CONSTRUCTION OF SPECIFIC DNA PROBE FOR THE DETECTION OF *SALMONELLA* IN FOOD

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Abstract. The *Salmonella* specific DNA fragment from genomic DNA of *S. typhimurium* ATCC 23566 was cloned in *E. coli* and successfully used as a digoxigenin labeled probe for detecting the presence of *Salmonella* serotypes in both artificially contaminated food and natural contaminated food samples.

INTRODUCTION

The potential pathogenicity of various enteric organisms and their presence in food is one of the major concerns to consumer. Among those organisms, *Salmonella* is one of the most prevalent enteric pathogen encountered in Thai seafood. At the present time, the identification of *Salmonella* sertotypes is made by using conventional microbiological methods which require many types of selective media and reagents for biochemical and serological tests. The process requires technical skill for initial recognition of the colony morphology and requires time of at least one week for the entire tests. This results in extended time for food storage which adds more expense to food producers.

Detection of *Salmonella* in food by specific probe has been reported in the past few years. Fitts (1985), Gopo *et al* (1988), Scholl *et al* (1990), Olsen *et al* (1991), Tsen *et al* (1991) and Bej *et al* (1994) have cloned *Salmonella* specific probes from genomic DNA of *Salmonella typhimurium*. The DNA probe has been considered valuable for detection of *Salmonella* contaminated in food samples by Fitts *et al* (1983). Comparative study of DNA hybridization method (DNAH) and the conventional culture method for detection of *Salmonella* in food was studied by Flowers *et al* (1987). The results indicated that the DNAH was as informative as the standard culture method. At present, the DNAH method was granted officially by the AOAC as an approved analytical method for the detection of *Salmonella* in food products (Flowers *et al*, 1987). In order to facilitate alternative technology for microbiological evaluation in food industries, this study was conducted to clone a DNA fragment specific to *Salmonella* spp and to develop a safe and sensitive non-radioactive digoxigenin labeled probe for detection of the *Salmonella* in food sample by manifold dot blot hybridization.

MATERIALS AND METHODS

Bacterial strains and plasmids

S. typhimurium LT2 ATCC 23566 was used as source of genomic DNA for probe construction. *E. coli* DH5α and plasmid pUC12 were used as host and vector in the cloning experiments. Chromosomal DNA of gram negative bacteria from clinical isolates of Ramathibodi Hospital were used for exclusion of the nonspecific fragments of probe by hybridization. The list of gram negative bacterial species is shown in Table 1. The total of 247 *Salmonella* strains used for determination of probe specificity were obtained from the National Salmonella-Shigella Center, Nonthaburi as shown in Table 2.

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Table 1

Clinical isolates of gram negative bacteria for exclusion of non *Salmonella* specific chromosomal DNA fragments in hybridization experiments.

Bacterial species	No. of strains
Acinetobacter calcoaceticus	2
A. lowfii	1
Aeromonas hydrophila	1
A. sobria	1
Citrobacter freundii	12
Enterobacter cloacae	1
Enterobacter spp	1
Escherichia coli	11
Klebsiella oxytoca	2
K. pneumoniae	1
Morganella morganii	1
Proteus mirabilis	1
P. vulgaris	1
Providensia rettgerri	1
Pseudomonas aeruginosa	1
P. maltophilia	1
Shigella dysenteriae	2
S. flexneri	11
S. sonnei	5
Vibrio spp (non agglutinating)	1
Yersinia enterocolitica	1
Total	59

Preparation of microorganisms from food samples

Small scale food sample was prepared by enriched 1 *g* of homogenized food in 9 ml of L broth or nutrient broth, mixed by vortex and then incubated at 37°C for 18-24 hours. After incubation, 1 ml of the preenriched sample was enriched in 9 ml of tetrathionate broth for 18-24 hours at 37°C. Then 10^0 to 10^{-3} dilutions of preenriched and enriched food samples were subjected to manifold filtration, 0.1 ml of 10^{-6} to 10^{-8} dilutions were subjected to gram negative count on McConkey agar and *Salmonella* count on Rambach agar (Rambach, 1990). One loopful of each culture was streaked on selective media *ie* Xylose Lysine Dextrose agar, Bismuth Sulphite agar, Salmonella Shigella agar, Brilliant Green agar and Rambach agar, for conventional culture method as described by AOAC.

DNA preparation

For chromosomal DNA preparation, 2 ml of overnight culture of *S. typhimurium* LT2 were harvested and resuspended in 200 µl of 25 mM Tris-HCl/5 mMEDTA pH 8.0 containing 0.2 mg of lysozyme. The cell suspension was incubated at 37°C for 30 minutes. Cells were lysed with 5 µl of 20% sodium dodecylsulfate. Protein was extracted twice with phenol. After removal of phenol residue by water saturated ether, chromosomal DNA was recovered by precipitation with 2 volumes of cold absolute ethanol, washed with 70% ethanol and resuspended in TE after drying. Plasmid DNA was isolated by the alkali-lysis method as previously described by Birnboim and Doly (1979).

Preparation of non radioactive labeled probe

To purify DNA fragment for probe preparation, randomly selected recombinant clones were digested with *Bam* HI restriction enzymes and electrophoresed in agarose gel to separate the inserted DNA from the vector DNA. The inserted DNA band was cut out from the gel and recovered by freeze-squeezing technique (Thuring *et al*, 1975), followed by phenol extraction and ethanol precipitation. The inserted fragment was labeled with 11-dUTP by using multiprimer method of Boehringer-Mannheim (Germany).

Filter preparation for hybridization

Colony, Southern or manifold dot blot hybridization was carried out by using filter paper or membrane. Colony hybridization was determined on Whatman 541 paper using the modification method of Sethabutr *et al* (1985). The colonies on paper were steamed for 5 minutes in lysing solution (0.5 M NaOH/1.5 M NaCl) and immersed in neutralizing solution (1M Tris HCl pH 7/2M NaCl) for 10 minutes. The filters were air dried for 1-2 hours, followed by proteinase K treatment (1 mg/ml) at 65°C for 1 hour. Southern transfer was achieved either on nitrocellulose or nylon membrane by the method of Southern (1975). Both the colony and Southern were mainly used to determine the *Salmonella* grown in media. Manifold dot blot was prepared by making 10 fold dilutions of bacterial cultures or food samples and spotted 100 µl of each dilution on nylon filters (Gibco/BRL "USA").

Table 2

Salmonella serotypes used in the determination of probe specificity.

Serotypes	Groups	No. tested	Serotypes	Groups	No. tested
S. aberdeen	F	4	*S. goldesberg*	N	1
S. agona	B	6	*S. halmstad*	E_2	2
S. alachua	O	4	*S. [illegible]adar*	C_2	6
S. albany	C_3	2	*S. havana*	G_2	2
S. anatum	E_1	5	*S. heidelberg*	B	2
S. augustenburg	C_1	2	*S. hvittingfoss*	I	3
S. bareilly	C_1	2	*S. infantis*	C_1	1
S. bere	X	2	*S. javiana*	D_1	3
S. blockley	C_2	5	*S. kentucky*	C_3	2
S. bovismorbificans	C_2	6	*S. krefeld*	E_4	4
S. bredeney	B	2	*S. kumasi*	N	1
S. brunei	C_3	2	*S. langensalza*	E_1	3
S. cerro	K	6	*S. lexington*	E_1	2
S. choleraesuis	C_1	4	*S. litchfield*	C_2	2
S. derby	B	5	*S. liverpool*	E_4	1
S. eastbourne	D	1	*S. livingstone*	C_1	1
S. emek	C_3	5	*S. london*	E_1	4
S. enteritidis	D_1	5	*S. mbandaka*	C_1	3
S. farmsen	G_2	2	*S. montevideo*	C_1	5
S. gaminara	I	1	*S. muenchen*	C_2	3
S. gera	T	1	*S. muenster*	E_1	2
S. newport	C_2	3	*S. stanley*	B	5
S. newington	E_2	1	*S. tennessee*	C_1	1
S. ohio	C_1	4	*S. thompson*	C_1	4
S. oranienburg	C_1	3	*S. typhi*	B	6
S. orion	E_1	2	*S. typhimurium*	B	7
S. panama	D_1	6	*S. urbana*	N	5
S. paratyphi A	A	5	*S. virchow*	C_1	5
S. paratyphi B	B	5	*S. wandsworth*	Q	2
S. poona	G_1	3	*S. washington*	G_1	1
S. portsdam	C_1	2	*S. welikade*	I	2
S. quakam	D	3	*S. weltvreden*	E_1	6
S. rissen	C_1	3	*S. worthington*	G_2	2
S. rubinlow	F	2	*S. arizonae*	-	2
S. ruiru	L	3	*S.I.4,5,12:-:-*	B	4
S. saintpaul	B	4	*S.I.35:-:-*	O	2
S. sandiego	B	4	*S.I.39:-:-*	Q	1
S. schwarzengrund	B	2	*S.I.4,12:-:-*	B	2
S. senftenberg	E_4	5	*S.IV.43:Z4,Z23:-*	U	4
S. singapore	C_1	1			
			Grand Total		247

Hybridization method and detection

Hybridization with biotinylated probe and detection were done as previously described (Sethabutr *et al*, 1985). Hybridization with digoxigenin together with colorimetric detection was performed according to the instruction manual of Dig Nucleic Acid Labelling and Detection Kit (Boehringer-Manheim, Germany).

Library construction of recombinant DNA

Genomic DNA from *S. typhimurium* LT2 ATCC 23566 was cloned into *E. coli* DH5α by ligating *Bam* HI fragments with the plasmid vector pUC12. Recombinant clones were recognized as colorless colonies on ampicillin 50μg/ml plus X-gal medium while non recombinant clones appeared blue.

RESULTS

Construction of probe and preliminary study for specificity

Plasmids isolated from seventy white *E. coli* transformants on X-gal plus ampicillin containing medium were subjected to *Bam* HI digestion and gel electrophoresis for size determination. Six clones which harbored inserted fragments of the size 4-7 kilobases were randomly picked up for further evaluation of their specificity as probes. These clones were designated, A18, A30, A32, A34, A36, and A37. Each clone was grown for a large scale plasmid preparation. The inserted fragments were then recovered from agarose gel and labeled with biotin 14-dATP, 11 dUTP and used as probes. These probes were used in colony hybridization with 59 strains of 21 species of gram negative bacteria (Table 1) and 91 strains of 35 *Salmonella* serotypes (Table 2). From these six probes, probe A34, A36, A37 showed positive hybridization reaction with majority of gram negative bacterial DNA, hence they were excluded for further study. The other three probes, A18, A30, A32 gave very weak hybridization (±) with all gram negative bacterial DNA except Shigellae. Two of these three probes; A18 and A30 could hybridize to all 91 strains of *Salmonella* serotypes (Table 3). The level of hybridization is 3+ to 4+. The probe A32 hybridized with only 45/91 strains of *Salmonella*, hence it was excluded for further study.

Trimming of pSA18 and pSA30 and selection of *Salmonella* specific probe

Plasmids harboring DNA from clones, A18 and A30 which were further designated as pSA18 and pSA30 were subjected to digestion with various enzymes *Acc* I, *Bam* HI, *EcoR* I, *Pst* I and *Sal* I. Using either single or a combination of these enzymes, the restriction maps were obtained (Fig 1). Digestion of pSA18 with *Acc*I, *Bam* HI, *Pst* I gave 6 smaller fragments whereas digestion of pSA30 with *Bam* HI, *EcoR* I and *Sal* I gave 4 smaller fragments (Table 4). Fragments No. 1 from both plasmids were inserted into puc12. Seven smaller fragments and the two original inserted fragments as shown in Table 4 were labeled with digoxigenin. Their specificities were determined by Southern hybridization with large amounts of DNA (approximately 3 mg DNA) extracted from 13 strains of gram negative bacteria which were shown to cross hybridize to the original probes: A18 and A30. The level of hybridization was determined after overnight incubation of membrane with dye solution. Grading was done by eye. Fragments A18: 4-5 and A18 : 6 cross-hybridized with several strains of gram negative bacteria and also failed to hybridize with one out of 4 strains of *S. paratyphi B* (1/4), *Salmonella* I:39:-:-(1/1) and *Salmonella* IV : 43 : Z4 : Z23:-(1/1) (data not shown). Cross hybridization to non-pathogenic bacterial DNA was observed in some fragments. Fragment A18:3-4; cross hybridized (4+) to *C. freundii*. Fragment A30 : 4 cross hybridized (3+) to *M. morganii*. Fragments A30:2 and A30:3 cross hybridized to DNA of some gram negative species. All of these fragments were therefore excluded. The fragment number 2 of pSA18 designated as A18:2 gave only weak hybridization to non-pathogenic gram negative DNA (1+), and to *Shigella* spp, but strongly hybridized (4+) to all 247 strains of 79 *Salmonella* serotypes shown in Table 2. The fragment A18:2 was therefore considered as the best for further development as a probe for the detection of *Salmonella* in food.

Sensitivity and specificity of A18:2 probe to detect *Salmonella* in broth culture

The sensitivity and specificity of the A18:2 probe was initially evaluated by determining its ability to detect *Salmonella* and gram negative bacteria in broth culture prior to its use in detecting

Table 3

Hybridization of *Salmonella* probes within the genus *Salmonella.*

Groups	Serotypes	No. tested	No. of positive hybridization of indicated DNA probes with *Salmonella* sp		
			A18	A30	A32
A	*S. paratyphi A*	2	2	2	2
B	*S. agona*	5	5	5	0
	S. derby	4	4	4	0
	S. paratyphi B	1	1	1	0
	S. saint-paul	1	1	1	1
	S. stanley	1	1	1	1
	S. typhimurium	5	5	5	5
C_1	*S. oranienberg*	1	1	1	0
	S. thompson	1	1	1	1
	S. virchow	1	1	1	1
C_2	*S. blockley*	5	5	5	0
	S. bovismorbificans	2	2	2	2
	S. newport	1	1	1	1
C_3	*S. emek*	1	1	1	0
D_1	*S. panama*	1	1	1	1
	S. typhi	4	4	4	4
E_1	*S. anatum*	4	4	4	0
	S. langensalza	1	1	1	0
	S. london	2	2	2	2
	S. weltvreden	3	3	3	3
E_4	*S. krefeld*	4	4	4	0
F	*S. aberdeen*	4	4	4	4
	S. rubislow	2	2	2	2
G_1	*S. poona*	2	2	2	0
G_2	*S. farmsen*	1	1	1	0
	S. havana	2	2	2	0
I	*S. hvittingfoss*	3	3	3	3
K	*S. cerro*	6	6	6	6
L	*S. ruiru*	2	2	2	0
N	*S. kumasi*	1	1	1	0
	S. rubana	5	5	5	5
O	*S. alachua*	4	4	4	0
Q	*S. wandsworth*	2	2	2	2
U	*IV 43:Z4 Z23:-*	2	2	2	0
X	*S. bere*	2	2	2	0
	Total	91	91	91	45

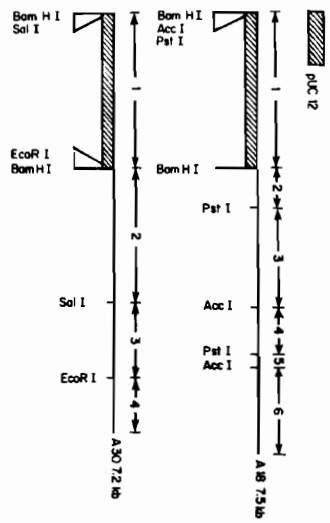

Fig 1–Restriction map of *Salmonella typhimurium* clone A 18 and A 13 following restriction enzymes digestion with *Acc* I, *Bam* HI, *EcoR* 1, *Pst* 1 and *Sal* 1.

Salmonella serotypes in food. *Salmonella typhimurium* ATCC 23566 and the gram negative bacteria initially known to cross hybridize to the probe were tested, using *Y. enterocolitica* as a negative control. Cells from 100 µl of ten fold dilution of 18 hours broth culture of various bacteria were filtered and fixed on membrane filters. The filters were treated, hybridized with digoxigenin labeled A18:2 probe and detected by colorimetric method. The level of hybridization was graded from 0, ± to 4 + by naked eye. The probe could detect *Salmonella* at $\geq 10^7$ cfu/dot and the hybridization level was 3+. Other gram negative bacteria at inoculum of 10^5-10^9 cfu/dot did not cross hybridize to the probe.

Sensitivity and specificity of probe in detecting *Salmonella* in food

To determine whether the nonradioactive digoxiginin labeled probe A18:2 can detect *Salmonella* in food samples against a background of a large number of gram negative bacteria in food, both artificially contaminated and natural contaminated foods were evaluated. In artificially contaminated food, shrimp were inoculated with a known number of *Salmonella* spp at the concentrations of 4, 40 and 400 cells/ml. In natural contaminated food, squid and shrimp which were documented for presence or absence for *Salmonella* by the Division of Food Analysis, Ministry of Public Health were used for study. After the process of preenrichment and enrichment as previously described, all samples from enriched media were used to detect *Salmonella* by manifold dot blot hybridization. Colony counts and identification of *Salmonella* and other gram negative bacteria isolated from food samples were also done. Pure cultures of each gram negative bacteria were subjected to colony hybridiza-

Table 4

DNA fragments which were selected as probes for hybridization with various Gram negative DNA.

Fragments No.		Designated	Restriction enzymes used to generate fragment	Size (kb)
pSA18	1	A18:insert	*Bam*H I	4.8
	2	A18:2	*Bam*HI - *Pst* I	0.7
	3 + 4	A18:3	*Pst* I	1.7
	4 + 5	A18:4	*Acc* I	0.8
	6	A18:6	*Acc* I	1.4
		A30:insert	*Bam*H I	4.5
pSA30	1	A30:2	*Sal* I - *EcoR* I	2.3
	2	A30:3	*Sal* I - *EcoR* I	1.3
	3	A30:4	*Sal* I - *EcoR* I	0.9
	4			

Table 5

Hybridization reaction of A18 and A30 derivative probes to gram negative DNA.

Probes	*S. typhimurium*	*A. sobria*	*C. freundii*	*E. coli*	*E. cloacae*	*Enterobacter* spp	*K. oxytoca*	*K. pneumoniae*	*M. morganii*	*P. rettgeri*	*S. dysenteriae*	*S. flexneri*	*S. sonnei*	*Y. enterocolitica*
A18 : insert	4+	-	4+	2+	3+	2+	1+	+	1+	-	1+	1+	2+	1+
A18 : 2	4+	-	1+	1+	-	-	-	+	±	-	1+	1+	1+	-
A18 : 3-4	4+	-	4+	2+	2+	1+	-	±	±	-	1+	1+	1+	+
A18 : 4-5	4+	-	1+	2+	1+	±	1+	1+	-	-	1+	1+	1+	-
A18 : 6	4+	-	1+	2+	1+	1+	±	1+	-	-	2+	2+	2+	-
A30 : insert	4+	2+	2+	2+	2+	2+	2+	2+	2+	1+	2+	3+	3+	-
A30 : 2	4+	1+	3+	2+	1+	1+	1+	1+	1+	±	3+	2	3+	-
A30 : 3	4+	±	2+	1+	1+	±	1+	1+	±	-	2+	1+	2+	-
A30 : 4	4+	±	2+	1+	1+	±	2+	2+	3+	-	2+	2	3+	-

Table 6

Specifity of A18:2 for detection of *Salmonella* in natural contaminated food and in *Salmonella* inoculated food.

Food		No. of *Salmonella*	No. of organisms after enrichment added	Hybridization Reaction	
				Mix culture	Pure culture
Types	Specification				
Artificial	Shrimp	4	*Salmonella* > 10^8	+	+
Contaminated			*E. coli* 2×10^8	-	-
			Citrobacter 1×10^8	-	+
			K. pneumoniae 1×10^7	-	-
	Shrimp	40	*S. typhimurium* > 10^8	+	+
	Shrimp	400	*S. typhimurium* > 10^8	+	+
Natural	Squid		*Salmonella* 2.2×10^8	-	+
Contaminated		-	*Citrobacter* spp 1×10^8	-	+
			Enterobacter spp 2×10^8	-	-
			K. pneumoniae 3×10^7	-	-
	Shrimp		*E. coli* 1.8×10^8	-	-
		-	*K. pneumoniae* 8.6×1^7	-	-

ND = not done

tion. The result showed that no cross hybridization occurred with gram negative bacteria either as pure culture or those contaminated in food. The probe could detect *Salmonella* in both artificially contaminated and natural contaminated food at $\geq 10^7$ cfu/ml which is always achievable following overnight incubation of enriched culture. The results are shown in Table 6.

Evaluation of A18:2 probe for *Salmonella* detection in preenriched food

In order to see if the detection of *Salmonella* in food samples can be shortened by the manifold dot blot method, overnight preenriched natural foods were subjected to manifold filtration and dot blot hybridization. These samples were previously analyzed for the presence or absence of *Salmonella* by the Division of Food Analysis, Ministry of Public Health. They were preenriched overnight and then ten-fold diluted to 10^{-1}. One hundred µl of each dilution was dot blotted on to membrane for hybridization. The probe detected only the culture containing *Salmonella*. Undiluted samples which contaminated $\geq 10^7$ cell/dot of *Salmonella* showed 4+ hybridization and the 10^{-1} dilution showed 3+ reaction.

DISCUSSION

Nucleic acid probes for rapid identification of microbial pathogens in food are currently being developed. In addition to *Salmonella*, probes have been developed for the detection of various pathogens, for example, *Shigella* spp (Lampel *et al*, 1990), foodborne enterotoxigenic *E. coli* (Hill *et al*, 1983a), *Yersinia enterocolitica* (Hill *et al*, 1983b: Jagow and Hill, 1986, 1988), *Listeria* spp (Datta *et al*, 1983: Klinger *et al*, 1988). *V. parahaemolyticus* (Kaper *et al*, 1984) and *Vibrio* spp (Kaper and Levine, 1981). Although many of these probes became commercially available, the majority of these probes are radioactively labeled which is discouraging for wide use, particularly in developing countries, due to the high cost of radioactive material and the risk of improper handling and process for discarding the radioactive material. Mostly the methods used for these probes were colony hybridization which was reported to be convenient with high sensitivity. The method, however requires addtional one day for growing the cells. We have cloned *Salmonella* specific DNA from genomic DNA of *S. typhimu-rium* ATCC 23566 and searched for clones harbored *Salmonella* fragments large enough to contain a specific gene for *Salmonella* serotypes. From these fragments, the best fragment was selected to be used as a probe and labeled with digoxigenin and used in manifold dot blot hybridization to shorten the detection time and to enable the handling of a large number of food samples.

We found that the 0.7 kb fragment of pSA18 *ie* pSA18-2 was the best. It gave strong hybridization with *Salmonella* and only weakly cross hybridized with certain other enteric bacteria encountered in food contamination. As the probe showed weak cross hybridization with large amounts of gram negative DNA, the background interference of gram negative bacteria which may be encountered in the dot blot hybridization was also determined together with sensitivity test of the probe. The A18:2 probe was able to detect *Salmonella* at 10^6-10^7 cfu/dot at the level of 3+ to 4+. The chemiluminesence method enhanced the detection of a lower inoculum (10^5 cell/dot) with stronger reaction. Weak cross hybridization (1+) of A18:2 to *E. coli* at $> 10^6$ cell/dot was rarely found. However this weak reaction is neglegible, since it can be differentiated easily from a positive signal of *Salmonella*. Thus, a gram negative background does not interfere with the interpretation of the presence of *Salmonella*. The sensitivity of this probe is 10^5-10^6 cell/dot, similar to the report of Dovey and Towner (1989), who found reliable dot blot detection by biotinylated probe is limitted at 10^5 cell/dot. Similarly, a ten times increased dot blot hybridization reaction using a ^{32}P labeled probe was a previously reported for *S. typhi* (Rubin *et al*, 1985). Thus we suggest that our nonradioactive probe is sensitive and specific enough for the detection of *Salmonella* in either preenriched or enriched food. Sequencing of this fragment was done and the determination of primers for the PCR amplification of target DNA confirmed that no cross amplification occurred with *Shigella* as seen in the DNAH method. This will enable the furture use of this technique for rapid detection of *Salmonella* in both food samples and human clinical specimens.

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