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Detection of *Salmonella* in Food Samples by Dot-ELISA using Polyclonal Antibody

Krisada Tuchinda[1], Boontharika Naknam[1], Patcharee Sunthornandh[1]*, Chaiwat Kittikul[1] and Ong-ard Lawhavinit[2]

ABSTRACT

The immunological method of dot-ELISA(enzyme linked immunosorbent assay) was performed using a polyclonal antibody produced in rabbits immunized with *Salmonella* ser. Anatum and *Salmonella* ser. Enteritidis. The purification of immunoglobulin G (IgG) was carried out by affinity chromatography and the purity was characterized by SDS-PAGE. The purified IgG was used to detect *Salmonella* by the dot-ELISA method. It was found that all *Salmonella* serovars representative of each group were positive. However, there was cross reaction with other enteric Gram-negative bacteria. Therefore, Rappaport Vassiliadis Soya (RVS) selective enrichment broth was used to select and increase the number of *Salmonella* (but inhibit the other enteric Gram-negative bacteria) which then were examined by the dot-ELISA method. It was found that this method was able eliminate the cross reaction as well. Detection of *Salmonella* from food samples using RVS broth followed by the dot-ELISA method, compared with a modified standard method (ISO 6579:2002) was performed. Forty-eight out of 175 food samples (27.43%) were positive for salmonellae detection which was the same result as by the standard method. Detection using RVS broth followed by the dot-ELISA method took only 24 h, which was shorter than the standard method. Moreover, determination by the dot-ELISA method was easy and used lower amounts of reagents, as well as being cheaper and highly sensitive.

Keywords: polyclonal antibody, dot-ELISA, *Salmonella*

INTRODUCTION

Salmonella is one of the important bacteria that cause diseases in the intestinal tract. It has more than 2,400 serovars (Chris and Kyriakides, 2002). *Salmonella* can be found in environments such as water, soil, plants and in many agricultural products, for example, meat, milk, eggs, vegetables and cooked-meat products. Diseases caused by *Salmonella* are generally called salmonellosis and include typhoid fever which is the most dangerous disease. Salmonellosis is a global disease but is mostly found in tropical countries, especially in south-east Asia, including Thailand. *Salmonella* cause diseases both in human and animals.

Health standards require that *Salmonella* must not be found in food produced for either domestic or exported consumption (FAO/WHO, 1997). It is essential to have a good detection method that provides corrective and accurate results and is highly specific and sensitive. Also,

1 Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fscipcs@ku.ac.th

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it is necessary to have a rapid detection method to prevent damage to the reputations of producers and for the health and safety of consumers. Detection of *Salmonella* is possible using international standard conventional methods that have been determined by accepted associations, such as the Association of Official Agricultural Chemists (AOAC), the Bacteriological Analytical Manual (BAM) and the International Organization for Standardization (ISO). These methods used selective broth to increase the number of *Salmonella* which provides accurate results; however it may take 3–4 d or more to confirm the detection. Therefore, an immunological method will become widely used if it is specific, highly sensitive and can detect a small amount of *Salmonella* in a sample. Ideally, it should also provide rapid results. Some methods may take only 2–3 h for analysis, such as the ELISA, immunomagnetic separation and the immunoprecipitation methods. Kit tests for some immunological methods are available. The dot-ELISA method is good because it is easy to use, economical and highly sensitive. In addition, it does not require special equipment and uses lower amounts of reagents. Detection of *Salmonella* using monoclonal antibody is specific to only one epitope. When it is used to detect *Salmonella* that has many serovars, it can detect only serovars that have an epitope that is specific only to that monoclonal antibody (Chaicumpa *et al*., 1995; Janyapoon *et al*., 2000). On the other hand, a polyclonal antibody that has antibodies against many epitopes helps to detect *Salmonella* in various serovars. The objective of the present study was to produce polyclonal antibody against *Salmonella*, and then separate pure immunoglobulin G (IgG) to use in the detection of *Salmonella* in samples using the dot-ELISA method.

The process of monoclonal antibody is quite complicated. It is costly, requires specific equipment and an expert producer. On the other hand, polyclonal antibody processing is cheaper and easier.

MATERIALS AND METHODS

Preparation of polyclonal antibody

Salmonella ser. Anatum and *Salmonella* ser. Enteritidis were used as antigens for immunization. Bacteria were prepared for immunization at 1×10^9 CFU/mL. The bacteria were inactivated with 0.5% formaldehyde then mixed with complete Freund's adjuvant. The rabbits used in the experiment were first immunized subcutaneously and subsequent immunizations were carried out at 1–2 w intervals. Blood was drawn and sera were separated and stored at -20 °C until used.

Determination of specific antibody titer in serum samples by direct agglutination

The assays were carried out using a microtiter plate and the sera of each blood sample were determined. The high titers of every sera were pooled for purification of IgG.

Purification of IgG of rabbit antisera immunized with salmonellae

Pooled sera were precipitated by $(NH_4)_2SO_4$ and purified by affinity chromatography using a protein G-sepharose affinity column, and the pooled fractions of IgG were lyophilized. The purity of IgG was determined by SDS-PAGE and protein concentration was carried out by Lowry's method (Lowry *et al*., 1951).

Preparation of *Salmonella* antigens for the dot-ELISA method and enumeration of *Salmonella* by dilution plate count

The cultures of *Salmonella* on TSA slant after incubation for 18 h at 37 °C were diluted with 0.85% NaCl to a turbidity of $OD_{600} = 0.1$. After boiling at 100 °C for 10 min to extract the antigen, followed by centrifugation, the

supernatant was used as the antigen for the dot-ELISA method. The enumeration of *Salmonell*a was carried out by dilution plate count at the same time in order to determine the number of *Salmonella* at $OD_{600} = 0.1$. Briefly, the *Salmonella* suspension at $OD_{600} = 0.1$ was serially diluted and spread on plate count agar, incubated at 37 °C for 24 h and then the CFU/plate were counted and the CFU/mL were calculated. The other enteric Gram-negative bacteria (*E. coli, Enterobacter* sp. and *Shigella* sp.) were enumerated in the same way as *Salmonella* so that the antigens of these bacteria could be prepared for use in the dot-ELISA method in order to determine the cross reaction.

Determination of specificity of purified IgG to *Salmonella* by the dot-ELISA method

The preparation of the *Salmonella* antigen at approximately 1×10^8 CFU/mL as described, was spotted onto nitrocellulose (NC) membrane and incubated at room temperature until dry. The dried NC was placed in a chamber to which was added blocking solution composed of 2% skimmed milk powder in phosphate buffered saline-Tween 20 (PBS-T) and then rotated for 30 min. After washing five times with PBS-T at intervals of 3 min, the diluted antibody (purified IgG) was added into the chamber and rotated for 90 min. After washing with PBS-T, goat anti-rabbit IgG horseradish peroxidase conjugate was added, rotated for 60 min and washed with PBS-T, then tetramethylbenzidine (TMB) substrate was added into the chamber, incubated for 2 min and the reaction was halted using distilled water.

Optimization of concentration of purified IgG and conjugate (goat anti-rabbit IgG HRP conjugated) by the dot-ELISA method

The purified IgG was prepared at concentrations of 50, 80, 100 and 312 μg/mL and the conjugate was diluted at 1/5,000, 1/10,000 and 1/12,000 and determined by the dot-ELISA method, as described above.

Determination of specificity of purified IgG to various serovars of *Salmonella* in different groups by the dot-ELISA method

The optimal concentrations of purified IgG and conjugate were used to determine the specificity to several serovars of *Salmonella* in different groups by the dot-ELISA method (Table 3). In addition, the other enteric Gram-negative bacteria (*E. coli, Enterobacter* sp. and *Shigella* sp.) were tested to determine the cross reaction by the same method.

Utilization of selective enrichment Rappaport-Vassiliadis Soya (RVS) broth to solve the problem of cross reaction

RVS broth is a selective enrichment broth that supports the growth of salmonellae and inhibits other enteric Gram-negative bacteria as well. RVS broth is also used in standard microbiological methods approved by BAM and ISO. The representative salmonellae and other enteric Gram-negative bacteria were inoculated in RVS broth starting with 1×10^3 and 1×10^4 CFU/mL, incubated at 42 °C for 18 h and then determined by the dot-ELISA and dilution plate count methods.

Detection of *Salmonella* in artificially contaminated foods by the dot-ELISA method

Three groups of mixed bacteria (Table 1) were prepared and included in three kinds of food (chicken sausage, milk sausage and pork ball) to make artificially contaminated foods with starting numbers of *Salmonella* of 3×10^3, 3×10^4 and 3×10^5 CFU/g. The mixed bacteria and food samples were combined using a stomacher and about 0.1 mL of the mixture was inoculated in RVS broth at 42 °C for 18 h and then determined by the dot-ELISA method.

Detection of salmonellae in food samples from markets by the dot-ELISA method compared with a standard microbiological method

Table 1 Groups of mixed bacteria used to make artificially contaminated food.

Group	Microorganisms
1	*Shigella sonnei*
	Escherichia coli
	Enterobacter aerogenes
2	*Salmonella* ser. Aberdeen
	Escherichia coli
	Enterobacter aerogenes
3	*Salmonella* ser. Anatum
	Escherichia coli
	Enterobacter aerogenes

Several food samples from markets selling both raw and ready-to-eat foods were analyzed for detection of salmonellae by the dot-ELISA method and a standard method (ISO 6579:2002). Briefly, food samples were inoculated in RVS broth and after incubation at 42 °C for 18 h, 0.1 mL of RVS broth was spread on selective xylose lysine deoxycholate (XLD) agar. The remaining RVS broth was boiled at 100 °C to extract the *Salmonella* antigen for determination by the dot-ELISA method. Well-isolated, typical colonies of *Salmonella* on XLD agar were collected to determine some typical biochemical characteristics.

RESULTS AND DISCUSSION

Determination of specific antibody titer in crude sera by direct agglutination

Crude sera from the 4th to the 12th bleeding were analyzed to determine the antibody titer. The results revealed that the average antibody titer was about 1/512 to 1/1,024.

Purification of IgG

The sera of high antibody titers were pooled for purification by affinity chromatography using a protein G-sepharose column. After purification, the purity of IgG was determined by SDS-PAGE, which showed two bands of 50 kDa and approximately 25 kDa which were heavy and light chains of IgG, respectively.

Preparation of *Salmonella* antigens and enumeration of *Salmonella* by dilution plate count

Suspended *Salmonella* on TSA slant was mixed with 0.85% NaCl to produce a turbidity of OD_{600} = 0.1 for extraction of the antigen and enumeration by the dilution plate count method, which showed that the amount of *Salmonella* at OD_{600} = 0.1 was about 1×10^8 CFU/mL. The numbers of the other enteric Gram-negative bacteria (*E. coli, Enterobacter* sp. and *Shigella* sp.) were determined in the same way as for *Salmonella*. The results indicated that the numbers of these other bacteria (about $1.1–2.7 \times 10^8$ CFU/mL) were similar to the numbers of *Salmonella*. The same growth rate could be explained by the fact that they were in the same enteric group. Willey *et al*. (2009) reported that the generation times of these bacteria were about 21–25 min.

Determination of specificity and optimization of concentration of purified IgG and conjugate (goat anti-rabbit IgG HRP conjugated) by the dot-ELISA method

The dot-ELISA method was carried out as described, using the purified IgG to test the responses to *Salmonella* and the extracted antigen from *Salmonella* ser. Anatum. The purified IgG was specific to *Salmonella*. The optimum concentration of IgG was 80 μg/mL with the conjugate at 1/10,000, showing a clear blue color on the nitrocellulose membrane (Table 2).

Determination of specificity to various serovars of *Salmonella* and determination of cross reaction to other enteric Gram-negative bacteria by the dot-ELISA method

The dot-ELISA method was used to determine the specificity to various serovars of *Salmonella* and tested for cross reaction with other enteric Gram-negative bacteria (*E. coli*, *Enterobacter* sp. and *Shigella* sp.). The results were positive for both salmonellae and the other enteric Gram-negative bacteria (Table 3), for which an explanation could be that whenever a polyclonal antibody is used which has antibodies against several epitopes on the cells of bacteria, then a cross reaction could occur with other bacteria within the same group. In this case, the other tested enteric Gram-negative bacteria were in the same family. They had some epitopes that were similar to *Salmonella*, (called a common epitope in this paper) which caused the cross reaction with the antibody against *Salmonella*. *E. coli* and *Enterobacter* sp. were chosen in the experiment because these bacteria are commonly found in many kinds of food and drink and specimens from patients, so it was necessary to solve this problem by using selective enrichment broth to enrich and select the *Salmonella*, but to be able to inhibit the other bacteria before detection by the dot-ELISA method.

Table 2 Concentrations of immunoglobulin G (IgG) and conjugate producing different blue color intensities on nitrocellulose membrane (with the optimum combination being purified IgG of 80 µg/mL and conjugate at 1/10,000).

Dilution of conjugate	Concentration of purified IgG (µg/mL)			
	50	80	100	312
1/5,000	-		-	-
1/10,000				
1/12,000	-		-	-

Table 3 Detection of various serovars of *Salmonella* and other enteric Gram-negative bacteria by the dot-ELISA method (+ indicates detected).

Microorganism	Group	Result	Microorganism	Group	Result
S. ser. Paratyphi A	A	+	*S.* ser. Aberdeen	F	+
S. ser. Paratyphi B	B	+	*S.* ser. Yoruba	I	+
S. ser. Derby	B	+	*S.* ser. Hvittingfoss	I	+
S. ser. Stanley	B	+	*S.* ser. Urbana	N	+
S. ser. Cholerae-suis	C1	+	*S.* ser. Waycross	S	+
S. ser. Virchow	C1	+	*E. coli*		+
S. ser. Enteritidis	D1	+	*Enterobacter* sp.		+
S. ser. Typhi	D1	+	*Shigella* sp.		+
S. ser. Dublin	D1	+			

Utilization of selective enrichment Rappaport-Vassiliadis Soya (RVS) broth to solve the problem of cross reaction

To address the problem of cross reaction with other enteric Gram-negative bacteria, selective enrichment RVS broth was used for pre-enrichment and selected *Salmonella*. *Salmonella*, at 1×10^3 CFU/mL were incubated in RVS broth at 42 °C. After 18 h of incubation, the concentration of the bacteria was $3.7–5.6 \times 10^7$ CFU/mL (by dilution plate count), while the other enteric Gram-negative bacteria were inhibited. Fries and Steinhof. (1997) and Hammack *et al.* (2001) found that the RVS broth could be used to inhibit the growth of other bacteria and enrich the *Salmonella* present in food samples. When determined by the dot-ELISA method after culturing in RVS broth, the results for *Salmonella* were positive, while the other enteric Gram-negative bacteria results were negative. Thus, it was shown that the RVS broth could be used to inhibit other enteric Gram-negative bacteria in order to prevent a false positive by the dot-ELISA method. Another reason for choosing RVS broth was because it is used in the standard method ISO 6579:2002 and in BAM.

Detection by the dot-ELISA method of *Salmonella* in artificially contaminated foods

Three groups of mixed bacteria were prepared and added to food samples, group 2 and group 3, which represented *Salmonella* ser. Aberdeen and *Salmonella* ser. Anatum, respectively, and could be detected by the dot-ELISA method after culturing in RVS broth for 18 h, while group 1 of the mixed bacteria which did not include *Salmonella* showed a negative result by the dot-ELISA method (Table 4). The results indicated that the RVS broth was a good selective-enrichment broth to pre-enrich and select salmonellae in food samples and that it inhibited the other enteric Gram-negative bacteria before detection by the dot-ELISA method.

Detection of salmonellae in food samples from markets by the dot-ELISA method compared with a standard microbiological method (ISO6579:2002)

In total, 175 food samples from many places were tested for the detection of salmonellae by culturing in RVS broth at 42 °C for 18 h. This was followed by dot-ELISA and spreading on selective XLD agar to pick typical colonies of *Salmonella* to characterize using some typical biochemical tests. Of the 175 samples, 48 (27.43%) tested positive with the dot-ELISA method, as well as by the standard method.

Table 4 Detection results of *Salmonella* in artificially contaminated food by the dot-ELISA method (+ indicates detected, - indicates not detected).

Food Sample	Original amount of bacteria (CFU/g)	Result of dot-ELISA		
		Group 1	Group 2	Group 3
Chicken sausage	3×10^3	-	+	+
	3×10^4	-	+	+
	3×10^5	-	+	+
Milk sausage	3×10^3	-	+	+
	3×10^4	-	+	+
	3×10^5	-	+	+
Pork ball	3×10^3	-	+	+
	3×10^4	-	+	+
	3×10^5	-	+	+

CONCLUSION

Detection of salmonellae was carried out using a combination of methods involving culturing in selective enrichment RVS broth at 42 °C for 18 h, followed with the dot-ELISA method using polyclonal antibody (purified IgG). The optimum concentration of purified IgG of 80 μg/mL with optimum conjugate at 1/10,000 were used in the dot-ELISA method which was able to detect amounts of *Salmonella* in contaminated food samples to at least levels of 1×10^2 to 1×10^3 CFU/mL. Cross reaction with other enteric non-salmonellae—namely *E. coli, Enterobacter* sp. and *Shigella* sp. did not occur because the growth of these bacteria could be inhibited by the RVS broth before detection by the dot-ELISA method, which took only 24 h and so was 3–4 times faster than a standard microbiological method. In addition, the dot-ELISA method was easy, used lower amounts of reagents, did not require expensive equipment and could be performed in a general laboratory. When the dot-ELISA method was compared with the PCR method (kit test) or other methods that use several pieces of equipment and reagents, detection by the dot-ELISA method was found to be cheaper and to cost only THB 20 per test.

Finally, the results of this study indicated that it was possible to use a polyclonal antibody and the dot-ELISA method for the detection of *Salmonella* in food samples, water and other drinks, and in specimens from patients.

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