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Direct Detection of *Salmonella* spp. in Estuaries by Using a DNA Probe

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A method for direct detection of *Salmonella* spp. in water was developed by using a commercially available DNA probe. Particulate DNA was extracted from 500- to 1,500-ml water samples collected from New York Harbor and Chesapeake Bay and used as a substrate for a salmonella-specific DNA probe in dot blot assays. The method detected salmonellae in water samples from 12 of 16 sites, including 6 sites where salmonellae could not be cultured. The specificity of the probe was evaluated, and cross-hybridization, although negligible, was used to set detection limits for the assay. *Salmonella* DNA bound the probe quantitatively, and from these results *Salmonella* DNA in the total particulate DNA in environmental samples could be estimated. The data obtained in this study indicate that *Salmonella* spp. often are not detected in water samples by culture methods, even when they are present in significant numbers.

With increased demands on water resources, problems of contamination of surface water and groundwater by enteric microorganisms have become an increasing concern. Although enumeration of coliforms and fecal coliforms is used to assess the quality of water used for drinking, shellfish harvesting, and recreation, the validity of methods for estimating the presence of human pathogens has been questioned (14, 20). Direct detection of waterborne pathogens, rather than indicator organisms, has not been adopted as a standard method in the United States (2) because, in general, enumerative schemes for enteric pathogens involve time-consuming, expensive methods which often yield equivocal results.

Salmonella is a prime example of a water- and shellfish-transmitted pathogen which is difficult to culture from environmental samples, although many different culture media and enrichment regimes have been proposed (1, 2, 15, 16, 23, 24, 35). One of the factors contributing to this difficulty is the ability of *Salmonella* spp. to enter a viable but nonculturable state after lengthy exposure to river water and seawater, under ambient conditions of temperature and low nutrient concentration (28, 29). Other investigators have described sublethal injury subsequent to exposure to the aquatic environment, when the organism remains culturable but the efficiency of recovery in culture is decreased (3, 6). Culture methods for detection and enumeration of *Salmonella* spp. in aquatic systems are not only unreliable but also tedious, requiring incubation in many enrichment media for several days before presumptive salmonellae can be isolated (7, 16, 23, 24, 29, 35).

Development of immunodetection systems and DNA probes has facilitated the detection of *Salmonella* spp. in contaminated samples. However, the bulk of the work has been carried out with food samples (10) and involves culture of samples prior to testing with antibody or a DNA probe. Immunofluorescence methods (34) and enzyme immunosorbent assays (4, 19) have been successfully used to detect salmonellae in primary enrichment broths. The fluorescent-antibody technique is recommended as a standard method for direct detection in water and wastewater (2).

DNA probes specific for *Salmonella* spp. have been developed by several investigators (9, 11, 30, 36). Each of these probes is composed of one or more chromosomal DNA fragments targeted to unique regions of *Salmonella* chromosomal DNA. The probe developed by Fitts et al. (9), a mixture of several cryptic fragments of *Salmonella typhimurium* DNA, is commercially available (Gene-Trak Inc., Framingham, Mass.) in a kit for detecting *Salmonella* spp. in food. Another approach to development of a probe-based assay for salmonellae is to use rRNA as a target for synthetic oligodeoxynucleotide probes. Gene-Trak has recently developed such a probe for use in a colorimetric assay for detection of *Salmonella* spp. in food.

Advantages of DNA probe assays and immunoassays include their high specificity and rapid assay time, reducing the total time for positive identification from several days to 1.5 days (10). Direct application of the available DNA probe methods to analysis of environmental water samples provides only mixed success because the numbers of *Salmonella* spp. in most waters are generally below the detection limits of the assays, necessitating enrichment of the sample by culture. Since the efficiency of culturing *Salmonella* spp. from aquatic samples is low, detection by methods which rely upon culture will result in underestimations of the number of salmonellae present.

Previously, we reported a simple, rapid method for concentrating bacteria in water samples and extracting their nucleic acids (33). The nucleic acid extracts were of sufficient quantity and purity for molecular cloning and hybridization with DNA and RNA probes. We have applied the DNA extraction method to obtain target DNA directly from filter-concentrated water for hybridization with a commercially available, radiolabeled DNA probe for *Salmonella* spp. This permits detection of *Salmonella* spp. in water and treated wastewater, with no culture procedures required.

MATERIALS AND METHODS

Sampling sites. Water samples were collected on 14 and 15 September 1988 at 14 sites in New York Harbor, the Hudson and East Rivers, and offshore in the Hudson River plume.

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TABLE 1. Locations of sampling sites and sample characteristics

Site designation	Latitude (N)	Longitude (W)	AODC[a] (cells/ml, 10^6)	Vol filtered[b] (ml)	Salinity (‰)	Water temp (°C)
NYA	40° 34′ 07″	73° 53′ 34″	5.81	1,000	30.8	17.8
NYB	40° 32′ 07″	74° 06′ 07″	9.53	600	26.4	19.8
NYC	40° 38′ 27″	74° 02′ 29″	2.61	1,000	23.2	20.7
NYD	40° 38′ 32″	74° 07′ 40″	5.77	1,000	22.0	20.9
NYE	40° 42′ 28″	73° 58′ 49″	5.24	950	24.4	20.8
NYF	40° 47′ 11″	73° 55′ 23″	6.68	920	22.6	23.1
NYG	40° 47′ 54″	73° 50′ 37″	5.00	940	23.4	22.1
NYH	40° 47′ 01″	73° 53′ 31″	7.72	1,000	23.8	22.1
NYI	40° 48′ 05″	73° 53′ 11″	5.77	800	23.6	22.0
NYJ	40° 43′ 47″	73° 58′ 05″	4.06	800	23.6	21.9
NYK	40° 56′ 18″	73° 54′ 26″	4.73	800	8.6	21.9
NYL	41° 13′ 13″	73° 57′ 44″	5.06	800	3.4	24.7
NYM	40° 41′ 05″	74° 02′ 39″	4.03	1,000	21.8	20.9
NYN	40° 23′ 22″	73° 51′ 51″	3.32	1,600	31.0	19.7
CPP	39° 02′ 30″	76° 12′ 10″	ND[c]	1,000	12.5	10.9
CMP	39° 08′ 14″	76° 04′ 15″	ND	500	10.6	11.2

[a] AODC, Acridine orange direct count.
[b] Volume of sample pumped through each filter.
[c] ND, Not done.

Twelve sites were located near treated sewage outfalls (designated NYA through NYL), one sampling site was located at a dredge spoils dump site (NYN), and one, chosen at random, was not near any known source of sewage contamination (NYM). Water samples also were collected on 14 November 1988 at two sites in the Chester River in the upper Chesapeake Bay (CPP and CMP). Table 1 lists the coordinates of each site and physical data collected at the time of sampling.

Water samples were collected, in sterile 8-liter polypropylene bottles (Nalge Co., Rochester, N.Y.) released from the research vessel R/V *Ridgely Warfield*, at a depth of 1 m below the surface. All samples were immediately processed on board the research vessel. At each site, water temperature and salinity were measured and three 5-ml portions of each water sample were Formalin fixed (final concentration, 0.1%) for determinations of total cell counts (13).

Culture of *Salmonella* spp. from water samples. Samples were preenriched with buffered peptone water (BPW) (7) so that injured salmonellae might recover before inoculation into selective broth. A 100-ml portion of each water sample was inoculated into 20 ml of 6× BPW and incubated for 4 h at 25°C (16). Portions (10 ml) of the preenriched broth were inoculated into 90 ml of each of three selective enrichment broths: Rappaport broth, with the modifications of Vassiliadis et al. (37) (RV broth); RV broth supplemented with 10 μg of novobiocin per ml (23) (RVN broth); and dulcitol-selenite broth (proteose peptone, 4.0 g; dulcitol, 4.0 g; sodium selenite, 5.0 g; yeast extract, 1.5 g; Na_2HPO_4, 1.25 g/liter) (DS broth). Selective-enrichment broths were incubated at 43°C for 24 h.

Each enrichment culture was subsequently streaked onto three agar media (21, 22): xylose lysine decarboxylase agar (Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of novobiocin per ml (XN agar); tryptic soy brilliant green agar (TSBG agar); and TSBG agar containing sucrose but no lactose (TSBG-S agar). Plates were incubated at 43°C, and salmonella-like colonies were picked at 24 and 48 h and inoculated into triple sugar iron agar slants. The slants were incubated at 43°C for 24 h, and isolates which fermented only glucose were inoculated onto API 20E test strips (Analytab Products, Plainview, N.Y.) for identification to the genus level.

Culture of *Salmonella* spp. from concentrated water samples. Microorganisms in water samples were concentrated in Sterivex filter units (Millipore Corp., Bedford, Mass.) as previously described (33). Water was filtered through each Sterivex unit until the 0.22-μm-pore-size membrane became occluded. The volume of water filtered varied among sites, from 500 ml to 1.5 liters per filter, depending upon the turbidity of the water (Table 1). Three filters were prepared at each site as follows. After the desired volume of water was filtered, water remaining inside the filter housing was forced out with a 50-ml syringe, and 2 ml of BPW was added into the housing with a 20-ml syringe equipped with a 25-gauge, 5/8-in. [1.6-cm] needle. The entry and exit ports of the filter unit were capped, and the filter unit was incubated at 25°C for 4 h. After preenrichment, 1 ml of the BPW was drawn off each filter and replaced with 1 ml of either 2× RV broth, 2× RVN broth, or 2× DS broth. After incubation at 43°C for 24 h, a loopful of each enrichment broth was streaked onto XN agar, TSBG agar, and TSBG-S agar and *Salmonella* spp. were isolated as described above. Also, the cells from each enrichment broth were harvested for extraction of nucleic acids as described below.

Nucleic acid extraction from enrichment broths. Total nucleic acids were extracted from 1.8 ml volumes of enrichment broths incubated in Sterivex units and purified by methods similar to those previously described (33). Cells were harvested by centrifugation at 5,000 rpm for 5 min in an Eppendorf microcentrifuge. The broth was decanted, and the pellet was suspended in 1 ml SET buffer (20% [wt/vol] sucrose, 50 mM EDTA, 50 mM Tris hydrochloride [pH 7.6]). Cells were again pelleted by centrifugation, and the supernatant was decanted. The cell pellet was suspended in the residual SET buffer in the microcentrifuge tube and held at −20°C for 30 min. Cell suspensions could be held at −20°C for up to 2 weeks before further processing. They were thawed at 37°C, and 1 ml of SET buffer and 30 μl of lysozyme solution (5 mg/ml in TEN buffer [10 mM Tris hydrochloride, 1 mM EDTA, 10 mM NaCl]) were added. The solution was incubated on ice for 15 min. Cell lysates were deproteinized by sodium dodecyl sulfate and proteinase K treatment and then precipitated with ammonium acetate as described elsewhere (33). Nucleic acids were precipitated with ethanol, and the pellets were washed three

times with 70% ethanol and once each with 95% and 100% ethanol before being dried in a vacuum desiccator (18). The dried pellets were suspended in 300 μl of TE buffer (10 mM Tris hydrochloride [pH 7.6], 1 mM EDTA) and further purified by a second ammonium acetate precipitation followed by ethanol precipitation and ethanol washings as described above. The final nucleic acid pellets were suspended in 200 μl of TE buffer and stored at −20°C until utilized for gel electrophoresis and dot blot analysis.

Direct extraction of nucleic acids from filtered samples. At each site two Sterivex units were prepared for direct extraction of cellular nucleic acids as previously described (33). After the desired volume of water was filtered, water remaining in the filter unit was forced off and 10 ml of SET buffer was forced through the filter. The filter units were stored at −20°C until the nucleic acids were extracted and purified; details of these procedures have been described previously (33).

Agarose gel electrophoresis of nucleic acid extracts. Extracts were analyzed by loading 10 μl onto a 1.5% agarose gel and electrophoresing at 75 to 100 V in TAE buffer (18) for 1.5 to 2.5 h. The gels were stained with ethidium bromide as described elsewhere (18) to confirm visually that sufficient DNA was extracted before the preparations were applied to the dot blot.

Dot blot and colony blot hybridizations with the *Salmonella* probe. After electrophoresis the remaining nucleic acid extract was denatured and bound to nylon membranes (Gene-Screen; Du Pont, NEN Research Products, Boston, Mass.) by using a dot blot apparatus (Minifold; Schleicher & Schuell, Inc., Keene, N.H.) as recommended by the manufacturer. Serial dilutions of *S. typhimurium* DNA were applied to the membrane in duplicate as homologous controls, and samples of *Escherichia coli* and *Citrobacter freundii* were applied in duplicate as heterologous controls.

Colony blots were prepared by inoculating broth cultures of isolates onto nylon membranes (Colony/Plaque Screen; DuPont, NEN), which had been placed on the surface of LB agar (Difco) plates. The plates were incubated at 37°C until the colonies became visible (6 to 12 h), after which the membranes were removed and colony lysis was performed by the procedure of Grunstein and Hogness (12) with the modifications of Maas (17).

Colony blots were soaked in a prewash solution (1 M NaCl, 0.1% sodium dodecyl sulfate, 10 mM EDTA) at 45°C with gentle shaking for 1 h to remove residual colony debris. Both colony blot membranes and dot blot membranes were prehybridized at 65°C for 3 to 4 h in heat-sealed bags (Seal-a-Meal; Sears Roebuck Co.) with 2 ml of hybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, 0.5% nonfat dry milk) per 10-cm^2 membrane. The solution was removed and replaced with 0.75 ml of fresh hybridization solution per 10-cm^2 membrane. The *Salmonella* probe is produced by Gene-Trak by using a primer extension method to radiolabel several cloned salmonella-specific chromosomal DNA fragments (25). The ^{32}P-labeled probe was added at 0.5 μCi/ml of hybridization solution, and the bag was sealed. Hybridization was carried out for 16 to 20 h at 65°C with gentle shaking. Membranes were washed four or five times in a solution of 2× SSC–0.1% sodium dodecyl sulfate at 65°C for 5 min and air dried. Results were visualized by autoradiography, and the dots were excised from the membrane and quantitated by liquid scintillation counting.

Specificity and sensitivity testing. Colony blots of American Type Culture Collection strains and strains isolated from estuarine waters were prepared for hybridization with the Gene-Trak probe. The strains tested are listed in Table 2. Hybridizations and washes were performed as described above.

TABLE 2. Strains used to determine the specificity of the Gene-Trak *Salmonella* probe

Species	Source	Hybridization with probe
Proteus vulgaris	ATCC 13315	−
Proteus mirabilis	Water	−
Proteus mirabilis	Water	−
Providencia stuartii	Water	−
Serratia marcescens	ATCC 13880	−
Shigella flexneri	ATCC 12022	−
Vibrio cholerae	ATCC 14035	−
Plesiomonas shigelloides	ATCC 14029	−
Enterobacter sakazakii	Water	−
Enterobacter aerogenes	ATCC 13048	−
Enterobacter cloacae	Water	−
Klebsiella pneumoniae	Water	−
Klebsiella oxytoca	Water	−
Klebsiella pneumoniae	ATCC 13883	−
Citrobacter freundii	ATCC 8090	−
Citrobacter freundii	Water	−
Escherichia coli	ATCC 25922	−
Escherichia coli	ATCC 11303	−
Escherichia coli	Lab strain HB101	−
Bacillus subtilis	Lab strain	−
Staphylococcus aureus	Lab strain	−
Staphylococcus epidermidis	Lab strain	−
Streptococcus faecium	Lab strain	−
Streptococcus durans	Lab strain	−
Vibrio cholerae (non-O1)	Clinical	−
Vibrio natriegens	ATCC 14048	−
Vibrio nereis	ATCC 25917	−
Vibrio alginolyticus	ATCC 17749	−
Vibrio diazotrophicus	ATCC 33466	−
Vibrio parahaemolyticus	ATCC 17802	−
Vibrio fluvialis	ATCC 33812	−
Vibrio vulnificus	ATCC 27562	−
Psuedomonas aeruginosa	ATCC 10145	−
Photobacterium angustum	ATCC 25915	−
Aeromonas salmonicida	ATCC 14174	−
Aeromonas caviae	ATCC 15467	−
Aeromonas hydrophila	ATCC 7966	−
Photobacterium leiognathi	ATCC 25521	−
Salmonella paratyphi A	ATCC 9150	+
Salmonella paratyphi A	Water	+
Salmonella salamae	ATCC 6959	+
Salmonella typhimurium	ATCC 14028	+
Salmonella gallinarum	ATCC 9184	+
Salmonella arizonae	ATCC 13314	+
Salmonella typhi	ATCC 6539	+
Salmonella enteritidis	ATCC 13076	+
Salmonella choleraesuis	ATCC 13312	+
Five *Salmonella* strains	Water	+

DNA from four *Salmonella* species (*S. paratyphi* A ATCC 9150, *S. arizonae* ATCC 13314, *S. typhimurium* ATCC 14028, and *S. salamae* ATCC 6959) was isolated and used to determine the amount of probe bound per nanogram of DNA over a range of DNA concentrations in a dot blot assay. DNA was isolated from pure cultures of the *Salmonella* strains by using the Sterivex units, details of which have been described previously (33). Twofold serial dilutions of each DNA preparation were denatured and applied to a nylon membrane, and a dot blot hybridization assay with the *Salmonella* probe was performed as described above. The dots were excised from the membrane, and counts per

TABLE 3. Isolation of *Salmonella* spp. from water samples collected from New York Harbor and Chester River

Sample site	Culture in Sterivex units[a,b]	Culture in bottles[a,c]	Hybridization of presumptive isolates with probe
NYA	–	–	ND[d]
NYB	–	–	–
NYC	–	–	–
NYD	+	–	+
NYE	–	–	–
NYF	–	+	+
NYG	+	–	+
NYH	+	–	+
NYI	+	–	+
NYJ	+	–	+
NYK	–	–	–
NYL	–	–	ND
NYM	–	–	ND
NYN	–	–	ND
CPP	–	–	ND
CMP	–	–	ND

[a] Positive if any of the broth and agar medium combinations yielded *Salmonella* spp.

[b] The volume of water which was concentrated onto the Sterivex units varied between sample sites from 550 to 1,500 ml (Table 1).

[c] Sample volumes were 100 ml for each enrichment culture.

[d] ND, Not done, because no presumptive salmonellae were isolated from the site.

minute of probe bound per dot were determined by liquid scintillation counting. The correlation between counts per minute of probe bound and nanograms of DNA was calculated for each preparation, and plots of the nanograms of DNA applied to each dot versus the counts per minute of probe bound were used to determine the mean counts per minute of probe bound per nanogram of DNA for each *Salmonella* species.

To test whether the concentration of heterologous DNA affected the amount of probe bound in the hybridization assay, DNA from *E. coli* ATCC 11303 and *C. freundii* ATCC 8090 was extracted and purified by a procedure for large-scale chromosomal DNA isolation (27). Heterologous DNA was subjected to twofold serial dilutions from 100 μg to 5 ng, denatured, and applied to a nylon membrane as described above. Serial dilutions of *S. typhimurium* DNA from 10 to 0.3 ng were also applied to the membrane. Hybridization with the Gene-Trak probe and washes were performed as described above. Results were visualized by autoradiography.

Seeding experiments were conducted with Chesapeake Bay water collected at two unpolluted, clean-water sites in April 1989. *S. typhimurium* ATCC 14028 was grown in LB broth to mid-log phase (6.6×10^7 CFU/ml), and water was seeded to a concentration of 2.6×10^4 CFU/ml. Four 500-ml samples of seeded water and four unseeded water samples were filtered through Sterivex units, and the nucleic acids were extracted from cells on the filters as described previously (33). Extracts were applied to nylon membranes by the dot blot technique, hybridized, and washed as described above.

RESULTS

Culture of *Salmonella* spp. *Salmonella* spp. were cultured from Sterivex-concentrated samples at five sites and from unconcentrated samples at only one site (Table 3). This was probably due to the ability to culture much larger samples by using the Sterivex units. Sampling large volumes of water with the Sterivex units and culturing the microflora inside the units proved to be an efficient, facile alternative to culture of unconcentrated samples and to conventional sample concentration onto flat filters (16). There was no single broth and agar medium combination which was superior for isolating *Salmonella* spp. Of the 18 isolates recovered, 10 were isolated from RVN broth, 6 were isolated from RV broth, and only 2 were isolated from DS broth. TSBG and TSBG-S agars proved equally effective plating media, but only 2 of the 18 isolates were recovered on XN agar. The DNA probe hybridized with colony blots of all 18 of the isolates but did not hybridize with any presumptive *Salmonella* colonies which were later identified as nonsalmonellae by the API 20E tests (Table 3).

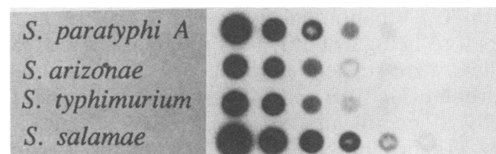

FIG. 1. Dot blot hybridization of DNA prepared from four *Salmonella* species by using a *Salmonella*-specific DNA probe. Each row contains twofold serial dilutions of DNA, with the highest concentration for each of the species shown as follows: *S. paratyphi* A, 68.5 ng; *S. arizonae*, 16.1 ng; *S. typhimurium*, 16.1 ng; *S. salamae*, 64.1 ng.

Probe specificity and sensitivity. The Gene-Trak probe proved to be highly specific for the genus *Salmonella* under the hybridization and wash conditions used. Table 2 lists the results of colony blot hybridizations of the probe with a battery of organisms considered likely to be found in estuarine water and wastewater. The probe did not cross-hybridize with any of the strains tested but did hybridize with all of the *Salmonella* species tested.

Dot blots of serially diluted nucleic acids, prepared from four *Salmonella* species, bound probe DNA quantitatively (Fig. 1). In Fig. 2 is shown counts per minute of probe bound versus the amount of DNA (nanograms) applied to the membrane for each species tested. The correlation coefficients for each species and the slope of the regression line for each plot were as follows: *S. paratyphi* A, 0.964 and 10.3

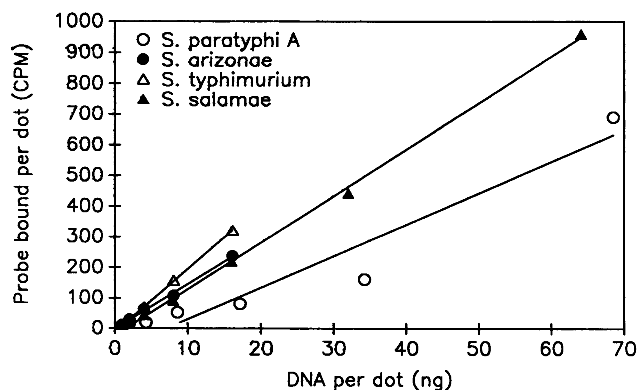

FIG. 2. Counts per minute of probe bound per dot versus amount of DNA per dot for DNA prepared from four *Salmonella* species. Data were generated from the dot blot in Fig. 1.

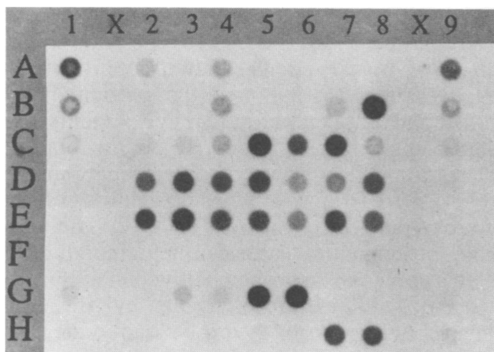

FIG. 3. Dot blot hybridization of nucleic acid extracts with a salmonella-specific DNA probe. See the text for an explanation.

cpm/ng; *S. arizonae*, 0.998 and 14.6 cpm/ng; *S. typhimurium*, 1.00 and 20.7 cpm/ng; and *S. salamae*, 0.999 and 15.2 cpm/ng. The mean ± standard deviation of counts per minute of probe bound per nanogram of DNA was 15.2 ± 4.27.

The heterologous control DNAs (*E. coli* ATCC 11303 and *C. freundii* ATCC 8090) on some dot blots bound probe DNA sufficiently to yield counts per minute above background levels, with perceptible darkening of the autoradiographs. The mean counts per minute bound to the heterologous control dots and their standard deviations were used to generate 99 and 99.9% confidence intervals for each blot, the upper limits of which were used to determine the detection limits for the assay (32). If the amount of probe bound (counts per minute) by the DNA sample was greater than the upper limit of the 99.9% confidence interval of the amount bound by the heterologous control DNA, the sample was scored positive. If the amount of probe bound by the sample was less than the upper limit of the 99.9% interval but greater than the upper limit of the 99% interval, the sample was scored plus/minus. All DNA samples which bound probe at levels below the upper limit of the 99% confidence interval of the heterologous controls were scored negative.

The detection limits (i.e., the nanograms of *Salmonella* DNA required for a positive score) for each dot blot were calculated by dividing the counts per minute value which defined the upper boundaries of the 99.9% confidence limit of the heterologous control DNA by the mean counts per minute of probe bound per nanogram of the *S. typhimurium* control DNA. The detection limits of the dot blots ranged from 7 to 15 ng, meaning that this was the minimum amount of *Salmonella* chromosomal DNA required for detection by the probe.

When heterologous DNA was applied to membranes in amounts approaching the DNA-binding capacity of the membrane (100 μg), the amount of probe bound in the hybridization assay did not increase (data not shown). The heterologous control background signal was therefore considered independent of the amount of DNA applied to the membrane.

Detection in water samples by using the probe. Figure 3 is an autoradiograph of one of the dot blots of nucleic acid extracts from concentrated water samples after hybridization and washing. Columns 1 and 9 (positions A to E) contain twofold serial dilutions of *S. typhimurium* DNA from 16 to 1 ng. Columns 1 and 9 (positions G and H) contain 1.1 μg of *C. freundii* DNA and 1.6 μg of *E. coli* DNA, respectively. Columns 2 to 8 contain extracts from samples collected in New York Harbor and the Chester River. These include extracts from culture-enriched Sterivex filters as well as filters which were extracted directly, without culture, prior to extraction. Columns marked X contain no DNA. In general, it was not difficult to distinguish positive hybridization signals from background by visual inspection of the autoradiograph. In some cases, however, the film was exposed slightly and it was not possible to determine whether the signal was due to nonspecific background or to low levels of target DNA in the sample (e.g., Fig. 3, position 7B).

TABLE 4. Results of dot blot analyses of nucleic acid extracted from water samples collected from New York Harbor and Chester River

Sample site	Hybridization of probe with:		Estimated *Salmonella* DNA/ml of sample filtered (ng)
	Broth extract[a,b]	Direct extract[b,c]	
NYA	±	+	0.0088
NYB	−	+	0.0350
NYC	+	+	0.0045
NYD	+	+	0.0126
NYE	+	+	0.0111
NYF	+	+	0.0026
NYG	+	+	0.0031
NYH	+	+	0.0081
NYI	+	+	0.0118
NYJ	+	±	ND[d]
NYK	±	±	ND
NYL	−	±	ND
NYM	−	±	ND
NYN	−	+	0.0116
CPP	−	+	0.0214
CMP	−	+	0.0128

[a] Total nucleic acids extracted from cell suspensions cultured in Sterivex units.

[b] Symbols: +, counts per minute of probe bound were above the 99.9% detection limit; ±, counts per minute were between the 99 and 99.9% detection limits; −, counts per minute were below the 99% detection limit. See the text for an explanation of detection limits.

[c] Total nucleic acids extracted directly from cells concentrated in Sterivex units.

[d] ND, Not done, since the amount of probe bound was below the 99.9% detection limit.

Quantitative analysis of the dots by using a liquid scintillation counter permitted us to use detection limits, calculated for each dot blot as described above, to determine whether signals from such samples were significantly above background. Table 4 summarizes the results of the dot blot assays. Results of hybridization of the probe with nucleic acids which were extracted directly after concentration and with nucleic acids extracted from selective enrichment broths are both shown. The mean counts per minute of probe bound to the direct extracts from each site were divided by the mean counts per minute above background bound per nanogram of *S. typhimurium* DNA to estimate the amount of *Salmonella* DNA recovered from the site. These results, adjusted for the volume of sample filtered at each site, are also reported in Table 4.

Figure 4 shows results of a dot blot assay of nucleic acid samples prepared from unpolluted Chesapeake Bay water samples. Column 1 (positions A and B) shows controls containing *S. typhimurium* and *E. coli* DNA, respectively. Columns 2 and 3 contain extracts from water collected at two sites in the Chesapeake Bay. Columns 2 and 3 (positions B and D) contain extracts from 500 ml of water to which *S. typhimurium* was added to a final concentration of 2.6×10^4 CFU/ml. These extracts hybridized strongly with the probe

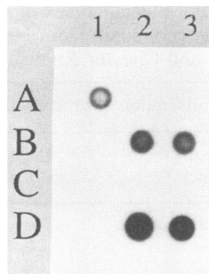

FIG. 4. Dot blot hybridization of nucleic acid extracts (obtained from unpolluted water) with a salmonella-specific DNA probe. See the text for an explanation.

as expected. Columns 2 and 3 (positions A and C) contain extracts from 500 ml of unpolluted water to which no additions were made. The extracts of water samples from these two sites did not hybridize with the probe, indicating that *Salmonella* spp. were absent or were present below detectable levels in the water.

DISCUSSION

The Gene-Trak probe was selected for direct detection of *Salmonella* spp. in water because it has proven useful in detecting *Salmonella* spp. in food (8, 10, 31) with acceptable specificity (9). The specificity of the probe was confirmed in our laboratory by using both the colony blot and the dot blot formats for hybridization.

A major drawback in adapting the Gene-Trak assay system to analysis of water samples, however, is the requirement of the assay for culture of salmonellae from the sample. Samples are enriched in broth culture before cell lysis and hybridization with the probe (Gene-Trak Systems Inc., technical literature). Attempts to use the Gene-Trak assay to detect salmonellae in laboratory microcosms seeded with *Salmonella* cultures and environmental samples resulted in an unacceptable number of false-negatives (data not shown). This observation is consistent with the observations of other investigators, namely, that salmonellae are difficult to culture from aquatic samples (7, 16, 23, 24, 29, 35) and a negative result from culture methods should be regarded as provisional (2).

We have previously reported details of a method for the concentration of microorganisms from aquatic samples and extraction of their nucleic acids (33), providing a convenient means of obtaining target DNA for hybridization studies. When this method was used to obtain DNA for hybridization with the salmonella-specific probe, DNA extracts from samples collected at 12 of the 16 sites included in this study bound the probe at counts per minute levels above the 99.9% detection limit (Table 4). Conversely, *Salmonella* spp. were cultured at only five of these sites (Table 3). Although it is not surprising that *Salmonella* spp. at some sites were detected by the probe but not by culture, it is somewhat surprising that at four sites sampled in this study (NYB, NYN, CPP, and CMP), direct extracts of cells concentrated in Sterivex units were probe positive but DNA extracted from cell suspensions in Sterivex units which had been enriched by the addition of culture media were negative (Table 4).

A possible explanation for this observation is that the salmonellae present in the sample were in the nonculturable state and were unable to replicate when incubated at elevated temperatures and in the presence of selective reagents and competing microflora. Poor recovery of enteric bacteria from aquatic samples under selective conditions has been observed by others (6, 26), and the particular effects of high-salinity and low-temperature environments upon culture of salmonellae in selective-enrichment media have been reported (5, 16). All but two of the samples in the study reported here were from sites where conditions of salinity or temperature would be expected to make the culture of salmonellae problematic (Table 1), including all of the sites from which samples were probe positive but culture negative (Tables 3 and 4). These observations and the convincing results of specificity tests of the probe lead to the conclusion that at some sites, the gene probe detected *Salmonella* spp. in water samples from which the organism could not be cultured.

The ability to detect DNA from *Salmonella* spp. in the nucleic acid extracts of cells concentrated from water samples without first culturing the cells makes it possible to quantitate relative amounts of target in the water samples by measuring the amount of probe bound by the nucleic acid extracts. Since, for each *Salmonella* species tested, the counts per minute of probe bound were directly proportional to the nanograms of DNA (Fig. 1 and 2), a binding ratio (mean counts per minute of probe bound per nanogram of DNA) for each species can be calculated. The binding ratios were similar among the species tested (Fig. 2), and therefore the binding ratio of the positive control DNA on a dot blot could be used to estimate the nanograms of *Salmonella* DNA in the unknown extracts.

In this study *S. typhimurium* DNA was used as the positive-control DNA. Since the probe is constructed of cloned *S. typhimurium* DNA fragments, its DNA binds more probe per nanogram than all the other tested species do (Fig. 2). Because environmental samples should contain other *Salmonella* species, which have a lower affinity for the probe than *S. typhimurium* does, the amounts of *Salmonella* DNA in nucleic acids extracted from water samples (Table 4) are most probably underestimations. When the binding ratio of probe to a high-affinity substrate, i.e., the *S. typhimurium* DNA, is used to estimate amounts of lower-affinity substrates, i.e., DNA from a mixed *Salmonella* population, the results underestimate the actual amounts of lower-affinity substrates. Thus, the amounts of *Salmonella* DNA per milliliter of water sample filtered (Table 4) should be regarded as minimum estimates.

It is difficult to translate the amounts of *Salmonella* DNA detected in the nucleic acid extracts of water samples into the number of *Salmonella* cells per milliliter of water sample. One of the reasons is that estimates of DNA yield obtained by using the Sterivex method for recovering cellular nucleic acids from water samples vary over an order of magnitude (33). One can expect DNA yields of ca. 1 ng/10^5 cells from an overnight culture of *S. typhimurium*, but the yield from environmental samples will depend upon the physiological state of the salmonellae in the sample, e.g., whether the cells are actively dividing. The site with the lowest estimated level of *Salmonella* DNA per milliliter of sample filtered was NYF (Table 4). If a yield of 1 ng of DNA per 10^5 cells is used to estimate the number of cells per milliliter detected at this site, the result is 2.6×10^3 cells per ml. This number is reasonable considering the volume of water filtered at this station (920 ml) and the detection limits of the probe. We have found that DNA from ca. 10^6 cells of *S. typhimurium* is required for detection. Experiments are in progress to determine the DNA yield from viable but nonculturable salmo-

nellae, and these data should be helpful in improving the accuracy of cell number estimates in environmental samples.

The *Salmonella* DNA probe has proven useful in assessing the presence of this organism in water samples collected from the environment. The method described here could be completed within 2 days and was specific for the detection of salmonellae. The advantages of this approach are obvious. An extensive survey of the public health quality of waters of the Chesapeake Bay and environs has been undertaken, the results of which will be published.

The utility of this method for estimating the public health safety of wastewaters and of shellfish-harvesting waters is such that the uncertainties of the coliform tests for estimating the presence of waterborne pathogens, such as *Salmonella* spp., can be overcome by direct detection of these organisms in water samples with a minimum of delay. Obviously, more work will have to be done to determine the validity of this method for highly turbid waters and for sediment samples.

It is expected that the greatest utility of this method will be in determining the potability of groundwaters and drinking-water supplies. However, the application of the gene probe for detection of *Salmonella* spp. in estuarine and marine waters at ocean outfalls and, most importantly, at shellfish-harvesting sites will be of great benefit in overcoming the inadequacies of the coliform test.

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