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Rapid Detection of Chlorine-Induced Bacterial Injury by the Direct Viable Count Method Using Image Analysis

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A modified direct viable count method to detect living bacteria was used with image analysis for the rapid enumeration of chlorine-injured cells in an *Escherichia coli* culture. The method was also used for determining chlorine-induced injury in coliform isolates and enteric pathogenic bacteria. Cultures were incubated in phosphate-buffered saline, containing 0.3% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.03% yeast extract, and optimal concentrations of nalidixic acid. Samples were withdrawn before and after incubation and stained with acridine orange, and cell lengths and breadths were measured by computerized image analysis. After incubation, cells which exceeded the mean preincubation length (viable cells) were enumerated and the results were compared with those obtained by the plate count method. Injury in the chlorine-exposed cell population was determined from the difference in viable count obtained with a nonselective Casamino Acids-yeast extract-nalidixic acid medium and a selective Casamino Acids-yeast extract-nalidixic acid medium containing sodium deoxycholate or sodium lauryl sulfate. The levels of injury determined by the direct viable count technique by using image analysis were comparable to those determined by the plate count method. The results showed that image analysis, under optimal conditions, enumerated significantly higher numbers of stressed *E. coli* than the plate count method did and detected injury in various cultures in 4 to 6 h.

One of the major problems in studying indicator and pathogenic bacteria within aquatic environments, including drinking water, is their accurate enumeration. Enumeration of indicator bacteria is routinely done to assess the microbiological quality of water, and the presence of indicator bacteria in excessive numbers signals the possible existence of pathogens. Quantitative recovery of pathogens from the source water may be important in understanding the nature of waterborne disease outbreaks. These microbiological tasks are complicated by drinking water environments that are generally nutritionally limiting and that cannot support the growth of coliform and enteric pathogenic bacteria. Also, bacteria in treated drinking water are exposed to both physical and chemical stress factors (4–6). Chlorine and other stressors, at low levels, are lethal only for a small number of exposed cells but induce injury in a large proportion of the remaining bacterial population (4, 26). As a result, injured bacteria become sensitive to selective agents that are commonly incorporated in media used for their routine enumeration and are unable to form colonies (6, 15, 18). Thus, injury can lead to a significant underestimation of indicator bacteria, since more than 90% of the population in drinking water may become injured (19). The media currently used for quantitative recovery of coliforms from drinking water, such as m-Endo agar, have been associated with low detection rates of injured bacteria as well as poor differentiation of coliforms from noncoliform bacteria (17, 24). More recently, m-T7 medium was developed for the improved detection and enumeration of injured coliforms from chlorinated drinking water (14). However, these procedures require an incubation period of 24 h.

In 1979, Kogure et al. (13) described a direct viable count (DVC) method for the detection of living bacteria that were present in natural waters by incubating samples with low levels of nutrients and nalidixic acid. Although high concentrations of nalidixic acid (≥50 μg/ml) interfere with several metabolic activities of gram-negative bacteria (8), a moderate concentration specifically inhibits bacterial DNA synthesis without affecting other metabolic activities of the cell (7, 12). Thus, in the presence of suitable concentrations of nalidixic acid and nutrients, viable gram-negative bacteria continue to grow without cell division, resulting in the formation of elongated cells. The enlarged bacteria counted by epifluorescence microscopy, after acridine orange staining, represent the viable proportion of the total bacterial population. This method has been used by several investigators for the enumeration of actively growing cells as well as physiologically dormant bacteria in the environment (22, 23, 27). The major disadvantages of this method is the tedious and subjective process of visually differentiating normal cells from marginally elongated cells by conventional microscopy. Recent applications of image analysis permit fast and accurate determination of morphological measurements (1) and biochemical changes associated with bacterial growth (10). Using image analysis and a modified yeast extract-nalidixic acid method, Singh et al. (25a) have described an efficient and quantitative method of counting viable bacteria in a relatively short time.

Here we report on a modification of the yeast extract-nalidixic acid method in which conditions for enumerating chlorine-injured bacteria were optimized and made compatible with image analysis. Chlorine-induced injury was assessed by determining the difference in DVCs that was obtained after the cells were incubated in both nonselective and selective media. This is a rapid and efficient procedure for enumerating injured bacteria and assessing the injury levels in bacterial cultures. The results compared favorably with the traditional plate count method.

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MATERIALS AND METHODS

Bacterial cultures. The following cultures used in this study either were obtained from the culture collection of the Department of Microbiology, Montana State University, or were obtained earlier from different sources. *Escherichia coli* C1 and C2 were previously isolated from the Gallatin River in the Bozeman, Mont., area, and *Enterobacter cloacae* and *Klebsiella pneumoniae* were isolated from urban distribution water samples. *Salmonella typhimurium* SL3201 was obtained from B. A. D. Stocker (Department of Medical Microbiology, Stanford University, Stanford, Calif.), *Yersinia enterocolitica* O:8 was supplied by D. A. Schiemann (Department of Microbiology, Montana State University), and *Vibrio cholerae* strains were obtained from M. M. Levine (Center of Vaccine Development, University of Maryland, Baltimore). Stock cultures were stored at −70°C in 1% peptone water containing 20% glycerol.

Preparation of injured cells. Cultures of *E. coli* and *Y. enterocolitica* were grown in tryptic soy broth without glucose supplemented with 1% lactose and 0.3% yeast extract (TLY); *V. cholerae* was grown in Casamino Acids (Difco Laboratories, Detroit, Mich.) containing 0.3% yeast extract, 0.6% Na_2HPO_4, 0.12% $NaH_2PO_4 \cdot H_2O$, and 0.85% NaCl (CAA); *E. cloacae* and *K. pneumoniae* were grown in tryptic soy broth without glucose; and *S. typhimurium* was grown in minimal medium containing 0.2% glucose; 0.01% $MgSO_4 \cdot 7H_2O$; 0.1% $(NH_4)_2SO_4$; 0.01% sodium citrate (dihydrate); 0.12% $NaH_2PO_4 \cdot H_2O$; 0.6% Na_2HPO_4; and 5 mg each of leucine, cysteine, and histidine per 100 ml (MM). After 24 h of incubation at 35°C, cells from each culture were harvested by centrifugation, washed twice with sterile, reagent-grade water (Milli Q water system; Millipore Corp., Bedford, Mass.), and suspended in Milli Q water; the *V. cholerae* cells, however, were washed and suspended in sterile saline solution (0.85% NaCl). Bacterial concentrations in the washed suspensions were adjusted to obtain approximately 10^9 viable cells per ml.

To induce chlorine injury, cells were exposed to different chlorine concentrations (0 to 0.8 mg/liter) for 10 min at 4°C as described earlier (26), and then the chlorine was neutralized with sodium thiosulfate solution (final concentration, 0.01%).

Assessment of viability and injury by the plate count method. Injury in chlorine-exposed cultures was assessed by determining the difference between the number of CFU per milliliter on nonselective and selective media. The nonselective and selective media used for *E. coli* and *Y. enterocolitica* were TLY agar (TLY containing 1.5% agar) and TLYD agar (TLY agar containing 0.1% sodium deoxycholate), respectively; for *V. cholerae* they were CAA agar and CAAD agar (CAA agar containing 0.01 to 0.05% deoxycholate), respectively; for *E. cloacae* and *K. pneumoniae* they were tryptic soy agar and tryptic soy agar containing 0.05% sodium deoxycholate, respectively; and for *S. typhimurium* they were MM agar and MML agar (MM agar containing 0.1% sodium lauryl sulfate). All plates were counted after 24 to 48 h of incubation, and results were expressed as the percentage of the total population enumerated on nonselective media, as described previously (17).

Assessment of viability and injury by image analysis. Viability of the cultures was determined by the modified method of Kogure et al. (13) as described earlier (25a). Briefly, the washed bacterial cultures were incubated at 35°C in phosphate-buffered saline containing 0.3% Casamino Acids, 0.03% yeast extract, and appropriate concentrations of nalidixic acid (CA-YE-NA medium). Samples were withdrawn at timed intervals and processed by the method described by Hobbie et al. (9), with minor modifications. The withdrawn samples were fixed immediately with Formalin (final concentration, 4%), heated in a boiling water bath for 5 min, and cooled rapidly by immersing them in ice cold water. Samples were filtered through polycarbonate (pore size, 0.2 μm; diameter, 25 mm; black membranes; Nuclepore Corp., Pleasanton, Calif.). The membranes were moistened with filter-sterilized water, and a low filtration pressure was applied to ensure uniform spreading of bacteria on the filter. The retained bacteria were washed with 5 ml of water and stained by slowly filtering through the filters 3 to 5 ml of 0.02% acridine orange solution to allow 2 to 3 min of contact and then by filtering through the filters 3 to 5 ml of water to remove unbound stain. The wash water, Formalin solution, staining solution, and incubation medium were all filtered through 0.2-μm-pore-size membranes before use. Each stained membrane was air dried and placed on a drop of immersion oil on a glass slide. Another drop of immersion oil was then placed on the filter, and a cover slip was firmly pressed over it. The slides were examined under oil immersion by using an epifluorescence microscope connected to an image analyzer.

Image analysis was done with an image analyzer (Q10; Cambridge Instruments, Buffalo, N.Y.) equipped with a processor image analyzer unit (68,000; Cambridge Instruments), four independent binary image planes, and a CP-M controlling unit (64,000 RAM). The system was integrated with an epifluorescence microscope (model BH-2; Olympus). The analyzer had the capability of digitizing images in 0 (black) to 64 (white) grey levels of variable slices into a binary image with 512 by 480 pixels. Cellular measurements of individual features such as length and breadth were made in 10 microscopic fields. Field measurements, including the total number of features detected, average feature size, standard deviation, and frame area, were also made by the computer. The system allowed editing of each image frame displayed on the screen by the addition of a feature that was not detected by the system but that was visible through the microscope or by separation of the images that were produced by closely placed and entangled cells before final measurements were made. In addition, outputs of measurements of different distribution histograms at 0.5-μm intervals were calculated and printed. Some of the field measurements and typical distribution histograms of an *E. coli* culture at time zero and after 4 h of incubation are shown in Fig. 1A and B, respectively. DVCs were calculated from the numbers of elongated cells after incubation in CA-YE-NA medium, and calculations were made as follows: DVC/ml = (average elongated cells/field) × (filter area [μm^2]/frame area [μm^2]) × dilution.

Injury in chlorine-exposed cultures was assessed by determining the difference between the number of viable (enlarged) cells in a nonselective CA-YE-NA medium and a selective CA-YE-NA medium containing sodium deoxycholate or sodium lauryl sulfate. The nonselective medium supported the growth of both uninjured and injured bacteria, whereas the selective medium allowed only uninjured cells to grow. The results were expressed as percent injury in the test population.

MIC of nalidixic acid. Two methods for estimating the MIC of nalidixic acid were used. (i) In the microdilution broth procedure (11), washed cell suspensions were used to inoculate Mueller-Hinton broth tubes (10^6 to 10^7 cells per ml). The tubes were incubated at 35°C for 18 h and examined

TABLE 1. Effect of nalidixic acid on the length and breadth of *E. coli* C2 after incubation at 35°C

Incubation time (h)	Nalidixic acid concn (μg/ml)	Length (μm)	Breadth (μm)
0	10	1.97 ± 0.82	1.38 ± 0.54
4	10	6.05 ± 4.20	1.88 ± 0.84
4	20	5.76 ± 3.63	1.60 ± 0.51
4	40	4.49 ± 2.30	1.69 ± 0.53
8	10	6.77 ± 4.60	1.86 ± 0.81
8	20	6.05 ± 4.06	1.79 ± 0.74
8	40	4.12 ± 2.31	1.62 ± 0.53
12	10	6.78 ± 4.91	1.83 ± 0.90
12	20	6.25 ± 4.20	1.82 ± 0.95
12	40	4.51 ± 2.78	1.65 ± 0.74

visually to determine the MIC. (ii) For the disk agar diffusion test, antibiotic-impregnated disks were prepared by soaking sterile blank disks (diameter; 1/4 in. [7 mm]; Difco) in solutions (20 μl per disk) of the desired concentrations of nalidixic acid. The disks were completely air dried before use. The tests were performed by the procedure of the National Committee for Clinical Laboratory Standards (21) by using Mueller-Hinton agar.

Statistical analysis. Regression analysis was performed by using the MSUSTAT statistical analysis package, microcomputer version 4.10, developed by Richard E. Lund (Montana State University).

RESULTS

Determination of optimal conditions for DVC. The optimal recovery and the assessment of injured bacteria depend on the efficiency of determination of a differential count of viable bacteria on nonselective and selective media. Thus, conditions for the DVC method were optimized and made compatible with image analysis. Previous results (25a) indicated that the concentration of nalidixic acid (20 μg/ml) suggested by Kogure et al. (13) causes an underestimation of viable cells in uninjured cultures. To determine the effect of incubation time and nalidixic acid concentrations on cell enlargement, unexposed cultures of *E. coli* C2 were used. Cells (10^7/ml) were incubated in CA-YE-NA medium containing different concentrations of nalidixic acid. The results (Table 1) show that 10 μg of nalidixic acid per ml caused approximately 1.3- and 3-fold increases in breadths and lengths of cells, respectively, after 4 h of incubation. Further incubation for up to 12 h did not cause a substantial increase in the mean cell enlargement. Nalidixic acid concentrations lower than 10 μg/ml allowed cell division of *E. coli* C2 cultures and were not suitable for determining viability. However, higher concentrations (>10 μg/ml) yielded lower mean cell lengths (Table 1) and may result in underestimation of viable cells, as reported elsewhere (25a).

Determination of MICs and optimal levels of nalidixic acid for different cultures. The MICs of nalidixic acid were determined for both unexposed and chlorine-exposed cultures in an attempt to find the optimal levels for use with the DVC method. The results are shown in Table 2.

Preliminary trials indicated that the MICs of nalidixic acid obtained by the microdilution or the disk agar diffusion method did not cause optimal enlargement of incubated cells. Thus, different concentrations of nalidixic acid close to the MICs were used to determine the optimal levels that were required for maximal cell enlargement in unexposed and chlorine-exposed cultures (data not shown) by using a procedure similar to that used for *E. coli* C2 cultures. The MIC data provided range-finding values which facilitated determination of optimal concentrations of nalidixic acid (Table 2) for the image analysis method. We also measured the length and breadth distribution of bacteria in each sample (data not shown) before they were incubated in CA-YE-NA medium. After incubation, cells longer than the mean length at 0 h were considered viable, as shown in Fig. 1A and B for *E. coli* C2 cultures. Other useful modifications of this procedure have been described elsewhere (25a).

Comparison of viable cell enumeration methods in chlorine-injured cultures of *E. coli* C2. Comparisons of viable cell enumerations done by plating and image analysis methods were made in serially diluted strain C2 cultures that were chlorine injured (82 to 87% injury). The viability of cells was determined by both the plate count method by using nonselective TLY agar and the DVC method by using CA-YE medium containing 10 μg of nalidixic acid per ml. The results obtained by these two methods are given in Fig. 2. Linear regression analysis showed that both image analysis and the plate count method can be used for the determination of relative numbers of viable cells in serially diluted chlorine-injured cell cultures. Further analysis with a reduced model showed that a single regression line with one slope and one intercept was insufficient to fit all the datum points generated by the two methods ($P = 0.00165$). Thus, these regression lines represent different levels of viable cell detection. Figure 2 and statistical analysis indicated that higher numbers of chlorine-injured *E. coli* cells could be determined by the image analysis technique than by the spread plate method.

TABLE 2. MICs of nalidixic acid for uninjured and chlorine-injured cultures and optimal levels used in the DVC method

Organism	MIC by:				Optimal levels (μg/ml) used for DVC of the following cells:	
	Tube dilution (μg/ml)		Disk diffusion (μg/disk)			
	Uninjured	Chlorine-injured	Uninjured	Chlorine-injured	Uninjured	Chlorine-injured
Salmonella typhimurium	16	8	16	16	10	10
Yersinia enterocolitica	16	8	8	4	20	10
Escherichia coli C1	8	8	32	16	10	10
Escherichia coli C2	16	8	32	16	10	10
Vibrio cholerae CVD101	8	8	4	2	2.5	2.5
Vibrio cholerae 569B	8	4	2	1	5	2.5
Enterobacter cloacae	16	4	32	16	10	2.0
Klebsiella pneumoniae	4	4	32	16	10	2.0

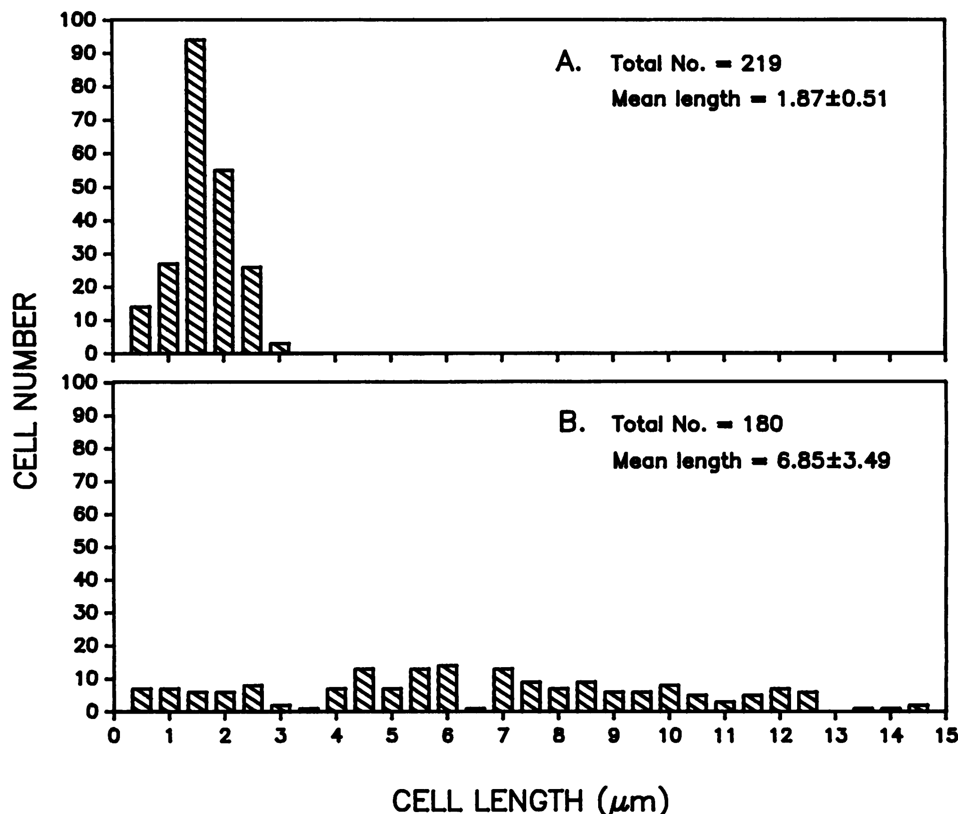

FIG. 1. Computer measurements of cell lengths and distribution histograms of an *E. coli* C2 culture at time zero (A) and after 4 h of incubation (B) in CA-YE-NA medium.

Also, the initial cell density (10^5 to 10^9/ml) did not influence the efficacy of either the plate count method or the DVC technique by using image analysis for enumerating stressed cells.

Detection of chlorine-induced injury by image analysis epifluorescence microscopy. Chlorine-induced injury in different cultures was assessed after washed cells were incubated in nonselective and selective media. The concentrations of selective agents (0.05 to 0.1% sodium deoxycholate or 0.1% sodium lauryl sulfate) caused minimal inhibition of the unexposed cultures but revealed maximal levels of injury in the chlorine-exposed cells. The results are shown in Table 3. Analysis of variance and paired t test showed no significant difference (at the 0.05 level of significance) in percent injury detected by the plate count method and image analysis in unexposed (P = 0.70) and chlorine-exposed (P = 0.115) cultures. However, for image analysis considerably less time (4 to 6 h) was needed to obtain the results.

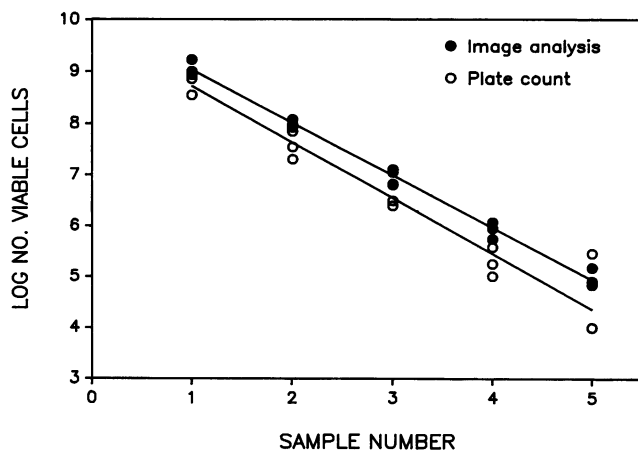

FIG. 2. Comparison of viable cells detected in decimally diluted samples of chlorine-injured *E. coli* C2 cultures determined by plate count (○) and image analysis (●).

DISCUSSION

The modified DVC method for determining cell viability by image analysis demonstrated the importance of determining the optimal concentration of nalidixic acid for each culture. Prolonged incubation with concentrations higher than the optimal levels has been shown to affect protein and RNA synthesis, and to inhibit DNA synthesis, in *E. coli* (8). Liebert and Barkay (16) have reported that lower levels of nalidixic acid are unsuitable for DVC methodology since they allow multiplication of mercury-resistant bacteria during incubation of aquatic samples. The optimal concentration of nalidixic acid determined by those investigators was fivefold greater (100 μg/ml) than that suggested for use in the method of Kogure et al. (13). Several investigators used 20 μg of nalidixic acid per ml in the DVC method for determining the number of viable bacteria in aquatic environments (13, 22, 23, 27). However, the results of this and other investigations (16, 25a) showed the enumeration of maximal numbers of viable bacteria by the DVC technique by using optimal concentrations of nalidixic acid. The determination of optimal concentrations was facilitated by the computer-

TABLE 3. Detection of injury in unexposed and chlorine-exposed bacterial cultures by using the spread plate count and image analysis methods

Organism	Chlorine concn (mg/liter)	No. of replicates	% Injury by:	
			Plate count method (24 h)	DVC (image analysis) (4 h)
Salmonella typhimurium	0	3	19.6 ± 6.5	24.6 ± 6.4
	0.4	3	90.0 ± 11.5	89.6 ± 7.5
Yersinia enterocolitica	0	4	20.0 ± 10.2[a]	25.5 ± 8.5[b]
	0.8	4	87.0 ± 9.6[a]	88.5 ± 9.0[b]
Escherichia coli C1	0	4	19.5 ± 13.5	24.0 ± 5.3
	0.3	4	88.2 ± 6.1	79.7 ± 5.8
Vibrio cholerae CVD101	0	3	37.6 ± 2.5	36.7 ± 6.1
	0.8	3	98.0 ± 1.7	93.3 ± 4.2
Vibrio cholerae 569B	0	3	35.3 ± 5.5	26.0 ± 4.6
	0.6	3	91.3 ± 4.0	92.0 ± 3.0
Enterobacter cloacae	0	2	11.0 ± 15.5	12.5 ± 17.6
	0.7	1	89.0	68.8
Klebsiella pneumoniae	0	2	33.0 ± 8.5	30.5 ± 19.1
	0.5	2	72.5 ± 3.5	65.0 ± 7.1

[a] Colonies were counted after 48 h of incubation at 35°C.
[b] Cultures were incubated for 6 h before DVC by image analysis.

ized image analysis technique for studying cell elongation (Table 1) in response to concentrations near the MICs (Table 2).

We distinguished elongated cells objectively by image analysis by measuring cell lengths before and after incubation. Cells that exceeded the mean preincubation length (rounded to the next 0.5-μm interval) after incubation were considered viable (Fig. 1A and B). Although the use of preincubation mean cell length as a criterion for viability assessment was somewhat arbitrary, it appeared to be effective. This was done to minimize the inherent problems of counting a few nonviable bacteria which initially exceeded the mean preincubation length and not counting cells which elongated but did not exceed the mean preincubation length (2).

Sublethal injury is a reversible process, and recovery follows after injured cells are placed in a suitable medium. The recovery of an injured enterotoxigenic *E. coli* strain, determined by the plate count method, was reported after 6 h of incubation in a defined amino acids medium (25). The CA-YE-NA medium used in the image analysis method was also found to be suitable for the recovery of chlorine-injured cultures of *E. coli* since optimal numbers of enlarged cells were formed after 4 h of incubation. However, the time for optimum enlargement of uninjured cells was shorter than that for chlorine-injured cultures, which also displayed an extended lag phase (data not shown). We noted that during growth, injured bacteria exhibited an extended lag phase, which has also been reported by others (3, 25). This prolonged lag phase may be responsible for the longer time required for optimal cell enlargement of chlorine-injured cultures. Some chlorine-injured cultures also exhibited increased sensitivity to nalidixic acid (Table 2). The reason for this is not known. However, it was possible to detect significantly more viable cells after chlorine injury by this method than by the plate count method (Fig. 2). These results are in agreement with those reported earlier for uninjured cultures (25a). A higher yield of viable bacteria may be due, in part, to the more precise and quantitative nature of the image analysis technique.

Detection of bacterial injury induced by chlorine and other stressors in aquatic environments is important. A large number of sublethally injured bacteria may remain undetected by routine analysis and provide false information on the effectiveness of a treatment that is intended to reduce the bacterial load in water (20). The information is also valuable in formulating selective media for the resuscitation of injured bacteria (14). By using image analysis, it was possible to rapidly and quantitatively determine the injured subpopulation in chlorine-exposed suspensions. However, the detection levels were comparable to those obtained by the plate count method. The detection of higher levels of injury was not expected because the image analysis technique detects higher numbers of both uninjured (26) and chlorine-injured bacteria when compared with those determined by the plating method (Fig. 2). Thus, both methods gave similar estimates of the bacterial subpopulation that was injured.

The use of image analysis for enumerating and detecting injured bacteria in stressed cultures is an improvement over conventional plate count methods. Although it requires initial optimization of the nalidixic acid concentration, incubation time, and concentration of a selective agent, the use of image analysis with the DVC method is efficient and relatively rapid compared with the commonly used plate count method. The method described here could be used for quantitative assessment of the viability of slow-growing bacteria which form visible colonies on solid media only after extended periods of incubation. This procedure also facilitates the collection of sufficient objective data for rigorous statistical analysis with much less effort than that required with conventional microscopic methods.

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LITERATURE CITED

1. **Adam, H. L., and C. R. Thomas.** 1988. The use of image analysis for morphological measurements on filamentous microorganisms. Biotechnol. Bioeng. **32**:707–712.
2. **Al-Hadithi, S. A., and R. Goulder.** 1989. An alternative approach to the yeast extract-nalidixic acid method for determining the proportion of metabolically active aquatic bacteria. Lett. Appl. Microbiol. **8**:87–90.
3. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. **29**:186–194.
4. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37**:633–641.
5. **Egan, A. F.** 1978. Enumeration of stressed cells of *Escherichia coli*. Can. J. Microbiol. **25**:116–118.
6. **Fujioka, R. S., H. H. Hashimoto, E. B. Siwak, and R. H. F. Young.** 1981. Effect of sunlight on survival of indicator bacteria in seawater. Appl. Environ. Microbiol. **41**:690–696.
7. **Goss, W. A., W. H. Deitz, and T. M. Cook.** 1964. Mechanism of action of nalidixic acid in *Escherichia coli*. J. Bacteriol. **88**: 1112–1118.

8. **Goss, W. A., W. H. Deitz, and T. M. Cook.** 1965. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. **89:**1068–1074.
9. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
10. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosomes in human monocytes. J. Cell Biol. **99:**1936–1943.
11. **Jones, R. N., A. L. Barry, T. L. Gavan, and J. A. Washington II.** 1985. Susceptibility tests: microdilution and macrodilution broth procedures, p. 972–987. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
12. **Kantor, G. J., and R. A. Deering.** 1968. Effect of nalidixic acid and hydroxyurea on division ability of *Escherichia coli* Fil^+ and Lon^- strains. J. Bacteriol. **95:**520–530.
13. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
14. **LeChevallier, M. W., S. C. Cameron, and G. A. McFeters.** 1983. New medium for improved recovery of coliform bacteria from drinking water. Appl. Environ. Microbiol. **45:**484–492.
15. **LeChevallier, M. W., and G. A. McFeters.** 1985. Enumeration of injured coliforms in drinking water. J. Am. Water Works Assoc. **77:**81–87.
16. **Liebert, C., and T. Barkay.** 1988. A direct viable counting method for measuring tolerance of aquatic microbial communities to Hg^{2+}. Can. J. Microbiol. **34:**1090–1095.
17. **McFeters, G. A., S. C. Cameron, and M. W. LeChevallier.** 1982. Influence of diluents, media, and membrane filters on the detection of injured waterborne coliform bacteria. Appl. Environ. Microbiol. **43:**97–103.
18. **McFeters, G. A., and A. K. Camper.** 1983. Enumeration of indicator bacteria exposed to chlorine. Adv. Appl. Microbiol. **29:**171–193.
19. **McFeters, G. A., J. S. Kippin, and M. W. LeChevallier.** 1986. Injured coliforms in drinking water. Appl. Environ. Microbiol. **51:**1–5.
20. **McFeters, G. A., M. W. LeChevallier, A. Singh, and J. S. Kippin.** 1986. Health significance and occurrence of injured bacteria in drinking water. Water Sci. Technol. **18:**227–231.
21. **National Committee for Clinical Laboratory Standards.** 1984. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
22. **Peele, E. R., and R. R. Colwell.** 1981. Application of a direct microscopic method of enumeration of substrate-responsive marine bacteria. Can. J. Microbiol. **27:**1071–1075.
23. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30:**334–338.
24. **Schiff, L. J., S. M. Morrison, and J. V. Mayevx.** 1970. Synergistic false-positive coliform reaction on m-Endo MF medium. Appl. Environ. Microbiol. **20:**778–781.
25. **Singh, A., and G. A. McFeters.** 1986. Recovery, growth, and production of heat-stable enterotoxin by *Escherichia coli* after copper-induced injury. Appl. Environ. Microbiol. **51:**738–742.

25a. **Singh, A., B. H. Pyle, and G. A. McFeters.** 1989. Rapid enumeration of viable bacteria by image analysis. J. Microbiol. Methods **10:**91–101.

26. **Singh, A., R. Yeager, and G. A. McFeters.** 1986. Assessment of *in vivo* revival, growth, and pathogenicity of *Escherichia coli* strains after copper- and chlorine-induced injury. Appl. Environ. Microbiol. **52:**832–837.
27. **Xu, H.-S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8:**313–323.