รายงานการวิจัยฉบับสมบรูณ์

โครงการวิจัยการเตรียมโมโนโคลนอลแอนติบอดีต่อแบคทีเรียก่อโรคอาหารเป็นพิษ:

***Salmonella* Typhimurium**

Preparation of monoclonal antibodies against foodborne bacteria:

***Salmonella* Typhimurium**

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บทคัดย่อ

เตรียมโมโนโคลนอลแอนติบอดีต่อ *Salmonella* Typhimurium โดยการหลอม P3X myeloma cells กับ เซลล์ม้ามของหนู BALB/c mice ที่ได้รับการปลูกภูมิคุ้มกันด้วย *S.* Typhimurium ATCC 13311 ในรูปแบบที่เซลล์ถูกฆ่าด้วยความร้อน หลังการหลอมเซลล์สี่ครั้งและทำการคัดเลือกทำให้ได้โมโนโคลนอลแอนติบอดี 7 โคลนและแบ่งได้สี่กลุ่มตามความจำเพาะต่อ serovar typing ของ *Salmonella* sp.เมื่อทดสอบด้วยวิธี dot blotting มีเพียง โมโนโคลนอลแอนติบอดีในกลุ่ม 2, 3 และ 4 ที่แสดงปฏิกิริยาจำเพาะ ต่อ *Salmonella* sp. ที่ใช้ทดสอบและไม่แสดงปฏิกิริยาข้ามต่อแบคทีเรียสกุลอื่นๆ โมโนโคลนอลแอนติบอดี ST 2 และ ST 4 มีความไวดีที่สุดในการตรวจหา *S.* Typhimurium ATCC 13311 ที่ความเข้มข้น 2.0×10^3 CFU μl^{-1} ด้วยวิธี dot blotting (2.0×10^6 CFU ml^{-1})

Abstract

Monoclonal antibodies (MAbs) against *Salmonella* Typhimurium were produced by fusion of P3X myeloma cells with spleen cells of BALB/c mice immunized with heat-killed forms of *S.* Typhimurium ATCC 13311. After primary and secondary screening the seven MAbs obtained from four fusions were divided into four groups according to their specificities to different serovar typing of *Salmonella* sp. as determined by dot blotting. Only MAbs in Groups of 2, 3 and 4 were specific to *Salmonella* sp. without any cross-reactivity to other bacteria tested. MAbs, ST2 and ST 41 show the highest sensitivity for detection of S. Typhimurium ATCC 13311 at the concentration of 2.0×10^3 CFU μl^{-1} by dot blotting (2.0×10^6 CFU ml^{-1}).

Theoretical background

Thailand is a national agricultural industry and farming. Most of the exported product is agricultural and meat products. The important problem that affects the products is bacterial contamination. Especially, *Salmonella* spp., one of the most dangerous foodborne enterobacteria that can cause severe foodborne diseases in human (Schneid et al., 2005).

Salmonella enterica serovar Typhimurium, a pathogenic Gram-negative bacteria with rod shape, motile and grow very well at 37°C under facultative anaerobic condition. *S.* Typhimurium is a common cause of gastroenteritis in human and animal. Approximate cases ~40-70% of human salmonellosis with symptoms of diarrhea, fever, vomiting and abdominal pain were recorded (Leon-Velarde et al., 2004). *S.* Typhimurium is among the most extensive serovar in Europe and America and growing importantly in Southeast Asia, Africa, and the Western Pacific (Herikstad et al., 2002). In Thailand several reports have demonstrated the presence of *Salmonella* spp. in chicken meats, chicken egg , pork, and pork products obtained from general markets(Padungtod and Kaneeene, 2006).

In 2006, fresh vegetables were banned because of the detection of *Salmonella* in coriander imported from Thailand, leading to the decline of imports by Finland. At the same time, United Kingdom also found *Salmonella* spp. in morning glory imported from Thailand (ThaiEurope, 2006). Contamination of products and exported product with *Salmonella* is not only a domestic public health problem but also an international problem. Due to vegetable and meat products, exported poultry and their products are one of the major economics of Thailand (Boonmar et al., 1998). In addition food safety has recently been concerned globally. Thus, determination of *S.* Typhimurium in foods is worldwide perception. Recently, detection of *S.*

Typhimurium in food products based on isolation in pure culture, biochemical and serological test as known as conventional method consumes several days and requires specialised skill (FDA, 1998). Moreover, molecular methods such as PCR method is accurate and high accuracy (Takeuchi, A. and Sode, K., 2000) but requires a specific tool, expensive and specialised skill. Immunological method using monoclonal antibody against any epitope of *S.* Typhimurium especially surface antigen has been shown to present high specificity and sensitivity (Sojka et al., 1996) to detect *S.* Typhimurium contaminated in food successfully. Detection of *S.* Typhimurium before export Thai agricultural products should routinely be practised as internal audit in order to confirm our safe food items and reduce barrier of inter-trade.Therefore, this study aims to develop monoclonal antibodies specific to of *S.* Typhimurium that can identify and differentiate *S.* Typhimurium accurately from other groups of *Salmonella* and other bacteria. Our monoclonal antibodies against *S.* Typhimurium will be applicable for use as rapid test kit in the future.

Material and Methods

Bacterial preparation

Salmonella Typhimurium ATCC 13311 and other bacteria. *S.*Typhimurium ATCC 13311 from stock culture were inoculated into tryptic soy broth (TSB) and cultured at 30 °C for 24 h with agitation. Other bacteria were cultured at 37 °C for 24 h with agitation in either TSB or TSB supplemented with 2% (w/v) NaCl for the growth of *Vibrio* species. Bacteria were harvested by centrifugation at 8,000 rpm for 10 min at 4 °C and washed twice with 0.15 M phosphate buffered saline (PBS) pH 7.2. The pellets were resuspended in PBS, and then the absorbance of the final bacterial suspensions was adjusted to the optical density (OD) of 1.0 at 660 nm. The

number of viable cells were determined as CFU by spread plates on tryptic soy agar (TSA). This was equivalent to approximately 10^9 CFU ml^{-1}. All bacteria used in this experiment are indicated in Table 1.

Antigen preparation

S. Typhimurium ATCC 13311 were prepared as antigen for immunization in heat-killed form. Bacterial suspension was heat-killed at 60 °C for 60 min to inactivate the bacteria, and then cooled to 4 °C. Heat-killed forms of antigens were divided into several aliquots and stored at -20 °C for later use.

Immunization

Four female BALB/c mice 6-8 weeks old were immunized by intraperitoneal injection (i.p.) with 50 μl of the antigen, emulsified in equal volumes of Freund's complete adjuvant. Thereafter, mice were boosted with different times in 2 weeks using the same amount of antigen emulsified in equal volumes of Freund's incomplete adjuvant. One week later, blood was taken from each mouse by tail bleeding, and then centrifuged at 10,000 rpm for 5 min at 4 °C for collecting the serum. The antibody response was tested against *S.* Typhimurium ATCC 13311 by dot blotting. Three day before fusion, the mouse showing the highest titer was final booster with the same antigen without adjuvant to use as spleen donor for hybridoma production. Serum samples were obtained before first immunization to be used as negative control.

Hybridoma production

Hybridomas were produced according to the method developed by Köhler and Milstein (1976) with modifications described by Mosmann et al. (1979). P3X myeloma cells were fused with spleen cells of the selected mouse using 50% polyethylene glycol (PEG) as a fusogen.

Myeloma cell lines

The P3X myeloma cell line were used as fusion. They were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified 5% CO_2 incubator at 37 °C. The myelomas were maintained in exponential phased growth by subculturing for one week prior to fusion. Approximately 10^7 cells of NS-1 myeloma were added into a 50 ml of a polypropylene tube and then centrifuged at 1,500 rpm for 5 min. After the supernatant was discarded, RPMI 640 medium supplemented with 0.2 mg ml^{-1} gentamicin was added and then placed in a humidified incubator to use in the fusion step.

Spleen cells

S. Typhimurium ATCC 13311 immunized mice was anesthetized with diethyl ether before blood drawn by cardiac puncture, and their serum were pooled and stored at -20 °C to be used as positive control in the immunological assay. The spleen was collected aseptically and gentle washed in sterile Petri dishes containing RPMI 1640 medium supplemented with gentamicin to minimize the chance of contamination. Then the connective tissues were carefully removed as much as possible during extensive soak in the medium. A single cell suspension was prepared by cutting the spleen into small pieces and then crushing by use a 10 ml syringe plunger through a sterile grid into the medium. Spleen cells were harvested by centrifugation at 1,500 rpm for 5 min and resuspended in 5 ml of the medium for fusion with myeloma cells.

Fusion procedure

Myeloma cells were added into a 50 ml of a polypropylene tube containing the spleen suspension at a 2:1 ratio. It was centrifuged at 1,500 rpm for 5 min and all of the supernatant was discarded as completely as possible. After mixing the cells by tapping the tube, 1 ml of pre-warmed (37 °C) 50% (w/v) polyethylene glycol (PEG)

was added dropwise to the cell suspension using a Pasteur pipette and simultaneously the tube was slightly agitated. This step was performed exactly for 1 min. After that, 30 ml of RPMI 1640 medium supplemented with 0.2 mg ml^{-1} gentamicin was added gently into the suspension, and mixed with the pipette. Pellet the cells by centrifugation and resuspended with the medium again in order to wash PEG from the cells. The fused cells were suspended in hypoxanthine-aminopterine-thymidine (HAT) selective medium supplemented with 20% FCS. The suspension was dispensed (200 μl $well^{-1}$) into twelve 96-well culture plates. All plates were placed in a humidified 5% CO_2 incubator at 37 °C.

Half medium of the wells was replaced by fresh HAT medium on day 7 post-fusion. When cells grow about two-thirds of the wells (10 to 12 days after fusion), the hybridoma culture supernatant in each well was screened for antibody activity against *S.*Typhimurium ATCC 13311. Cells were grown in HAT medium for 2 weeks and then HAT was substituted by HT medium.

Hybridoma screening

Primary screening against *S.* Typhimurium ATCC 13311 by dot blotting:Heat-killed form of *S.*Typhimurium ATCC 13311 approximately 10^9 CFU ml^{-1} used as antigen in immunization step was used for primary screening by dot blotting. Bacterial samples were applied to a nitrocellulose membrane as 1 μl $spot^{-1}$. Positive and negative controls, sera from immunized and nonimmunized mouse, respectively were also dotted onto the membranes. The membranes were dried at 37 °C for 30 min and blocked in 5% skim milk at room temperature (RT) for 30 min to prevent nonspecific binding of the antibodies. After washed with PBS, the membrane was dried and stored at -20°C to use subsequently.

The membranes were incubated in hybridoma culture supernatant diluted 1:2 in PBS from each culture for 2 h and then washed 3 times for 5 min each with 0.05% Tween-20 in PBS (PBST). After that, horseradish peroxidase-conjugates goat anti mouse IgG (GAM-HRP) diluted 1:3000 in PBS was added and incubated for 2 h. After washing as above, the membranes were treated with DAB substrate solution for 5 min and the color reaction was stopped by washing thoroughly in deionized water. All steps were performed at room temperature with continuous rocking. The positive wells which appeared as a dark spot were further screened by dot blotting against *S.*Typhimurium ATCC 13311 and other bacteria.

Secondary screening against *S.* Typhimurium ATCC 13311 and other bacteria by dot blotting:The positive wells in primary screening were tested for cross-reactivity by dot blotting against *S.* Typhimurium ATCC 13311 and other bacteria. The bacteria approximately 10^9 CFU ml^{-1} in heat-killed form were spotted onto nitrocellulose membrane as 1 μl $spot^{-1}$ in each block of 4x4 mm. The membranes were dried and blocked in 5% skim milk in PBS. After washed in PBS, the membranes were incubated in hybridoma culture supernatant which gave positive results in primary screening (1:2 dilution in PBS) at RT for 2 h and then processed as described above in primary screening section. Hybridomas from the wells showing antibody patterns of interest with strong signals were selected for subsequent cloning by limiting dilution method.

Cloning of hybridoma cell by limiting dilution

Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 20% FCS to ensure that a hybridoma is stable and single-cell

cloned. From each positive well, 10 μl of the cell suspension was diluted with the medium to obtain about 80 viable cells and then mixed with 10 ml of HT medium. One hundred microlitres of the mixture was dispensed into 96-well plate containing 100 μl of HT medium. The plates were placed in a humidified 5% CO_2 incubator at 37 °C for 10 to 12 days. Wells containing a single colony were tested for antibody production by dot blotting. The selected hybridomas were cloned a minimum of 3 times or continue repeating the cloning until every well tested is positive in order to achieve stable single clones producing only a homogeneous antibody.

Characterization of monoclonal antibodies

Specificity test: The specificity of the MAbs was determined for the reactivity of the antibody with *S.*Typhimurium ATCC 13311 and other bacterial species by dot blotting. Heat-killed bacteria samples (10^9 CFU ml^{-1}) listed in table 1 were spotted onto nitrocellulose membranes as 1 μl $spot^{-1}$. The bacteria were spotted in each block of 4x4 mm. The membranes were dried at 37 °C for 30 min and blocked in 5% skim milk in PBS at RT for 30 min. Subsequently, the membranes were reacted with various MAbs diluted 1:50 (or serum diluted 1:20,000) in 5% skim milk in PBST at RT for 2 h. The membranes were washed 4 times for 5 min each with PBST and then incubated in GAM-HRP diluted 1:3000 in PBS at RT for 2 h. After washing, DAB substrate solution was added to the membranes and reacted at RT for 5 min. The color reaction was stopped by washing the membranes in deionized water. The intensity of the reaction with each MAb was compared by eye and scored relative to the reaction against antigen.

Sensitivity test: Five-fold serial dilutions of heat-killed *S.* Typhimurium ATCC 13311from 10^9 to 10^5 CFU ml^{-1} diluted in PBS were prepared. One microlitre of each

dilution was spotted onto a nitrocellulose membrane and processed for dot blotting using various MAbs as described above in the specificity test section. The lowest bacterial cell concentration which gave positive reaction was observed as the detection limit of *S.* Typhimurium ATCC 13311 using dot blotting technique.

Isotype determination

Isotype of mouse immunoglobulins secreted by the hybridomas were determined based on sandwich ELISA using Sigma's Mouse Monoclonal Antibody Isotyping Kit (Sigma-Aldrich, USA) according to the manufacturer's instruction.

Experimental Diagram

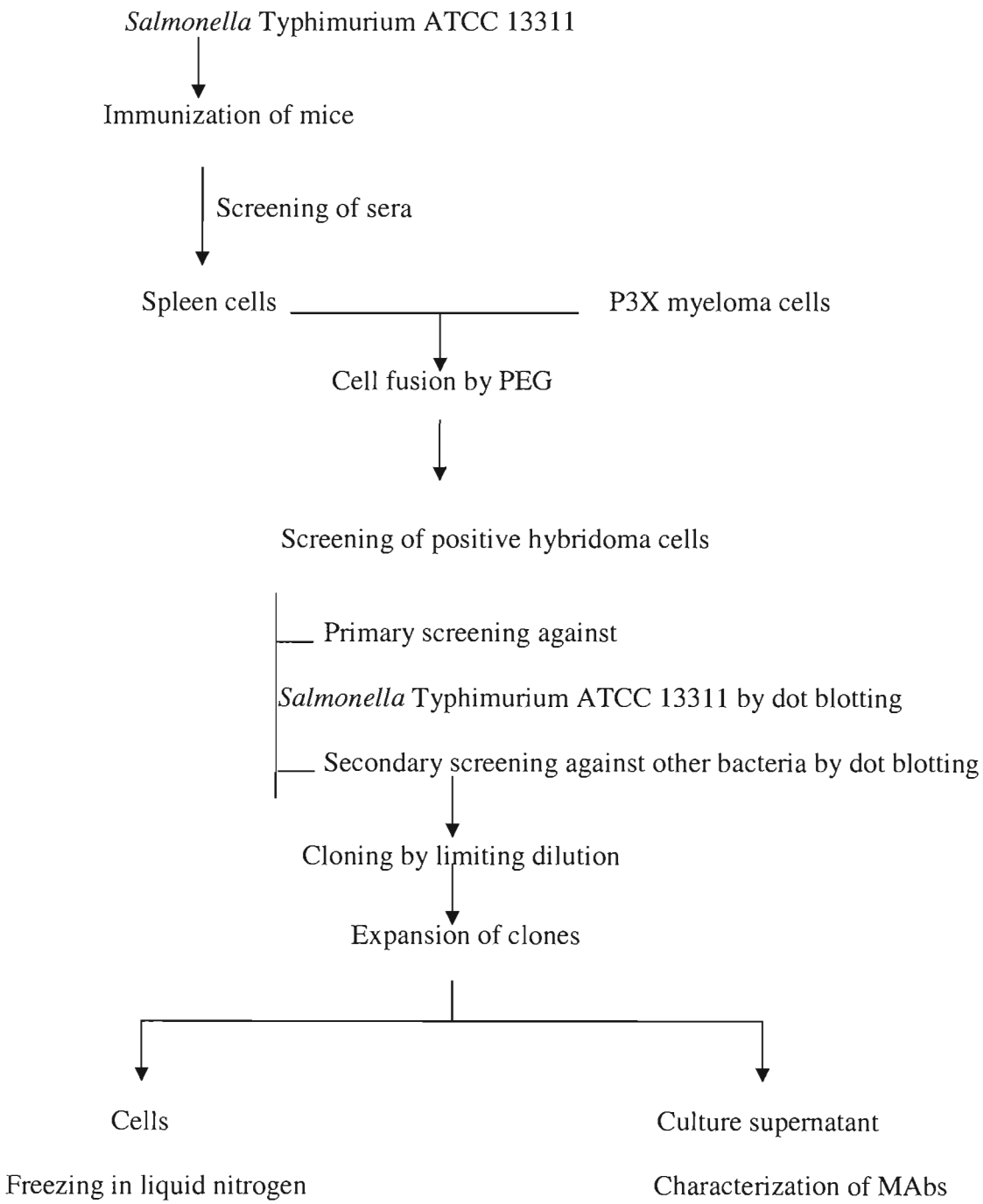

Table 1. List of bacterial strains and sources used in this study

Bacteria	
Salmonella	**Other Gram-negative bacteria**
Salmonella Typhimurium ATCC 13311	*Acinetobacter baumanii*
Salmonella Typhimurium DT 104	*Citrobacter freundii*
Salmonella Typhimurium ATCC 14028	*Edwardsiella tarda*
Salmonella Typhimurium (Copenhagen)	*Escherichia col*
Salmonella Typhimurium DMST 34036	*Enterobacter cloacae*
Salmonella Typhimurium DMST 34037	*Klebsiella pneumoniae*
Salmonella Typhimurium DMST 34038	*Proteus mirabilis*
Salmonella Typhimurium DMST 34039	*Providencia rettgeri*
Salmonella Typhimurium DMST 34040	*Pseudomonas aeruginosa*
Salmonella Typhimurium DMST 34041	*Serratia marcescens*
Salmonella Typhimurium DMST 34042	*Shigella flexneri*
Salmonella Typhimurium DMST 34043	*Vibrio parahaemolyticus*
Salmonella Typhimurium DMST 34044	*Yersinia enterocolitica*
Salmonella Typhimurium DMST 34045	*Yersinia frederiksenii*
Salmonella Typhimurium DMST 34046	*Yersinia kristensenii*
Salmonella Typhi	**Other Gram-positive bacteria**
Salmonella Paratyphi	*Listeria monocytogenes*
Salmonella Enteritidis	*Enterococcus faecalis*
Salmonella Enteritidis1773-72	
Salmonella Enteritidis 13076	

Results

1. Immunization of mice

One week after completion of the immunization, the sera from four BALB/c mice were collected to evaluate the *S.*Typhimurium ATCC 13311-specific antibody response against *S.* Typhimurium ATCC 13311. The results are shown in Figure 1.

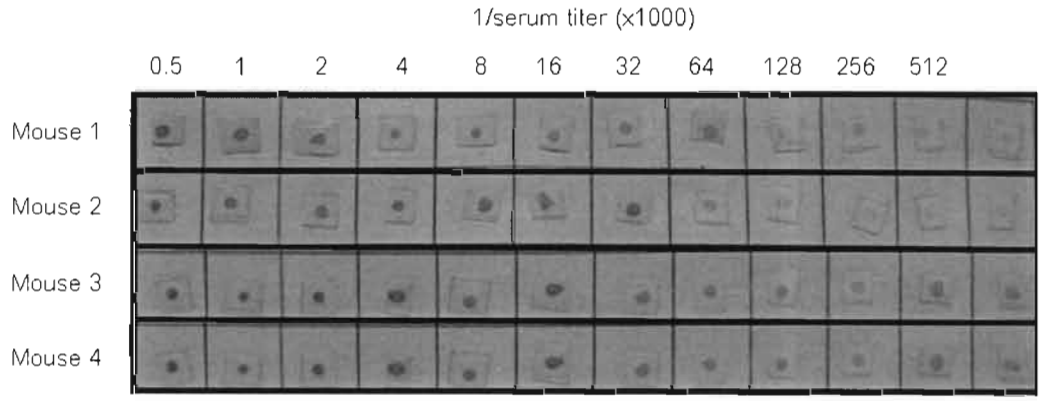

Figure 1. Dot blotting for specificity of mice antisera.The serum of immunized mouse number 1 , 2 , 3 and 4 were tested against heat-killed *S.* Typhimurium ATCC 13311

2. Production of monoclonal antibodies

Four fusions were performed after immunization with *S.*Typhimurium ATCC 13311. After primary and secondary screening by dot blotting, the hybridomas producing a strong immunoreactivity to heat-killed of *S.*Typhimurium ATCC 13311. were cloned. Finally, 7 monoclones were obtained and their respective MAbs subjected to further characterization. For each fusion, the percentage of the wells contained hybridoma clones, the wells which gave positive results after primary and secondary screening and the designation of monoclones were shown in Table 2.

Table 2. Hybridoma production for each fusion

Fusion no.	Mouse no.	Number of immunization	Hybridoma (%)	1st screen (wells)	2nd screen (wells)	Clone number
1	1	4	25	94	1	ST57
2	2	5	90	62	1	ST16
3	3	5	12	44	5	ST2,12, 39, 41
4	4	6	63	168	16	ST1

3. Characterization of monoclonal antibodies

Specificity test

The specificity of MAbs from 7 monoclones were determined by dot blotting with various heat-killed bacterial strains. These 7 MAbs can be divided into 4 groups according to their antigen specificities (Figure 2). The reactivities of MAbs in each group were as follows:

MAb in group 1 consisted of one clone, namely ST57. This MAbs reacted with *Salmonella* spp. Furthermore, it also cross-reacted with three isolate of *Yersinia* spp.

MAb in group 2 consisted of ST16. The result of this MAb differed from MAb in group 1 are that it gave stronger reactivity to *S.*Typhimurium. But this MAb could also cross-react with *S.* Enteritidis1773-72.

MAb in group 3 consisted of four clones, namely ST 2, ST 12,ST 39and ST41. These MAbs reacted with some strain of *S.* Typhimurium and showed cross-reactivity to *S.* Paratyphi , *S.* Enteritidis and *S.* Enteritidis1773-72

MAb in group 4 consisted of ST1. This MAb reacted with *Salmonella* spp.

except *S.*Enteritidis1773-72.

4. Sensitivity test

The sensitivity of MAbs were determined by dot blotting with 5-fold serial dilution of heat-killed *S.* Typhimurium ATCC 13311 depending on their specificity to the antigen were shown in Figure 3. The detection limit of ST57 was indicated as 2.6 x 10^5 by dot blotting (2.6 x 10^8 CFU ml^{-1}) of *S.* Typhimurium ATCC 13311, while the sensitivity of ST 16 , ST12 , ST 39 and ST 1 was 1 x 10^4CFU by dot blotting (1 x 10^7CFU ml^{-1}). ST2 and ST 41 show the highest sensitivity as 2.0x10^3 CFU by dot blotting (2.0x10^6 CFU ml^{-1}).

5. Isotype determination

Isotype determination of seven MAbs as follws: ST16 as IgG_1 , ST2 as lgG_{2a}, ST 41 and 57 as lgG_{2b}, ST 12 as lgG_3, and ST1 and 39 as Ig M were determined as shown in Table 3.

Table 3. The absorbance value of MAbs for isotype determination

Fusion	MAb	A_{462}[a]					
		IgG_1	lgG_{2a}	lgG_{2b}	lgG_3	lgA	lgM
1	ST57	0.293	0.131	**1.533**	0.180	0.151	0.197
2	ST 16	**2.788**	0.131	0.193	0.182	0.143	0.235
3	ST 2	0.123	**1.375**	0.156	0.173	0.113	0.271
	ST 12	0.124	0.127	0.217	**1.549**	0.129	0.728
	ST 39	0.126	0.122	0.197	0.239	0.126	**2.892**
	ST 41	0.535	0.149	**2.181**	0.240	0.161	0.658
4	ST 1	0.183	0.179	0.196	0.275	0.125	**2.962**

A_{462}[a] = absorbance at 462 nm. Value was based on duplicated experiments.

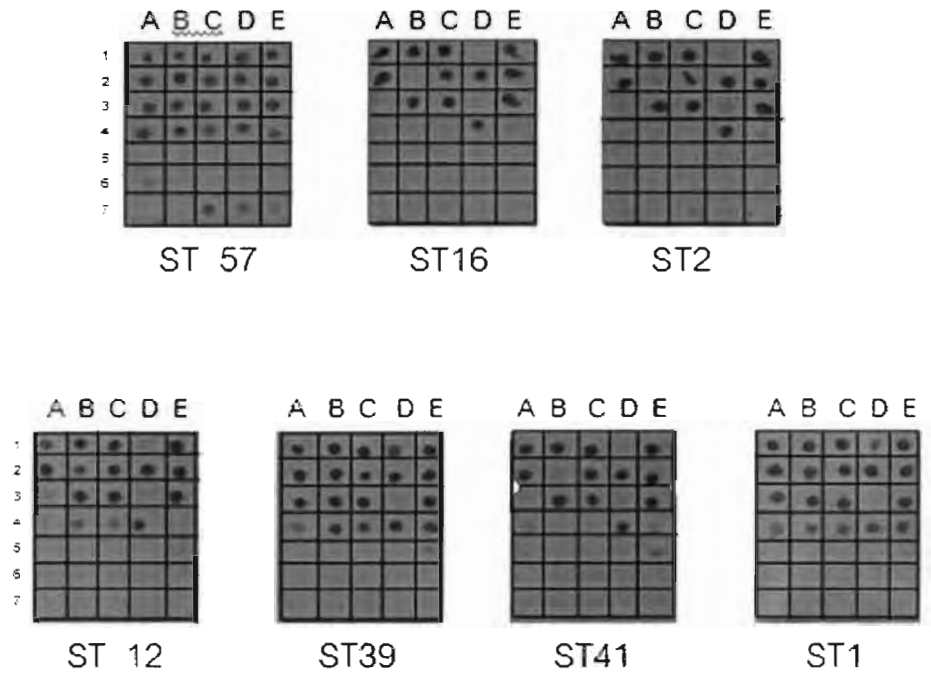

Figure 2. Specificity of MAbs assayed by dot blotting. Heat-killed bacteria (~10^9 CFU ml^{-1}) were spotted onto nitrocellulose membrane 1 μl $spot^{-1}$ and treated with MAbs from each group. Bacterium was spotted onto each block of the membrane as follows:

Row 1: (A) *S.*Typhimurium ATCC 13311; (B) *S.*Typhimurium DT 104 ; (C) *S.*Typhimurium ATCC 14028; (D) *S.* Typhimurium (Copenhagen); (E) *S.*Typhimurium DMST 34036

Row 2: (A) *S.*Typhimurium DMST 34037; (B) *S.* Typhimurium DMST 34038; (C) *S.*Typhimurium DMST 34039; (D) *S.* Typhimurium DMST 34040; (E) *S.*Typhimurium DMST 34041

Row 3: (A) *S.*Typhimurium DMST 34042; (B) *S.* Typhimurium DMST 34043; (C) *S.*Typhimurium DMST 34044; (D) *S.*Typhimurium DMST 34045; (E) *S.*Typhimurium DMST 34046

Row 4: (A)*S.* Typhi; (B) *S.* Paratyphi; (C) *S.* Enteritidis; (D) *S.* Enteritidis1773-72; (E) *S.* Enteritidis 13076

Row 5: (A) *Acinetobacter baumanii*; (B) *Citrobacter freundii*; (C) *Escherichia coli*; (D) *Enterobacter cloacae*; (E) *Enterococcus faecalis*

Row 6: (A) *Edwardsiella tarda*; (B) *Klebsiella pneuminoae*; (C) *Proteus mirabilis*; (D) *Providencia rettgeri*; (E) *Pseudomonas aeruginosa*

Row 7: (A) *Serratia marcescens*; (B) *Shigella flexneri*; (C) *Yersinia enterocolitica*; (D) *Yersinia frederiksenii*; (E) *Yersinia kristensenii*

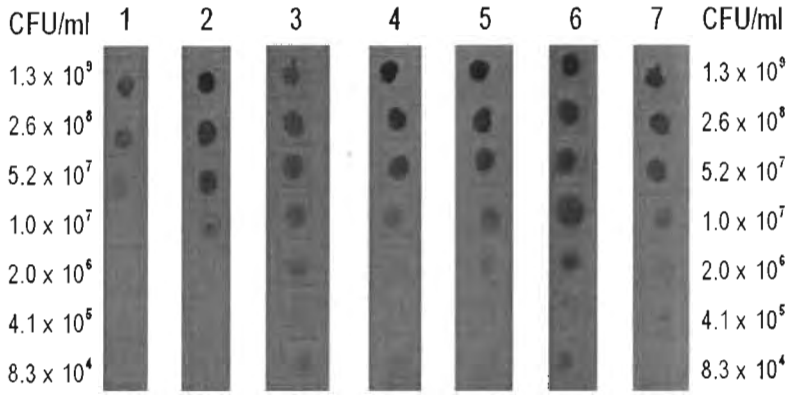

Figure 3. Sensitivity of the MAbs assayed by dot blotting. Five-fold serial dilution of heat-killed *Salmonella* Typhimurium ATCC 13311 was spotted onto nitrocellulose membrane as 1 μl spot^{-1} and then treated with MAbs: ST57 (1), ST16 (2), ST2 (3), ST12 (4), ST 39 (5), ST 41 (6) and ST 1 (7).

Conclusion

Seven monoclonal antibodies (MAbs) against *S.*Typhimurium were obtained from 4 fusion P3X myeloma cells with spleen cells of BALB/c mice immunized with heat-killed forms of *S.* Typhimurium ATCC 13311 and they can be divided into 4 groups according to their apparent specificities to different serovar typing of *Salmonella* sp. as determined by dot blotting. MAbs in Groups 2, 3 and 4 were specific only to *Salmonella* sp. without any cross-reactivity to other bacteria tested. Isotypes of all 7 monoclonal antibodies were IgG_1 , lgG_{2a}, lgG_{2b}, lgG_3 and lg M for ST 16, ST 3, ST41&ST57, ST12, and ST1&ST39, respectively. These MAbs could detect *S.* Typhimurium ATCC 13311 with different sensitivities ranging from approximately 10^4-10^6 CFU by dot blotting (10^7-10^9 CFU ml^{-1}). Among these two MAbs, ST 16 could detect 1 x 10^4 CFU by dot blotting (1 x 10^7 CFU ml^{-1}) of

*S.*Typhimurium ATCC 13311, while the sensitivity of ST 57 was 1 x 10^6 CFU by dot blotting (1 x 10^9 CFU ml^{-1}).

Next step of work are as follows:-

- To produce more MAbs against *Salmonella* Typhimurium by repeat immunization with *Salmonella* Typhimurium antigens by modifying antigen preparation procedure.
- To test the recognition of our MAbs *on Salmonella Typhimurium* ATCC 13311 antigens by Western blotting and SDS-PAGE analysis.
- To differentiate epitopes of antigen whether our MAbs recognize the same epitope or not.

Drawbacks of work

- Monoclones obtained in this study still can cross react with different serovar typing of *Salmonella* sp..
- Procedure of limiting dilution method took longer time than expected.

Acknowledgement

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