

## Fluorescent-Antibody Method Useful for Detecting Viable but Nonculturable *Salmonella* spp. in Chlorinated Wastewater

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An indirect fluorescent-antibody (IFA) technique, which employed adsorbed Behring polyvalent I O antiserum, was used to detect *Salmonella* spp. in environmental water systems. The IFA method used in this study detected 95% of *Salmonella* serotypes encountered in human infections in France, with a sensitivity threshold of  $7.5 \times 10^3$  bacteria per ml of wastewater. Specificity was assessed by testing IFA against *Salmonella*-free seawater and a variety of bacteria other than *Salmonella* spp. When used to examine raw and chlorinated wastewater over a 2-month period, the IFA method was successful in detecting *Salmonella* spp. in all 12 of the samples examined, with total numbers determined to be  $4.5 \times 10^5$  to  $3.3 \times 10^7$  salmonellae per 100 ml. In comparison, for the same samples, enumeration by culture, using the most-probable-number technique, was effective in detecting *Salmonella* spp. in only four of eight raw-water samples and one of four chlorinated water samples tested. Three samples were further tested by using the direct viable count procedure combined with IFA and results showed that 5 to 31.5% of the *Salmonella* spp. enumerated by this method in chlorinated water were substrate responsive.

Statistically significant correlations between microbial water quality, measured by conventional culture techniques, and illness in bathers have been established (3, 22, 23). A viable but nonculturable stage of bacteria has also been documented (8, 20), showing persistence of human pathogens in the aquatic environment. As a consequence, there is a need to clarify the actual health significance of these bacteria under environmental conditions. The direct detection of pathogens rather than reliance on culture methods may more accurately assess water quality.

The acridine orange stain has been suggested as useful in monitoring water quality by estimating the total number of bacteria present in water samples (20). Fluorescent-antibody assay (1, 2) and enzyme immunoassay (5, 19), on the other hand, can be usefully applied to both direct and specific detection of bacteria in environmental samples.

Direct viable counts (DVC), described by Kogure et al. (13, 14), permit discrimination between growing (elongating) and presumably dead (not elongating) bacteria, as observed by epifluorescent microscopy. Acridine orange staining, on the other hand, yields only total cell counts. Combining DVC with the indirect fluorescent-antibody (IFA) procedure permits the enumeration of specific viable populations of bacteria (1).

The effect of chlorine on the virulence of waterborne pathogens has been an issue of great interest. Chlorine-injured, enteropathogenic *Escherichia coli* has been shown to exhibit reduced ability to colonize the small intestine and to initiate disease (25). The findings of LeChevallier et al. (15, 16) are consistent with earlier findings that the virulence of *Salmonella* spp., *Yersinia* spp., and, more markedly, *E. coli* is reduced after exposure to chlorine (25). On the other hand, the study of Camper and McFeters (4) suggests that chlorination is inadequate for improving water quality since, after chlorination, surviving *E. coli* is capable of regrowth

subsequent to exposure to chlorine, with a short lag phase occurring before regrowth.

Furthermore, Singh et al. (24) showed that after chlorination treatment similar to that applied to drinking water, enteropathogenic *E. coli* is able to resume growth and demonstrate virulence within the mammalian gut. In addition, the experiments of Grimes and Colwell (8) showing pathogenicity of *E. coli* exposed to seawater indicate that salinity and chlorination together do not eliminate pathogenicity of *E. coli*.

In the present study, an IFA staining technique was designed for *Salmonella* spp. and was combined with the most-probable-number (MPN) culture technique (18) for monitoring *Salmonella* spp. in an experimental sewage treatment plant after chlorination of raw effluent.

### MATERIALS AND METHODS

**Bacterial strains.** The clinical strains of bacteria used were *Citrobacter freundii*, *Serratia marcescens*, *Hafnia alvei*, *Yersinia enterocolitica* (O:3, O:5-27, O:9), *Yersinia pseudotuberculosis* (O:1, O:2, O:3, O:4, O:5), *Salmonella typhimurium*, *Salmonella bovis-morbificans*, *Salmonella blockley*, *Salmonella dublin*, *Salmonella enteritidis*, *Salmonella infantis*, *Salmonella panama*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Salmonella typhi*, *Salmonella virchow*, *Salmonella london*, *Citrobacter diversus* (2 strains), *Shigella* spp. (5 strains), and *E. coli* (12 strains).

The environmental strains isolated from seawater that were also used included *Pseudomonas aeruginosa*, *Pseudomonas* spp. (12 strains), *Aeromonas hydrophila*, *Vibrio alginolyticus*, *Proteus* spp. (5 strains), and *Enterobacter* spp. (2 strains).

**Disinfection procedure.** This study was carried out by using samples of urban raw wastewater, principally sewage effluent. The raw effluent was chlorinated by the addition of a sodium hypochlorite solution. Before outflow to the sea, chlorine was neutralized by the addition of metabisulfite.

**Sample collection.** Seawater and wastewater samples from

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a sewer were collected in flasks that had been washed with sulfuric acid (36 N), rinsed with distilled water that had been passed through a filter (0.22- $\mu$ m pore size), and sterilized by autoclaving at 121°C. Samples of chlorinated water and sewer water outflowing to the sea were collected in bottles prepared in the same way but containing 15 mg of sodium thiosulfate per liter to eliminate residual chlorine. Samples were collected on 8, 15, and 27 July 1987; 11, 19, and 26 August 1987; 11 and 18 July 1988; and 24 August 1988. Samples included both untreated and chlorinated wastewater samples and seawater samples collected along the beach at a distance from the point of outflow to the sea. A sample of sewer outflow water was also collected on 19 August 1987. All samples were stored at 4°C during transport to the laboratory and were subjected to bacteriological examination within 2 h of collection.

**Acridine orange staining.** Wastewater samples were filtered by using polycarbonate filters (11) of 47-mm diameter and 0.2- $\mu$ m pore size (Nuclepore Corp., Pleasanton, Calif.) prestained with Irgalan black (26) (CIBA-GEIGY, Rueil-Malmaison, France). The filters were covered with 2 ml of acridine orange and allowed to react for 5 min. A 10-ml volume of distilled water, sterilized by filtration with a 0.2- $\mu$ m-pore-size filter, was used to rinse the filters.

**Antisalmonella serum.** *Salmonella* test serum polyvalent I (Behringwerke AG, Marburg, Federal Republic of Germany), obtained from immunized rabbits, contained agglutinins against somatic antigen of *Salmonella* groups A to E4.

**Serum adsorption.** A primary pool was prepared by mixing the following strains: *C. freundii*, *E. coli*, *S. marcescens*, *H. alvei*, *P. aeruginosa*, *A. hydrophila*, *V. alginolyticus*, and *Pseudomonas paucimobilis*. The *Salmonella* test serum was mixed with the strains and allowed to react for 2 h at 37°C and then was left overnight at 4°C. The mixture was centrifuged at 1,300  $\times$  g for 10 min. The supernatant was allowed to react with a second pool of bacterial strains which included *Y. pseudotuberculosis* (five serotypes) and *Y. enterocolitica* (three serotypes). The supernatant that was recovered after centrifugation was sterilized by filtering through a 0.2- $\mu$ m-pore-size filter (Millex GS; Millipore Corp., Bedford, Mass.) and stored at 4°C until needed. The adsorbed serum was diluted to a final concentration of 1:4 with phosphate-buffered saline. The phosphate-buffered saline consisted of 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na<sub>2</sub>HPO<sub>4</sub>, and 0.2 g of KH<sub>2</sub>PO<sub>4</sub> in 1 liter of distilled water, adjusted to pH 7.4 with 1.0 N NaOH (26).

**Fluorescent antibody.** Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Pasteur Production, Marnes la Coquette, France), diluted to a final concentration of 1:20 with phosphate-buffered saline, was used to react with the antisalmonella test serum.

**IFA technique.** Each water sample was filtered through polycarbonate filters (47-mm diameter, 0.2- $\mu$ m pore size; Nuclepore) prestained with Irgalan black. A 20- $\mu$ l sample of adsorbed *Salmonella* test serum was spotted inside an 8-mm-diameter circle marked on each membrane filter, which had been placed in a moist chamber. Each filter was incubated at 37°C for 30 min and then rinsed with 30 ml of phosphate-buffered saline. A 20- $\mu$ l sample of fluoroisothiocyanate conjugate was deposited on the spot where the drop of serum had previously been placed. Each filter was then incubated and rinsed as described above and then placed on a clean slide to which was added a drop of immersion oil manufactured specifically for fluorescence microscopy.

**Microscopic examination.** Filters were observed at  $\times$ 1,000 magnification by using a Nikon microscope equipped with a

100-W mercury lamp, a blue excitation filter (460 to 485 nm), and a yellow-green fluorescence barrier filter (520 to 560 nm). The degree of fluorescence of the cells stained with IFA was estimated on a scale ranging from 0 to 4+ (4+ representing bright fluorescence and 0 representing absence of fluorescence).

**Enumeration procedure.** Salmonellae were counted on a minimum of 20 fields per slide. If no salmonellae were observed on 20 fields, a total of 50 fields were examined to establish that a result was negative. Each enumeration was performed twice.

**Determination of viable bacteria.** Untreated and chlorinated wastewater samples (100 ml) were enriched with 0.25 mg of yeast extract (Difco Laboratories, Detroit, Mich.) per ml, after which 12  $\mu$ g of nalidixic acid (Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y.) per ml was added to each assay, and the mixture was incubated at 37°C for 6 h. A portion of each sample was stained by the IFA method as described above. Only swollen or elongated green cells were counted as viable, i.e., substrate responsive.

**Residual chlorine.** Residual chlorine was measured by the *N,N*-diethyl-*p*-phenylenediamine colorimetric method after potassium iodide treatment.

**Culture conditions.** Salmonellae in seawater, raw wastewater, and chlorinated wastewater were enumerated by the MPN method as follows. For each type of water, five-tube MPN series, consisting of 10 ml of buffered peptone water inoculated with 10-, 1-, 0.1-, and 0.001-ml volumes of the water under study, was incubated at 37°C for 2 h. After incubation, the inoculated tubes were transferred to 10 ml of double-strength selective Rappaport broth and incubated at 37°C for 2 h (6, 10). After incubation, 10  $\mu$ l of Rappaport broth was streaked onto Hektoen enteric agar (A.E.S. Laboratories, Cobourg, France), and the plates were incubated at 37°C for 24 h. Salmonellalike colonies were confirmed biochemically and serologically.

Lactose-fermenting members of the family *Enterobacteriaceae* (LFE) were isolated by inoculation on Drigalski agar medium (A.E.S.), and the plates were incubated at 42°C for 24 h before they were read.

## RESULTS

**Serum specificity.** Adsorbed *Salmonella* test serum showed no fluorescing cells when tested against the two pools of bacteria, i.e., *C. freundii*, *E. coli*, *S. marcescens*, *H. alvei*, *P. aeruginosa*, *P. paucimobilis*, *A. hydrophila*, *V. alginolyticus*, and *Yersinia* spp.

No fluorescing cells were observed when the serum was tested against *Proteus* spp., *Citrobacter diversus*, *Enterobacter* spp., *Pseudomonas* spp., and *Shigella* spp., which had not been used to adsorb the serum.

The control strains, *S. typhimurium*, *S. bovis-morbificans*, *S. blockley*, *S. dublin*, *S. enteritidis*, *S. panama*, *S. paratyphi* A, *S. paratyphi* B, *S. typhi*, *S. virchow*, and *S. london*, exhibited strong fluorescence, i.e., 4+.

**Sensitivity of the IFA technique.** To determine the threshold of sensitivity of the IFA technique, the minimal number of observed bacteria per field (e.g., one bacterium per 20 counted fields equaled a minimal number of 0.05 per field) was multiplied by the number of fields on a 47-mm-diameter filter, i.e.,  $1.5 \times 10^5$ . The number obtained was then divided by the filtered volume, which varied from 1 to 100 ml. The result obtained in this manner yielded a threshold of sensitivity of  $7.5 \times 10^3$  cells per ml for 20 counted fields when 1 ml of water was filtered, without concentration of the test samples.

TABLE 1. Residual chlorine in wastewater samples

Sampling date	Chlorine (mg/liter)
8 July 1987	5
15 July 1987	8
27 July 1987	0.2
11 Aug 1987	2.5
19 Aug 1987	0
26 Aug 1987	3
11 July 1988	1
18 July 1988	8
24 Aug 1988	0

**Residual chlorine in wastewater.** Residual chlorine was measured at the same time water samples were collected for bacteriological analyses (Table 1).

**LFE plate count.** The number of LFE isolated ranged from  $1.5 \times 10^4$  to  $3.1 \times 10^7$  CFU/100 ml in raw wastewater and from  $1 \times 10^1$  to  $1.9 \times 10^5$  CFU/100 ml in chlorinated wastewater (Fig. 1).

**MPN count for salmonellae.** Culturable salmonellae were detected in each raw wastewater sample tested, with the numbers ranging from  $6.4 \times 10^3$  to  $9.4 \times 10^4$  salmonellae per 100 ml. *Salmonella* spp. were cultured only once, on 27 July 1987, in chlorinated wastewater at a concentration of  $7.5 \times 10^3/100$  ml. Other chlorinated wastewater samples were negative for *Salmonella* cultures, even when viable *Salmonella* cells were detected (on 11 July and 15 and 25 August 1988) by the IFA technique combined with DVC. On 19 August 1987, *Salmonella* spp. were detected in raw wastewater ( $9.3 \times 10^4/100$  ml), no culturable *Salmonella* spp. were found in chlorinated wastewater, and  $1.5 \times 10^2$  salmonellae per 100 ml were isolated from samples collected at the outflow to the sea. No culturable *Salmonella* spp. were detected in any seawater sample collected at a distance from the outflow to the sea.

**Salmonella spp. isolated.** *Salmonella* spp. isolated during the study were *S. typhimurium* (27 July and 26 August 1987), *S. panama* (11 August 1987), *S. paratyphi* B (19 and 26 August 1987), and *S. bovis-morbificans* (19 August 1987) from raw wastewater and *S. typhimurium* (27 July 1987) from chlorinated wastewater. *S. panama* was isolated on 19 August 1987 from an outflow water sample. These strains correspond to serotypes commonly isolated in human salmonellosis (17).

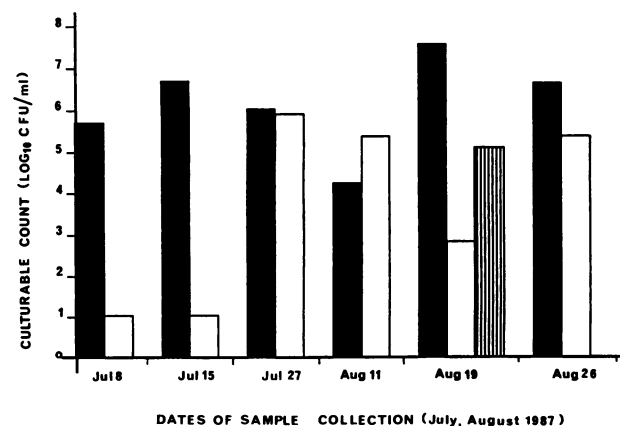


FIG. 1. Number of LFE isolated on Drigalski medium incubated at 42°C in raw water (■), chlorinated water (□), and water from outflow to the sea (▨).

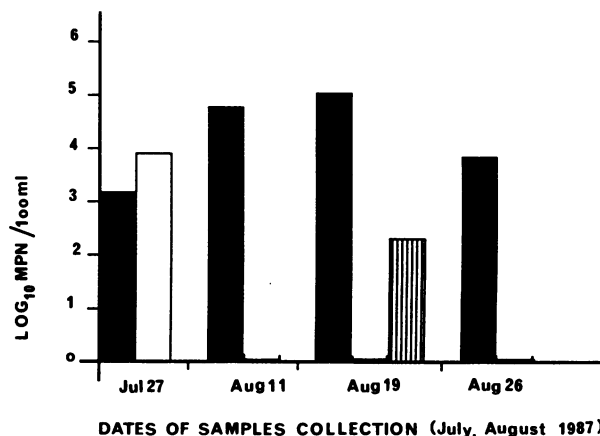


FIG. 2. MPN count of *Salmonella* spp. in samples of raw water (■), chlorinated water (□), and water from outflow to the sea (▨).

**Acridine orange count.** In seawater samples, the numbers of bacteria enumerated ranged from  $6.9 \times 10^7$  to  $4.6 \times 10^8/100$  ml. In raw wastewater, the numbers of bacteria ranged from  $4.0 \times 10^9$  to  $1.1 \times 10^{10}/100$  ml. In chlorinated wastewater,  $6.5 \times 10^8$  to  $9.4 \times 10^9$  bacteria per 100 ml were counted.

**IFA-TDC.** By using the IFA technique, *Salmonella* spp. were found in every sample of raw, chlorinated, or outflow wastewater (IFA total direct count [IFA-TDC]; Fig. 3). In raw and chlorinated wastewater samples, the numbers ranged from  $4.5 \times 10^5$  to  $3.3 \times 10^7$  and  $4.9 \times 10^5$  to  $7.1 \times 10^6$  salmonellae per 100 ml, respectively. In the outflow wastewater sample examined,  $2 \times 10^6$  salmonellae per 100 ml were enumerated. No *Salmonella* spp. were detected in any of the seawater samples collected at a distance from the outflow.

**IFA-DVC.** Viable *Salmonella* spp. were detectable in every wastewater sample examined (Fig. 4). In raw wastewater, the numbers ranged from  $7.2 \times 10^5$  to  $1.7 \times 10^6/100$  ml, i.e., from 19.4 to 62.9% of the corresponding IFA-TDC. In chlorinated wastewater, the number of viable, or substrate-responsive, bacteria ranged from  $7.5 \times 10^4$  to  $8.5 \times 10^5/100$  ml, i.e., 5 to 31.5% of the corresponding IFA-TDC enumeration. IFA-DVC was not successful in detecting *Salmonella* spp. in the seawater samples collected at a distance from the outfall.

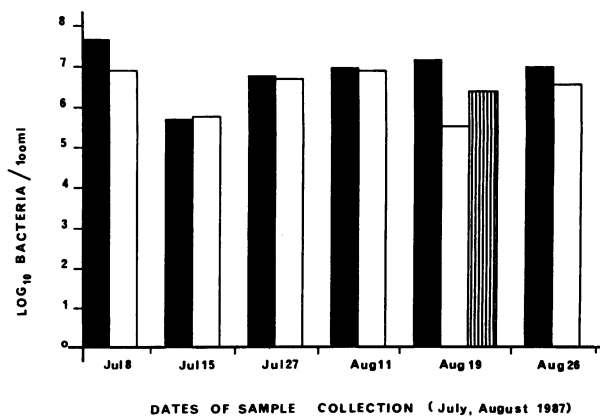


FIG. 3. IFA-TDC of *Salmonella* spp. in samples of raw water (■), chlorinated water (□), and water from outflow to the sea (▨).

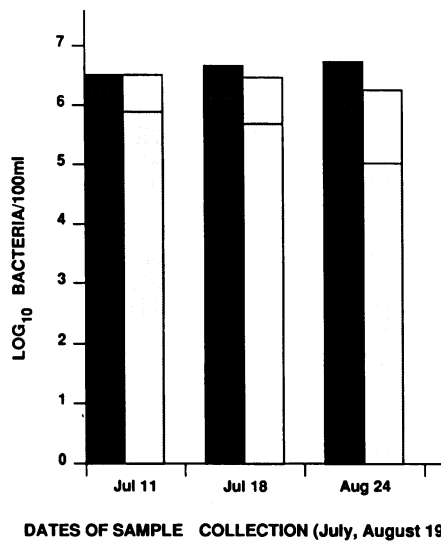


FIG. 4. IFA-DVC of samples with (■) and without (■) nalidixic acid treatment in raw water and with (▨) and without (□) nalidixic acid treatment in chlorinated water.

## DISCUSSION

From the results of this study, it can be concluded that O, H, and OH antisera can be used to detect *Salmonella* spp. in environmental water samples. The H antisera are preferred by some investigators (9) because no cross-reactions appear when H antisera are used. Cross-reactions occurring with O antisera can be eliminated by successive adsorptions to obtain a reaction-specific serum. In environmental samples, *Salmonella* spp. have adapted to the environmental conditions and, under such conditions, flagellar antigens are frequently undetectable (7). The OH antisera are of questionable value because they require the use of bacterial cells exhibiting well-developed flagella. For these reasons, the IFA technique used in this study was directed toward the O antigens. Cross-reactions occurring with the O antiserum were eliminated by successive adsorptions.

Cells binding the fluorescent antibody were observed to be intact or, at the least, to retain their O antigens. Such cells can be considered viable, i.e., substrate responsive (27). Bacteria in the environment, that is, bacteria not a part of the natural flora of the marine environment but exposed to seawater or other natural waters of aquatic ecosystems, may not demonstrate optimal flagellar development, i.e., that observed when cultured in the laboratory, or they may have lost part of an individual flagellum or all of their flagella. These bacteria are best tested with O group antiserum.

In the laboratory, *Salmonella* sera have proven useful for detection of *Salmonella* spp. by IFA. However, the specificity of a polyclonal serum is not extensive enough to detect all serotypes of *Salmonella* spp. In the present study, *Salmonella* species belonging to A, B, C<sub>1</sub>, C<sub>2</sub>, D, and E groups were detected by using adsorbed polyvalent I serum. These serogroups include most of the agglutinins (95%) encountered in human infections in France (17). Our IFA technique proved specific for *Salmonella* spp. when tested against a variety of bacteria, i.e., serological reactions were negative and no fluorescent bacteria were observed in seawater samples collected at a distance from the outfall, the point source of contamination. Only a relatively small volume of seawater was collected, however. If the seawater

samples had been concentrated by filtration of larger volumes of water, it is possible that fluorescent bacteria would have been detected by the IFA procedure. The threshold of sensitivity of IFA enumerations is relatively low, namely,  $7.5 \times 10^3$ /ml of sample. Dilution in seawater is concluded to have reduced the count below the threshold for direct examination.

The IFA method was found to be adequate for detecting *Salmonella* spp. even when the cells were rendered nonculturable by chlorine injury. The IFA and IFA-DVC techniques are rapid and are not as cumbersome as the MPN technique. Furthermore, samples can be collected, fixed, and stored for future analysis with the IFA and IFA-DVC techniques, unlike with the MPN procedure.

Culturable LFE enumerations were used as a reference in this study to establish the efficiency of the experimental treatment plant. Chlorination was concluded to be effective in reducing the number of culturable LFE to 0.01% in three of six samples collected (8 and 15 July and 19 August 1987). However, in the case of the three other samples collected, no reduction in count was observed. In addition, culturable *S. typhimurium* survived the chlorination process on 27 July 1987. Finally, when a water sample collected from the outflow to the sea was examined, both culturable LFE and *S. panama* were isolated in large numbers.

These data and the variability of residual chlorine in the effluent are consistent with a lack of control of influent overflows and of the chlorination process in the plant under study.

The results reported here agree with the hypothesis of Xu et al. (27) that there is a period during which bacteria exposed to natural aquatic systems may be viable but not culturable. In only a few cases in this study, *Salmonella* spp. were culturable when placed on a suitable medium after samples were transported to the laboratory and subjected to bacteriological testing.

Enumeration of salmonellae by the IFA method, however, provided evidence of the presence of *Salmonella* spp. in every raw and chlorinated wastewater sample tested. Although enumeration of salmonellae by the IFA-TDC method may overestimate the number of viable salmonellae, this technique offers a much greater advantage for detection of *Salmonella* spp. in chlorinated wastewater, especially when the MPN procedure gives only negative results because of its dependency on culturability of the organisms.

The IFA-DVC method showed that 19.4 to 62.9% of the *Salmonella* spp. enumerated by the IFA-TDC method were viable, or substrate responsive, in untreated wastewater and that 5 to 31.5% enumerated by IFA-TDC were viable in chlorinated wastewater.

In samples collected on 19 August 1987, the value of the IFA enumeration and DVC became evident, as the MPN culture failed to detect *Salmonella* spp. in chlorinated wastewater samples even when culturable *Salmonella* spp. were recovered downstream from the outflow to the sea. This observation supports results of previous studies showing that bacteria are capable of "resuscitation" when exposed to conditions suitable for growth (20, 21).

The present study provides results consistent with the conclusion that raw effluent chlorination does not eliminate all pathogens. Current U.S. practice consists of secondary treatment prior to chlorination. The findings of Kampelmacher et al. (12) suggest that the secondary treatment process is effective in reducing numbers of *Salmonella* spp. in wastewater. In France, it has been recommended that chlorination of raw effluents be abandoned. Thus, chlorination

would be applied only to secondary effluents (Conseil Supérieur d'Hygiène de France, section des eaux; Séance du 14 décembre 1987). Finally, direct detection of *Salmonella* spp. has proven useful, according to the results of the study reported here. The technique reported here is recommended for detection of pathogens in environmental water or wastewater samples, and it is suggested that culturing procedures for wastewater and environmental water quality testing be reevaluated. Further studies are needed to determine the health significance of viable but nonculturable bacteria, and such studies are in progress in our laboratories.

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