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Preparation of Monoclonal Antibody and Development of Enzyme-linked Immunosorbent Assay Specific for *Escherichia coli* O157 in Foods

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Objective To prepare monoclonal antibodies (MAb) and antisera specific for *Escherichia coli* (*E.coli*) O157, and to develop a sandwich enzyme-linked immunosorbent assay (ELISA) to detect *E.coli* O157 in foods. **Methods** Spleen cells from BALB/c mice immunized with the somatic antigen of *E.coli* O157:H7 were fused with murine Sp2/0 myeloma cells. The hybridoma cell line specific for *E.coli* O157 was established after having been subcloned. Antisera specific for *E.coli* O157 was prepared by intravenous injection into New Zealand rabbits with a stain of *E.coli* O157:H7. The sandwich ELISA was developed with the polyclonal antibody as the capture antibody and the MAb 3A5 as the detection antibody. The inoculated ground poultry meat and pasteurized milk were tested to confirm efficiency of the method. **Results** MAb 3A5 specific for *E.coli* O157 and O113:H21 belonged to subtype IgM. The ascetic titers of the antibody was $1:1\times10^6$. No cross-reactivity of the MAb was observed with strains of *Salmonella spp, Yersinia enterocolitica*, *Shigella dysenteriae*, *etc*. The purified polyclonal antibody had a titer of $1:1\times10^5$ with *E.coli* O157. The detection limit of this sandwich ELISA was 10^3-10^4 cfu *E.coli* O157/mL in pure culture with a high specificity, which was characterized by every non-O157 strain with negative response. With 10h enrichment procedure, *E.coli* O157:H7 recovered well from inoculated ground poultry meat and pasteurized milk at levels of 0.1 cfu/g and 0.1 cfu/mL. **Conclusion** MAb 3A5 specific for *E.coli* O157 and O113:H21 can be produced by immunizing BALB/c mice with a strain of *E.coli* O157:H7. Then a sandwich ELISA can be developed with the polyclonal antibody as the capture antibody and the MAb 3A5 as the detection antibody. The method is proved to be a sensitive and specific technique to detect low number of *E.coli* O157 in food.

Key words: *Escherichia coli* O157; Monoclonal antibody; Polyclonal antibody; ELISA

INTRODUCTION

Being recognized as a food-borne disease agent in 1982[1], *Escherichia coli* O157:H7 has been emerged as an important food-borne pathogen. *E.coli* O157:H7 and other serotypes belonging to enterohemorrhagic *E.coli* (EHEC) can cause hemorrhagic colitis and serious conditions such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in human beings. Most outbreaks have been linked to food and water. Many common foods such as beef, milk, poultry, fruit juice, vegetables have been implicated as source of infection in most of the outbreaks.

In order to isolate and identify the important human pathogen, *E.coli* O157 from food samples, a large number of methods were developed. The selectivity of selective agars and enrichment broths is based on the specific phenotypic characteristics of most *E.coli* O157:H7 strains, such as lack of sorbitol fermentation, failure to produce β-glucuronidase, resistance to antibodies and other inhibitory agents[2]. The method of PCR is based on amplification of specific gene fragments of virulence factors of EHEC or gene encoding protein specific for *E.coli* O157:H7[3-6]. Immunology-based assays are also developed for detecting antigens specific for *Escherichia coli* O157. The method of ELISA is accepted worldwide because of its high sensitivity. But problem of cross-reaction of specific antisera is always disturbing the evaluation of this method's specificity.

The objective of this study was to produce a monoclonal antibody (MAb) and a polyclonal antibody (PAb) highly specific for EHEC, and to develop a sandwich ELISA method for the rapid detection of *Escherichia coli* O157 in food samples.

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MATERIALS AND METHODS

Bacterial Strains

E.coli O157:H7 strain 882364 (isolated by Institute of Microbiology and Epidemiology, Chinese Center of Disease Control and Prevention) was used for mouse immunization for hybridoma production and as the positive-control antigen for MAb screening. *E.coli* O157:H7 strain ATCC 43895 (American Type Culture Collection) was used for producing rabbit antiserum. Other bacteria used to characterize MAb reactivity and specificity of double-antibody sandwich ELISA included 20 strains of other *E.coli* O157:H7, 2 *E.coli* O157 nonH7 strains, 30 other *E.coli* strains, 2 *Salmonella* sp, 3 *Yersinia* sp, 1 *Shigella dysenteriae*, 3 *Proteus* sp and 8 other bacteria strains.

Immunization of Mice

E.coli O157:H7 strain 882364 was grown in LB broth at 37℃ for 18-24 h. The cells were harvested by centrifugation (4000×g for 10 min), washed two times with 0.85% NaCl, and then boiled for 2 h. After boiling, the cells were harvested by centrifugation (4000×g for 10 min), washed two times with 0.9% NS and the concentration was adjusted to 10^9 cfu/mL. Five BALB/c mice (female, 6 to 7 weeks old) were immunized by intraperitoneal injection of 3×10^8 heat-treated cells. After 18 days, the mice received intraperitoneally the same number of heat-treated cells. Thereafter, the mice were given two intraperitoneal injections of 3.5×10^8 cells, 1 and 3 weeks after the injection. The final injection was given to the mice with 3.5×10^8 cells, then the mice were sacrificed 3 days later and their spleen cells were fused with myeloma cells.

Fusion, Cloning and ELISA Screening[7]

Briefly, spleen cells from immunized mice were fused with Sp2/0 myeloma cells in a ratio of 5:1. Putative hybrids were screened by enzyme-linked immunosorbent assay (ELISA) on supernatants for selecting the production of antibodies against *E.coli* O157:H7.

Enzyme immunoassay microtitration plates (Nunc, Nalge Nunc internatioal, Denmark) were coated with 100 μL bacterial cells (*E.coli* O157:H7) and *E.coli* O124:H32 for negative control (ca. 10^8 cells/mL) in 50 μL carbonate buffer (pH 9.6) and incubated at 4℃ overnight. After the wells were washed three times with 0.05 mol/L phosphate-buffered saline (PBS) (pH 7.4) plus 0.05% Tween-20 (PBS-T), the remaining binding sites were blocked with 6.67% bovine serum in PBS. After 1 h of incubation at 37℃, the blocking buffer was removed and 100 μL of hybridoma supernatant was added to the wells. The plates were incubated at 37℃ for 1 h, and then the wells were washed six times with PBS-T. Horse radish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (100 μL/well 1:1000 in PBS) was added and incubated at 37℃ for 1 h. After the wells were washed six times with PBS-T, 100 μL of O-phenylenediamine (OPD) substrate was added per well. After 20 min of incubation at 37℃, the reaction was stopped by adding 30 μL 2 mol/L H_2SO_4 per well. The OD at 490 nm of each well was measured with a microplate reader EL308 (Bio-Tek instruments). All hybrids were subcloned twice by limiting dilution to ensure stability prior to freezing cell samples or raising ascitic fluid.

Production of Ascitic Fluid

BALB/c mice were primed by intraperitoneal injection of 0.5 mL paraffin oil 1 week prior to the injection with 10^6 selected hybridoma cells. Mouse ascitic fluid was collected 7 days after injection. Cell debris and fibrin clots were discarded by centrifugation (1500×g, 10 min), and transparent supernatant was stored at -20℃.

Purification and Characterization of Monoclonal Antibody

MAb from ascitic fluid was purified by using the method of Xu[7]. Briefly, ascitic fluid was pre-treated by a 0.45 μm filter membrane. NaCl and $CaCl_2$ were successively added to the fluid to get the final concentration of 0.2 mol/L and 25 mmol/L respectively. After filteration with filter paper, the filtrate was dialyzed with distilled water at 4℃ for 15 h. After centrifugation (20 000×g, 30 min),the pellet was resuspended in 1 mol/L NaCl/0.1 mol/L Tris-HCL and again dialyzed as described above. The dialysate was collected and stored at -20℃ until use.

Protein concentration was determined with an ultraviolet spectrophotometer by the equation: protein concentration = OD280 nm×1.45-OD260 nm×0.74[7]. Immunoglobulin isotyping was done with ELISA using class-specific antiserum.

The activity and specificity of MAb were determined by direct ELISA . All the bacteria tested were grown in Luria-Bertani broth overnight. The cells were collected by centrifugation (2000×g, for 10 min) and resuspended in 0.85% NaCl. Cells were diluted appropriately in 0.85% NaCl. The actual concentration levels of *E.coli* O157:H7 were determined by culturing dilutions on LB plates at

37℃ for 24 h. All the heat-treated bacteria strains were adjusted to the concentration of 10^8cells/mL. Enzyme immunoassay microtitration plates were coated with 100 μL bacterial cells (10^8cells/mL) in order to determine the specificity of MAb by direct ELISA as described above.

Production of Rabbit Polyclonal Antibody

Two female New Zealand white rabbits, weighing 2.5 kg, were given seven intravenous injections of 0.5 mL of heat-treated *E.coli* O157:H7 strain ATCC 43895, once a day for 7 days. The doses of injections were 10^8, 2×10^8, 3×10^8, 4×10^8, 4×10^8, 4×10^8, and 5×10^8 cells. Seven days after the final injection, the rabbits were sacrificed and their blood was collected. Before each injection, about 1 mL blood was collected for examining the titer of polyclonal antibodies against *E.coli* O157: H7. The blood was kept at the room temperature for 1 h and at 4℃ for 2 h, and then the supernatant serum was collected. Purified polyclonal antibody was obtained from serum by the method of saturated ammonium sulfate. The titer of rabbit polyclonal antibody was measured by the similar method of ELISA as before. The purified polyclonal antibody was kept at -20℃.

Inoculation Studies

Fresh retail ground poultry meat and pasteurized milk were inoculated with different levels of *E.coli* O157:H7 to determine the sensitivity of this sandwich ELISA. *E.coli* O157:H7 ATCC 43895 was cultured in LB broth at 37℃ for 18 h. Cells were collected by centrifugation at 2000×g for 10 min, resuspended in 0.9% NaCl, and diluted appropriately in 0.85% NaCl. The actual inoculum levels of *E.coli* O157:H7 were determined by culturing dilutions of incula on LB plates at 37℃ for 24 h.

Fresh retail ground poultry meat and pasteurized milk were purchased from a retail market in Beijing. Prior to the inoculation of *E.coli* O157:H7, aerobic plate counts (APC) and total coliform counts of these food samples were determined by traditional method. The samples were examined for natural contamination with *E.coli* O157:H7 by SMAC-plating method in combination with enrichment with mEC+n or TSB+n at 42℃ for 16-18 h.

Each level of cell suspension, 0.5 mL, was spiked into 25 g ground poultry meat or 250 mL milk. For control uninoculated samples, 0.5 mL 0.85% NaCl was spiked into the food samples.

Two hundred and twenty-five mL mEC+n broth was added to each 25 g meat sample and 225 mL TSB+n broth was added to each milk sample, which was homogenized and incubated statically at 42℃ for 10 h. Two samples of each enrichment culture (1 mL for each) were taken afterwards. One sample was boiled for 10 min directly. The other was centrifug ated (16 000 rpm for 3 min). After supernatant was discarded, the pellet was resuspended with 1 mL 0.85% NaCl, and then the suspension was boiled for 10 min. The suspensions were used in the following procedure of sandwich ELISA for detection of the *E.coli* O157:H7.

Development of Sandwich ELISA

Each well was coated with 100 μL of purified polyclonal antibody (1:4000 or 8000 in 50 mmol/L carbonate buffer (pH9.6)) and kept at 4℃ overnight. After washing three times with PBS-T, the remaining binding sites were blocked with 150 μL 6.67% new-born bovine serum in PBS for 1 h at 37℃. The blocking agent was removed, and 100 μL enriched culture was added per well and incubated at 37℃ for 1 h. After the wells were washed six times with PBS-T, 100 μL of MAb 3A5 (purified immunoglobulin diluted at 1:10 000 in PBS) was added. The plates were kept at 37℃ for 1 h. Then the wells were washed six times with PBS-T, and 100 μL of goat anti-mouse IgG conjugated to horseradish peroxidase (diluted at 1:800 in PBS) was added and the culture was incubated at 37℃ for 1 h. After the wells were washed six times with PBS-T, 100 μL of OPD substrate was added per well and the plates were held at 37℃ for 20 min. The reaction was stopped by adding 30 μL 4 mmol/L H_2SO_4 per well and OD_{490} of each well was measured with a microplate reader. An OD_{490} of 0.2 above background was considered as positive. Enrichment culture of food samples without inoculated *E.coli* O157:H7 was used as control to establish background readings.

Decimal dilutions of pure culture of *E.coli* O157:H7 strain ATCC 43895 were used for determining the sensitivity of the method and 71 strains of *E.coli* O157:H7 and non-O157:H7 were measured to determine the specificity.

RESULTS

Production of Monoclonal Antibody

Only one hybridoma clone, named 3A5, produced antibodies that reacted specifically with all *E.coli* O157:H7 strains and enterohemorrhagic *E.coli* (EHEC) O113:H21, but had no cross-reaction with 46 non-O157 *E.coli* strain or other bacterial strains. Isotyping revealed that MAb 3A5 belonged to IgM class. The titer of the ascitic fluid primed by specific hybridoma was $1:10^6$. The purified monoclonal

antibody also had a high titer (1:2.5×10^5) and a work dilution of 1:2×10^4 (Fig. 1). The detection limit of the MAb 3A5 was 10^5-10^6 CFU/mL in pure culture *E.coli* O157:H7 using direct ELISA.

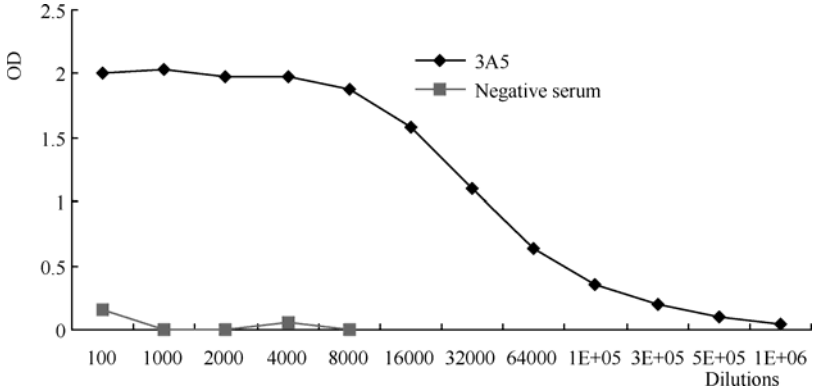

FIG. 1. Dilution curve of monoclonal antibody 3A5.

Production of Polyclonal Antibody

About 33 mL antiserum per rabbit was gained. The purified polyclonal antibody had a titer of 1:10^5 with *E.coli* O157:H7.

Development of Sandwich ELISA Method

The method had a high sensitivity with detection limit of 10^3-10^4 *E.coli* O157:H7 CFU/mL in pure culture, as shown in Fig. 2. High specificity was also observed. Only *E.coli* O157:H7 strains showed strong reaction with an OD>0.85. All other strains including *E.coli* O113:H21, other *E.coli* strains, *Salmonella* sp, *Y. enterocolitica*, *Shigella dysenteriae*, *Proteus* sp and other bacteria strains showed negative reaction with an OD<0.22 for all non-O157 strains tested.

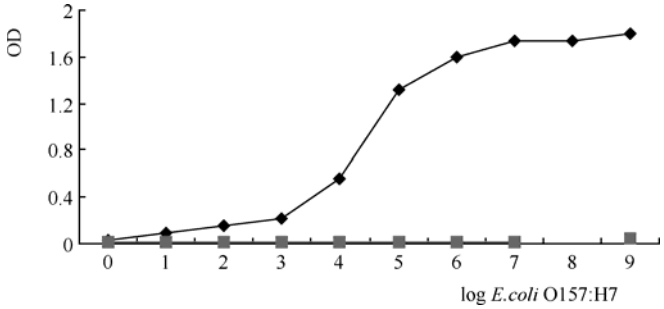

FIG. 2. Sensitivity of double-antibody sandwich ELISA technique for detecting *E.coli* O157:H7.

Detection of E.coli O157:H7 in Food Using Sandwich ELISA Method

The sandwich ELISA was developed for detecting *E.coli* O157:H7 in enrichment cultures of food samples. Several low dose levels of *E.coli* O157:H7 were inoculated into the ground poultry meat and pasteurized milk in order to determine the efficacy of enrichment-sandwich ELISA. Inoculation recovery studies showed that the procedure could detect as low as 0.1 CFU *E.coli* O157:H7 per gram ground poultry meat and 0.1 CFU per mL pasteurized milk. None of the products used for the research naturally contaminated *E.coli* O157:H7. The aerobic plate count (APC) and coliform count of ground meat before inoculation were 1.6×10^6 CFU/g and >2.4×10^4 CFU/g. The pasteurized milk samples had no contamination.

DISCUSSION

The ELISA described here is based on a double-antibody sandwich ELISA method in which a polyclonal antibody against *E.coli* O157:H7 somatic antigen is used as the capture antibody and a specific MAb for *E.coli* of O157 and O113:H21 is used as the detection antibody. The procedure for detecting *E.coli* O157:H7 is rapid, sensitive and specific. The sensitivity of the double-antibody sandwich ELISA is 100 times higher than that of the direct ELISA. As mentioned above, while the detection limit of direct ELISA with MAb 3A5 was 10^5-10^6 CFU *E.coli* O157/mL in pure culture, the double-antibody

sandwich ELISA could detect as low as 10^3-10^4 CFU *E.coli* O157/mL in pure culture. Specific MAb for *E.coli* O157:H7 reacted with another strain of EHEC O113:H21, but in the sandwich ELISA the cross-reaction disappeared. The sandwich ELISA had a high specificity for only *E.coli* O157 strains. Any non-O157 strains including O113:H21 had a negative reaction in the procedure. The reasons underlying the increase in sensitivity and specificity are possible influence of the capture antibody and polyclonal antibody on the filtration. The capture antibody can capture only *E.coli* O157 and strains containing common somatic antigen similar with *E.coli* O157. The rabbit antiserum has no cross-reaction with *E.coli* O113:H21, which strongly reacts with the detection antibody, MAb 3A5. On the other hand, because of the primary screen of the capture polyclonal antibody, a relatively higher concentration of the objective organisms is congregated at bottom of the wells of microtiter plates; as a result, more somatic antigens of the objective organisms are exposed to the specific detection antibody. Since more monoclonal antibodies conjugated with the specific antigens cause strong color reactions, a smaller number of *E.coli* O157 strains were recovered from samples by double-antibody ELISA than by direct ELISA with just one antibody, MAb 3A5.

The sensitivity of the assay is relatively higher when compared with another similar method developed by Kerr[8], which has a detection limit of 10^5 CFU/mL.

The double-antibody ELISA method could detect as low as 0.1 CFU per gram of ground poultry meat or mL of pasteurized milk in our inoculation recovery studies, suggesting that it is an effective method to detect low concentrations of the organism in foods. Another study showed that the duration of enrichment in enrichment culture before detection can be reduced to 8-10 h compared to the general 16-18 h. As the number of bacteria is sufficient for detection after the short time enrichment, the efficiency can not be increased by prolonging the enrichment (data not shown).

Different strains of *E.coli* O157:H7 have different immunity when the heat-treated strains were immunized intraperitoneally. We tested two strains of *E.coli* O157:H7 in experiment of production of MAb. One was 882364 strain isolated from a patient with diarrhoea by Institute of Microbiology and Epidemiology, Chinese Center for Disease Control and Prevention. The other was 933w strain from USA. The mice showed different immunocompetence against the two strains of *E.coli* O157:H7 after experiencing the same immunity dose and immunity procedure. Titer of mice antiserum immunized by strain 882364 was higher than that immunized by strain 933w (data not shown). Additionally, two methods for production of immunogen have been used. One is described above, and the other is formalin–treated cells described by Padhye[9]. We used the two immunity strains as described above with the two immunogen production methods and found no obvious difference between the two methods. As the method for formalin-treated cells was time consuming (for at least 7 days), the simple method for heat-treated cells was selected for the rest of the whole experiment.

The available methods for detecting the organism in foods are extremely time consuming or not highly specific or sensitive.

Unlike most *E.coli*, lack of sorbitol fermentation within 24 h is typically a specific phenotypic characteristic of *E.coli* O157:H7, so sorbitol MacConkey agar (SMA) culture has been used widely to isolate the organism[10]. *E.coli* O157:H7 strains lack the enzyme β-glucuronidase which can cleave substrate methylumbelliferyl-β-D-glucuronide (MUG)[10], and a selective agar, SMA containing 0.1% MUG (MUG- SMA) has been developed to detect *E.coli* O157:H7. The plating procedure is widely adopted in detecting the target organism. However, false-positive results are very common due to the presence of a large number of other sorbitol and MUG negative *E.coli* or other strains, and false-negative results can also be observed, because partial *E.coli* O157:H7 strains are variants with properties of fermenting sorbitol and MUG-positive *E.coli* strains. Immunoassay can overcome the weakness of plating procedure, which entirely depends on specific phenotypic properties. Method of immunology could save more troubles of confirmation procedures that are necessarily done in culture methods.

PCR is a sensitive method in detecting a smaller number of target bacteria, which is based on amplification of specific gene fragments of virulence factors of EHEC or genes encoding protein specific for *E.coli* O157:H7, such as *stx, eae, hly, uidA* gene[3-6]. But most virulence genes (*stx, eae, hly*, *etc.*) of PCR target genes are not specific for *E.coli* O157 exclusively[11-13], such as *E.coli* O127:H6, O114:H2, O86:H34, O55:H-, *C.freundii* biotype 4280. *Eae* gene of *E.coli* O55:H7 is almost the same of *E.coli* O157:H7 except for one base[11], and many non-O157 EHEC, EPEC strains have *stx* and *hly* genes[13]. Therefore PCR for these virulence genes-based has a low specificity. It was reported that only 9 of 49 *E.coli* O157:H7 strains isolated from China in 1997-1998 contained *stx* and *hly* genes, and no virulence gene was detectable in other 40 *E.coli* O157:H7 strains[14].

Specificity of the method remains to be evaluated. The MAb specificity for *E.coli* O157:H7 with no cross-reaction with any other strains is very difficult to obtain. One reason for this situation is the presence of LPS in the immunizing antigen, which often yields LPS-directed antibodies that are not highly specific for *E.coli* O157:H7[9]. A monoclonal antibody to the long-chain LPS of *E.coli* O157:H7 has proved this point, and a sandwich ELISA based on MAb can react with O157 strains and *E.coli* O15, *Citrobacter freundii*, *Samonella urbana* and *Vibrio cholerae* O1 strains[8]. Sandwich ELISA based on this MAb has a relatively low specificity. A previous study has provided a method of sandwich ELISA using a monoclonal antibody specific to *Escherichia coli* O157:H7 and O26:H11[15]. Although this method can detect *Escherichia coli* O157:H7 in food with a high sensitivity, its specificity is not entirely evaluated, because only *E.coli* O157 with several H serotype strains, not including *E.coli* O26:H11 and other serotype strains could be measured. Another similar study, a sandwich ELISA based on a MAb by immunizing mice with a strain of O26, which is specific for eae gene, can react with partial eae-positive strains. A number of O26 strains are also present in the ELISA-negative group[16].

In order to elevate the detection sensitivity and specificity, sandwich ELISA combined with other technique is necessary. It was reported that the immunomagnetic separation (IMS) technique in combination with sandwich ELISA increases the sensitivity by about 100-fold that of the sandwich ELISA[17].

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