various monoclonal antibodies could increase the sensitivity and decrease detection time (Canfiel et al. 1985). Dot blot technique is still required to detect for bacteria contamination in food and clinical samples due to this technique is simple and cheap (Yoshimasu and Zawistowski 2001).

Conclusion

The twelve monoclonal antibodies against SE produced in this study were characterized and their specificities were tested. Those monoclonal antibodies gave cross-reactivity with *S.* Typhi (serogroup D) and *S.* Paratyphi A (serogroup A) (Jay 1992). Therefore, more monoclonal antibodies specific only to SE will be rescreened in the future.

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