Detection of *Salmonella* by Using the Colorimetric DNA/rRNA Sandwich Hybridization in Microtiter Wells

Takanori NAMIMATSU, Mika TSUNA¹⁾, Yasuo IMAI, Satoshi FUTO¹⁾, Shizuo MITSUSE¹⁾, Tetsuya SAKANO and Shizuo SATO

Zen-Noh Institute of Animal Health, 7 Ohja-machi, Sakura-shi, Chiba, 285-0043 and ¹⁾Nippon Flour Mills Co., Ltd. Central Laboratory, 5-1-3 Midorigaoka, Atsugi-shi, Kanagawa 243-0041, Japan

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ABSTRACT. A rapid and readily available DNA probe kit was developed for the detection of *Salmonella* spp. This kit utilized the colorimetric DNA/rRNA sandwich hybridization method in microtiter wells. Within 3 hr *Salmonella* spp. in selective enrichment broth cultures were detected by the DNA probe kit. The kit effectively identified all of 187 strains of *Salmonella* tested and yielded no false-positive reactions in the examination of 674 pure cultures of non-salmonellae. The DNA probe kit could detect 10^5 cfu/ml in pure culture. A total of 379 naturally contaminated samples (raw chicken meat, liquid egg, animal feeds, poultry feces and frozen foods) were tested, both by the standard culture method and the DNA probe kit. The 169 of these samples were culture positive and 210 were culture negative. The sensitivity of the DNA probe kit was 98.2% (166/169) and the specificity was 99.5% (209/210). These results show that the DNA probe kit is a useful tool to examine a large number of various samples for contamination by *Salmonella* spp. in food and livestock industry.—**KEY WORDS:** DNA probe kit, hybridization, rapid detection kit, rRNA, *Salmonella*.

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Salmonella spp. is one of the most important pathogenic bacteria for humans and animals [3]. Therefore, it is very important to detect for *Salmonella* spp. from diarrheal stools of salmonellosis in the humans and the animals, foods, feeds and environmental samples. For increase of detection rate of *Salmonella* spp. from these samples, the culture methods and the selective media were modified and developed [7, 14, 17]. But the culture methods for *Salmonella* spp. can take 5–6 days until complete judgement [19].

In the past years, DNA probes hybridizing to the variable region of bacterial ribosomal RNA (rRNA) have been developed to identify some bacterial species [6, 11, 20]. For the specific detection of *Salmonella* spp., we developed the visual colorimetric DNA/rRNA sandwich hybridization system derived from the 23S rRNA gene of *Salmonella* Typhimurium in microtiter wells [15]. This system can reduce the period of detecting *Salmonella* spp. from samples by up to 2 days.

In this paper, this hybridization system was examined for the specificity and sensitivity using pure culture samples and was compared with standard culture method for the detection of *Salmonella* spp. in many artificially and naturally contaminated samples.

MATERIALS AND METHODS

The visual colorimetric DNA/rRNA sandwich hybridization system assay: Selective enrichment cultures were probed according to the manufacturer's instructions [15]. Briefly, test samples were exposed to strong alkaline conditions (0.3N NaOH) to release rRNA from bacterial cells. The hybridization buffer containing detector DNA probes and the lysate were placed in microtiter plate wells

where capturing DNA for rRNA of salmonella were fixed. Following incubation for 1 hr at 37°C, wells were washed. The solution containing anti-digoxigenin antibodies coupled to the enzyme horseradish peroxidase were added to the wells. Following incubation for 1 hr at 37°C, wells were washed again. The substrate solutions consisting of hydrogen peroxide and tetramethylbenzidine were added to the wells. Following incubation for 15 min at room temperature, the color change of the substrate solutions were read visually or measured by a microplate photometer with 660-nm filter. A sample is considered positive for salmonella when blue color develops by the reaction between the enzyme conjugate and the substrate solution, or a sample with absorbance value greater than 0.05 when read against a negative control was identified positive for salmonella. Analytical steps of this DNA probe kit is performed within approximately 3 hr.

Determination of the Specificity for pure cultured bacteria: A total of 187 strains of *Salmonella enterica* representing 127 serovars of 34 somatic groups and 674 non-salmonellae strains (Table 1) were used to challenge the specificity of the DNA probe kits. All cultures were maintained in a semisolid agar medium stored at 4°C. Stock cultures in semisolid storage medium were inoculated in 10 ml of Trypto-Soy (TS) Broth (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan) and incubated at 37°C for 24 hr. Undiluted portions (0.3 ml) of individual culture preparations were assayed by the DNA probe kit.

Determination of the detection limit of *S. Typhimurium* L-417 in HTT broth: About 100 colony-forming unit (cfu) of *S. Typhimurium* L-417 were inoculated into 100 ml of the selective enrichment medium, Hajna Tetrathionate (HTT) broth (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan), and incubated. Portions (0.3 ml) for sensitivity test

were taken at different times along the course of incubation. Viable counts of *S. Typhimurium* L-417 in each portion were obtained by plating serial dilutions of the broth cultures on Trypto-Soy agar (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan) and incubating the plates at 37°C for 20 hr.

Comparison of the DNA probe kit and standard culture for the detection of salmonellae in artificially or naturally contaminated samples: Artificially contaminated samples were prepared as follow. *S. Typhimurium* L-417 was grown in TS broth (10 ml) at 37°C for 16 hr. The cultures serially diluted in sterile phosphate-buffered saline and were inoculated into 25 g of samples (raw broiler meat, liquid egg or swine feces) to final concentrations of 10^2 , 10^1 , 10^0 , 10^{-1} , 0 cfu/25 g. A total of 379 samples, including 95 raw broiler meat, 53 unpasteurized liquid whole egg, 90 poultry feces, 30 uncooked frozen foods and 111 animal feed samples were obtained as naturally contaminated samples. The raw broiler meat and the frozen food samples were collected from some supermarkets and poultry feces samples from broiler farms. The liquid egg and feed samples were supplied by the manufacturers. Samples were transported to the laboratory directly after collection where they were stored at 4°C or -80°C until testing.

Standard culture procedure: Twenty-five grams of sample except feces was mixed and homogenized in 225 ml of Buffered Peptone Water (BPW, Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37°C for 18 hr. A portion of 10 ml was transferred to 100 ml of HTT broth and incubated at 42°C for 24 hr.

A feces sample of 25 g was mixed and homogenized in 225 ml of HTT broth and incubated at 42°C for 24 hr.

A loopful of the enriched HTT broth was streaked onto Brilliant Green (BG) agar (BBL, Becton Dickinson and Company, Cockeysville, U.S.A.) and Desoxycholate Hydrogen sulfide Lactose (DHL) agar (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan). Following incubation at 37°C for 20 hr, suspect colonies from both plating media were purified on DHL agar. Purified strains were confirmed biochemically by Triple Sugar Iron (TSI) agar (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan), Sulfide Indole Motility (SIM) medium (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan) and Lysin Indole Motility (LIM) semisolid agar (EIKEN CHEMICAL Co., Ltd., Tokyo, Japan) [19].

Calculation and interpretation: The sensitivity and specificity of the DNA probe kit for naturally contaminated samples were compared with those of the standard culture method [1].

Statistical analysis: The McNemar's test [9] was used to evaluate differences in the results of the DNA probe kits and of the standard culture methods, and the significance of the test was established at $p < 0.05$.

RESULTS

Specificity of the DNA probe kits: Pure undiluted cultures

Table 1. Reactivity of the DNA probe kit to *Salmonella* and non-salmonellae bacteria

Test organism	No. of strain tested	No. of positive strains	No. of negative strains
<i>Salmonella</i>			
Group O 4	31	31	0
Group O 6, 7	32	32	0
Group O 6,8	15	15	0
Group O 9, 12	38	38	0
Group O 3, 10	13	13	0
Group O 1,3,19	7	7	0
Others	51	51	0
non-salmonellae			
<i>Enterobacteriaceae</i>			
<i>Citrobacter freundii</i>	29	0	29
<i>Proteus</i> spp.	82	0	82
Others	461	0	461
non- <i>Enterobacteriaceae</i>			
<i>Enterobacteriaceae</i>	102	0	102

of 187 strains of *Salmonella* and 674 strains of non-salmonellae were tested with the DNA probe kits. The color change of the substrate solutions in wells were read visually. Positive results were obtained with all strains of *Salmonella* spp. (Table 1). The DNA probe kits yielded no false-positive reactions in the examination of 674 pure cultures of non-salmonellae.

Limit of detection for *S. Typhimurium* L-417: The color change were read visually and measured by a microplate photometer. When there were 1.8×10^5 cfu of *S. Typhimurium* L-417/ml•HTT broth, the color of substrate solution was changed in blue and absorbance value is 0.067. When there were 3.4×10^4 cfu/ml•HTT broth, the color of substrate solution was not changed and absorbance value remained well below the threshold level of 0.05. The detection limit of the DNA probe kit was 1.8×10^5 cfu/ml•HTT broth in pure culture (Table 2).

Comparison of the DNA probe kits and standard culture method using artificially contaminated samples: The color change of the substrate solutions were read visually and measured. Raw broiler meat samples inoculated with *S. Typhimurium* L-417 at numbers from 6.0×10^1 to 10^2 cfu/

Table 2. Sensitivity of the DNA probe kit to *S. Typhimurium* in HTT broth

Viable cells (cfu/ml)	Result	
	Visually ^{a)}	Absorbance ^{b)}
1.0×10^6	+	0.764
8.8×10^5	+	0.243
1.8×10^5	+	0.067
3.4×10^4	-	0.002
1.1×10^0	-	0.001
NT ^{c)}	-	0.000

a) +: Substrate solution was changed in blue.

-: Substrate solution was colorless.

b) Absorbance (660 nm) was judged.

c) Not inoculated.

Table 3. Recovery of *S. Typhimurium* from the selective enrichment cultures of raw broiler meat, liquid egg or swine feces with different level of *S. Typhimurium* contamination by the DNA probe kit and standard culture

	Inoculated dose (cfu/25 g)	the DNA probe kit		Standard culture
		Visually ^{a)}	Absorbance ^{b)}	
Raw broiler meat	6.0×10^2	+	2.374	+
	6.0×10^1	+	1.024	+
	6.0×10^0	+	0.663	+
	6.0×10^{-1}	+	1.017	+
	NI ^{c)}	–	0.001	–
Liquid egg	1.2×10^2	+	>3.000	+
	1.2×10^1	+	>3.000	+
	1.2×10^0	+	>3.000	+
	1.2×10^{-1}	–	0.004	–
	NI	–	0.002	–
Swine feces	1.2×10^2	+	1.078	+
	1.2×10^1	+	0.651	+
	1.2×10^0	–	0.003	–
	1.2×10^{-1}	–	0.004	–
	NI	–	0.001	–

a) +: Substrate solution was changed in blue. –: Substrate solution was colorless.

b) Absorbance (660 nm) was judged.

c) Not inoculated.

25 g were positive by both the DNA probe kit and conventional culture method. Liquid egg samples inoculated at numbers from 1.2×10^0 to 10^2 cfu/25 g were positive by both method. Swine feces samples inoculated at numbers from 1.2×10^1 to 10^2 cfu/25 g were positive by both method. Liquid egg sample inoculated at numbers 1.2×10^{-1} cfu/25 g, swine feces samples inoculated at numbers 1.2×10^{-1} to 10^0 cfu/25 g and no inoculated raw broiler, liquid egg and swine feces samples were negative by both methods (Table 3).

Naturally contaminated samples: The color change of the substrate solutions were read visually when the results of the DNA probe kits were judged. Of 95 raw broiler meat samples, 44 samples were positive by both methods and 49 samples were negative by both methods. One sample was positive only by the DNA probe kits and one sample was positive only by culture method. The sensitivity of the DNA probe kit was 97.5%, and the specificity was 98.0%. Of 53 liquid egg samples, 16 samples were positive by both methods and 37 samples were negative by both methods. The sensitivity and specificity of the DNA probe kit were 100%. Of 90 poultry feces samples, 81 samples were positive by both methods and 8 samples were negative by both methods. One sample was positive only by culture method. The sensitivity of the DNA probe kit was 98.8%, and the specificity was 100%. Of 111 animal feed samples, 24 samples were positive by both methods and 86 samples were negative by both methods. One sample was positive only by culture method. The sensitivity of the DNA probe kit was 96.0%, and the specificity was 100%. Of 30 frozen food samples, one sample was positive by both methods and 29 samples were negative by both methods. The

sensitivity and specificity of the DNA probe kit were 100% (Table 4). As for the results by the DNA probe kits and culture methods, there were not the statistical significant difference by McNemar's test.

DISCUSSION

The DNA probe kits for rapid detection *Salmonella* spp. by the visual colorimetric DNA/rRNA sandwich hybridization system in microtiter wells [15] was examined for the specificity. The DNA probe kits reliably detected 187 strains of *S. enterica* representing 34 somatic groups and not reacted 674 non-salmonellae strains. Over 2,000 *Salmonella* serovars by the Kauffmann-White antigenic scheme were known [3]. In Public Health, *S. Typhi* and *S. Paratyphi* A and C caused typhoid fever, *S. Enteritidis* and *S. Typhimurium* caused gastroenteritis were most important serovars [8, 16]. In animal health, pathogenic and important serovars are *S. Dublin*, *S. Choleraesuis* [3], *S. Gallinarum*-*Pullorum* [18] *etc.* In this study, it was confirmed that the DNA probe kit detected these serovars. Several of the *Citrobacter freundii* strains were reactive with the commercial rapid detection kits for *Salmonella* spp. [2]. But in this study, none of the 29 *C. freundii* strains isolated from animal feces and feed produced false-positive reactions with the DNA probe kits. This result shows that the DNA probe kit is a useful tool to check *Salmonella* spp. in public health and animal health.

The detection limit for *S. Typhimurium* L-417 in HTT broth of the DNA probe kit was 1.8×10^5 cfu/ml. When samples were twice cultured for pre-enrichment and selective enrichment, the DNA probe kits and the standard

Table 4. Comparison of the DNA probe kit and standard culture method for detection of *Salmonella* spp. from naturally contaminated samples

Sample	Number of samples	kit+ •	kit – •	kit + •	kit – •	the DNA probe kit	
		culture+ ^{a)}	culture+ ^{b)}	culture– ^{c)}	culture– ^{d)}	sensitivity ^{e)}	specificity ^{f)}
Raw broiler meat	95	44	1	1	49	97.8%	98.0%
Animal feed	111	24	1	0	86	96.0%	100.0%
Poultry feces	90	81	1	0	8	98.8%	100.0%
Liquid egg	53	16	0	0	37	100.0%	100.0%
Frozen foods	30	1	0	0	29	100.0%	100.0%
Total	379	166	3	1	209	98.2%	99.5%

a) No. kit and culture positive samples.

b) No. kit negative and culture positive samples.

c) No. kit positive and culture negative samples.

d) No. kit and culture negative samples.

e) No. kit+•culture+ / (No. kit+•culture+ + No. kit– • culture+) × 100.

f) No. kit– • culture– / (No. kit+•culture– + No. kit– • culture–) × 100.

cultures were detected 6.0×10^{-1} or more and 1.2×10^0 or more cfu of *S. Typhimurium* L-417 in 25 g raw broiler meat and liquid egg, respectively. When samples were once cultured for selective enrichment, the both assays were detected 1.2×10^1 or more and 6.0×10^2 or more cfu of *S. Typhimurium* L-417 in 25 g swine feces and raw broiler meat (data not shown), respectively. The detection results of the DNA probe kits and the standard culture methods were equal at all. It was indicated that the DNA probe kits could be detected several number of *Salmonella* spp. in 25 g samples by pre-enrichment and selective enrichment cultured.

In many reports, the commercial rapid detection kits for *Salmonella* spp. were compared to standard culture methods by naturally contaminated samples [5, 10, 13, 21]. In this study, a total of 379 naturally contaminated samples were tested. Of these, 169 were culture positive and 210 were culture negative. The sensitivity of the DNA probe kit was 98.2% (166 / 169) and the specificity was 99.5% (209 / 210). There were no statistical significant difference by McNemar's test between the results by the DNA probe kits and culture methods. This comparative study shows that the DNA probe kit is equally as sensitive as standard culture method. And this kit can reduce the period of detecting *Salmonella* spp. from foods by up to 3 days.

Recently, a commercially colorimetric DNA probe kit derived from the 23S rRNA gene has been developed by Gene-Trak for the specific detection of *Salmonella* spp. in food samples [4, 21]. This kit used the dipstick format which is one of the solid-phase sandwich hybridization format [22]. In this format, sandwich hybridization of salmonella rRNA and DNA probes, combination of the DNA probes and antibodies conjugated with an enzyme and detection of enzyme bound to salmonella rRNA were carried out in glass tubes. But by the DNA probe kit described here, hybridization, combination and detection were carried out in a microtiter well. In detecting target from large numbers of samples, the sandwich hybridization in microtiter wells is more appropriate for handling and has

a number of advantages over other hybridization formats [12].

These results show that this DNA probe kit is a useful tool to examine a large number of various samples for contamination by *Salmonella* spp. in food and livestock industry.

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