Research Paper

Comparison of rapid methods to evaluate chlorine inactivation of the biological agent *E. coli* O157:H7

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ABSTRACT

Rapid viability tests of the Category B agent *Escherichia coli* O157:H7 were evaluated after disinfection with chlorine. The metabolic activity dyes ChemChrome V6, a modified fluorescein diacetate (FDA) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) were compared to standard plate counts. ChemChrome V6 results were obtained using a solid phase cytometer and CTC results by microscopic analysis. The water-borne bacteria *Legionella pneumophila* and *Mycobacterium avium* were also tested as positive and negative controls. Under the conditions tested, CTC provided more consistency with plate count estimates of viability than did ChemChrome V6.

Key words | ChemChrome V6, CTC, *E. coli* O157:H7, fluorescent, viability

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NOTATION AND ABBREVIATIONS

A_f	effective area of filter
CDFW	chlorine demand free water
Ct	*C* is the concentration of disinfectant (mg/L); *t* is exposure time (min)
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
d	dilution factor
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DPD	N,N-diethyl-*p*-phenylene diamine
FDA	fluorescein diacetate
G	number of grids
N	number of cells
OD	optical density
PBS	physiological buffered saline
UV	ultraviolet
V_f	volume of diluted sample filtered
VBNC	viable but not culturable

INTRODUCTION

Evaluation of disinfection effectiveness in a timely manner is critical for the Environmental Protection Agency (EPA). Methods to provide rapid diagnosis of water quality are needed. Assays that detect metabolic activity within cells are increasingly being employed as substitutes for growth in cultures in order to shorten the time required for analysis of disinfection (Parthuisot *et al.* 2000; Ericsson *et al.* 2000; Forsman *et al.* 2000; Queric *et al.* 2004).

Several new metabolic indicator compounds are now available. One of these indicator compounds is 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) which produces an insoluble brilliant fluorescent red when reduced, usually by components of the electron transport system (Rodriguez *et al.* 1992). Other indicators are found in the group of fluorophores, e.g. fluorescein diacetate (FDA). FDA inside an active cell is cleaved by esterase activity to release fluorescein or fluorescein derivatives (Parthuisot *et al.* 2000). However, FDA is not retained well inside the cell (Diaper & Edwards 1994) but a modified FDA, designated ChemChrome V6 (Chemunex, Paris, France), is retained more efficiently inside cells with intact membranes (Parthuisot *et al.* 2000). In this study, ChemChrome V6 and CTC measurements of viability were compared to

standard plate counts after disinfection of water suspensions of *E. coli* O125:H7, *Legionella pneumophila* and *Mycobacterium avium*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains, culture conditions and media used for *E. coli* O157:H7, *L. pneumophila* and *M. avium* are listed in Table 1.

Preparation of chlorine demand free water

Chlorine demand free water (CDFW) was prepared by adding 7 mL of a 1:20 dilution of reagent grade sodium hypochlorite (Sigma, St. Louis, MO) to 5 L of deionized water (Millipore Corp., Bedford, MA). The pH was adjusted to 7.0. The CDFW was boiled for 5 min, exposed for 48 h to ultraviolet light (UV), autoclaved for 30 min and passed through nitrocellulose filters of 47 mm diameter and 0.025 μm porosity (Millipore). Pure water rather than a buffer was used to prepare the CDFW in order to avoid reduction of chlorine demand due to the presence of organic or inorganic compounds. The CDFW and added reagents were adjusted to pH 7.0 so that the pH before and after chlorine addition would remain at 7.0.

Bacterial suspensions in CDFW

Cultured bacteria were transferred from growth plates to eppendorf tubes. The cells were washed 3 times in CDFW and the optical density (OD) of the suspension was adjusted to 1.0 at a wavelength of 595 nm (Spectronic 20, Thermo Spectronic, Rochester, NY). Aliquots of the 1.0 OD suspensions were added to 150 mL of CDFW to achieve the desired number of cells.

The bacterial suspensions were prepared for the experiment by thoroughly mixing and then dispensing 20 mL aliquots into 50 mL sterile polypropylene centrifuge tubes (Corning Inc., Corning, NY) for 3 chlorine treatments per experiment. A minimum of 3 separate experiments were performed for each bacterium with 3 replicates per treatment for a total of 9 chlorine treatments for each bacterium.

Chlorine treatment

The chlorine (Sigma, St. Louis, MO) was prepared as a 1:200 stock in CDFW. Chlorine concentration (mg/L) multiplied by exposure time (min) is designated as the *Ct* value. The *Ct* values used in this study were similar to those proposed in the guidance manual for surface water treatment for 0.5–1.0 $\log_{10}$ inactivation of *Giardia lambia* cysts (*Guidance Manual* 1991). The concentrations of chlorine added to *E. coli* O157:H7 ranged from 0.66–2.2 mg/L over periods of 14–17 min for *Ct* values extending from 10–95. *L. pneumophila* chlorine concentrations were from 0.47–1.79 mg/L with timeframes of 10–15 min, resulting in *Ct* values of 7–27. *M. avium* chlorine concentrations ranged from 0.44–2.04 mg/L with times of 10–30 min, giving *Ct* values of 6.6–61. The chlorine treated bacterial suspensions were held in a 5°C recirculating refrigerated water bath with gentle mixing for times ranging from 10–30 min after which chlorine measurements of 10 mL aliquots were determined by the N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric method (*Standard Methods* 1998). The remaining sample was treated with the addition of 100 μL of a 10% solution of sodium thiosulfate (pH 7.0) to stop the treatment.

Table 1 | Bacterial strains and source, media used and growth conditions for each species of bacterium

Bacterium and source	Media	Culture conditions
Escherichia coli O157:H7	Tryptic soy agar	37°C, aerobic, 24 h
Legionella pneumophila 33152	Buffered charcoal with yeast extract	35°C in 10% CO_2 for 3 d
Mycobacterium avium 33B	Middlebrook 7H10 agar with Middlebrook OADC	35°C aerobic for 6 weeks

Estimate of viability after chlorine disinfection using standard culture plates

Ten-fold dilutions of the bacterial suspensions were made before and after chlorine treatment. Three replicate viability test plates were inoculated by adding 0.1 mL of each of three dilutions onto the medium selected for each bacterium (Table 1). Colonies on transparent media were counted with a Quebec counter (Gallenkamp, Germany) and colonies on opaque media were counted with a dissection microscope (Leitz Inc., Welzlar, Germany).

Estimate of viability using ChemChrome V6

ChemChrome V6 was used to detect cell metabolic activity following the manufacturer's instructions. Briefly, samples were filtered through black filters of 25 mm diameter and 0.40 μm porosity (Chemunex) followed by the addition of 1 mL of CSE/2 solution (Chemunex) to mask the background material.The treated filters were placed on absorbent pads (Millipore, Bedford, MA) containing 600 μL of a 1:100 dilution of ChemChrome V6 in B16 buffer (Chemunex) and incubated at 30°C for 30 min. Black cellulose acetate support filters (0.45 μm porosity and 25 mm diameter) (Chemunex) were placed over 0.1 mL of B16 buffer in stainless steel holders.The stained filters were placed on top of the support filters and scanned by the ChemScan solid phase cytometer model Chemunex C (Chemunex). All fluorescent bacteria that fit the specified discriminates such as intensity, size and shape were enumerated using the ChemScan solid phase cytometer. All cells were validated by microscopy when counts were less than 100. For numbers greater than 100, the ratio of targets to particles and unknowns was calculated and used to obtain the total number of cells found on the filter. Instrument controls included checking the peak of intensity obtained from standard fluorescent beads (Chemunex) to ensure these readings fell within the established range for the instrument.

Estimate of viability after chlorine disinfection using CTC

A 50 mM stock solution of CTC was prepared by filtering through a 0.22 μm syringe filter (Millipore) and used to give a final concentration in the samples of 5 mM (Rodriguez *et al.* 1992). Microtiter tubes containing CTC samples were mixed at 16 rpm on a mini-rotator wheel (Glas-col, Terre Haute, IN) for 4 h at room temperature in the dark for *E. coli* O157:H7. However, *M. avium* and *L. pneumophila* were incubated for 4 h at 35°C in a 5% carbon dioxide incubator.

After incubation, the samples were filtered through black Chemunex filters. A glycerol-carbonate buffer was prepared as follows: part A 3.7 g $NaHCO_3$, 0.48 g Na_2CO_3, 99 mL water; part B glycerol. One mL of part A was added to 9 mL of part B. The support filter was placed on a drop of the buffer and the Chemunex filter was placed on the support filter. The stainless steel holder with filters was placed on a Nikon microscope stage. CTC positive responses were counted using a 10 × ocular, a non-oil 100 × objective and a Nikon microscope filter block with an excitation of 450 nm and emission of 650 nm (D450 class 50 ×, barrier 480 DCLP emission D36/60M). Ten fields were counted when the number of fluorescent formazan crystals within the cells was 70 or more per field. For counts less than 70 per field, 20 fields were counted. Calculations of counts per mL were made as previously described (Kepner & Pratt 1994). Bacteria (cells per mL) $= (N \times A_f)/(d \times V_f \times G \times A_g)$, where N is the number of cells counted, A_f is the effective area of the filter (mm^2), d is the dilution factor, V_f is the volume of diluted sample filtered (mL), G is the number of grids and A_g is the area of the counting grid (mm^2).

Estimating total cell counts using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)

Total bacterial counts were measured by DAPI staining (Sigma, St. Louis, MO). The DAPI stock solution was prepared by dissolving 0.001 g in 1 mL of 100% methanol. The working solution consisted of 100 μL of the stock solution added to l liter of phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in l liter water). The pH was adjusted to 7.4 with HCl.

One L dilutions were treated with 100 μL of 30% (v/v) paraformaldehyde for 15 min before adding 100 μL of the DAPI working solution. The tubes were incubated at room temperature for 1 h while mixing on a rotating wheel at 16 rpm. The stained cells were filtered through the black Chemunex filters described above. Stained cells were

counted with a Nikon 600 eclipse microscope containing a filter block (excitation D360/40 ×; beamsplitter 400 through DCLP; emission D460/50). Calculations of cells per mL were determined by the Kepner & Pratt formula described above for CTC stained cells.

Statistical analysis

The results from the three independent experiments for each assay type (standard plating, CTC and ChemChrome V6) were compared by means of the Kruskaal–Wallis (KW) one-way test (Hollander & Wolf 1999). An alpha level of 0.05 was utilized to determine the significance of differences, while actual p values were based on the KW test.

RESULTS

Tables 2, 3 and 4 represent the $\log_{10}$ reduction of counts for each bacterium after chlorine treatment. Inactivation of *E. coli* O157:H7 ranged from 1.6 $\log_{10}$ to 6.2 $\log_{10}$ for V6 counts. Inactivation of *L. pneumophila* ranged from 1.5 $\log_{10}$ to 2.8 $\log_{10}$. The initial concentrations of viable

Table 2 | $\log_{10}$ reduction of *Escherichia coli* O157:H7 counts by assay after chlorine treatment

Ct (mg × min/L)	ChemChrome V6	CTC[1]	Plate[2]	DAPI
10	1.98	≥6.42	≥5.06	0.91
11	3.18	≥5.45	≥7.05	0.65
16	1.55	≥4.98	≥6.16	0.03
23	2.72	≥6.42	≥5.06	1.05
26	3.16	≥5.45	≥7.05	0.12
31	3.43	≥6.42	≥5.06	0.95
33	6.19	≥5.45	≥7.05	0.28
38	2.51	≥4.98	≥6.16	0.03
95	4.33	≥4.98	≥6.16	0.04[3]

[1]The numbers for the CTC assay represent $\log_{10}$ of initial counts where no activity was found for chlorine treated samples.
[2]The numbers for the plate assay represent $\log_{10}$ of initial counts where no growth was found for chlorine treated samples.
[3]This number represents an increase from the non-chlorinated count.

Table 3 | $\log_{10}$ reduction of *Legionella pneumophila* counts by assay after chlorine treatment

Ct (mg × min/L)	ChemChrome V6	CTC[1]	Plate[2]	DAPI
7	1.48	≥5.08	≥5.72	0.18
8	2.23	≥5.97	≥4.65	0.12
13	2.11	≥5.97	≥4.65	0.05[3]
16	1.85	≥5.23	≥5.98	0.08
19	2.14	≥5.08	≥5.72	0.21
19	2.23	≥5.08	≥5.72	0.17
20	2.54	≥5.97	≥4.65	0.18[3]
21	2.79	≥5.23	≥5.98	0.25
27	2.61	≥5.23	≥5.98	NC[4]

[1]The numbers for the CTC assay represent $\log_{10}$ of initial counts where no activity was found for chlorine treated samples.
[2]The numbers for the plate assay represent $\log_{10}$ of initial counts where no growth was found for chlorine treated samples.
[3]This number represents an increase from the non-chlorinated count.
[4]NC represents no change.

Table 4 | $\log_{10}$ reduction of *Mycobacterium avium* counts by assay after chlorine treatment

Ct (mg × min/L)	ChemChrome V6	CTC	Plate	DAPI
7	0.2	0.49	0.43	0.10[1]
10	0.2	0.98	0.44	0.21[1]
10	0.52	0.43	0.01	0.34
16	0.21	0.17[1]	0.04	0.16
18	0.07	0.19	0.32	0.10
21	0.4	NC[2]	0.09[1]	0.41
43	0.3[1]	0.16	0.47	0.08
52	0.08[1]	0.17	0.43	0.30
61	0.27[1]	0.30	0.93	0.32

[1]This number represents an increase from the non-chlorinated count.
[2]NC represents no change.

Table 5 | Measurements of viability, activity and total numbers of *Escherichia coli* O157:H7, *Legionella pneumophila* and *Mycobacteriuim avium* before and after chlorine disinfection

	Activity estimate	Viability estimate		Total cell estimate
	V6	CTC	Plate	DAPI
	Log_{10} concentration of viable cells per ml			
Escherichia coli				
Without chlorine[1]	6.5 ± 0.5[2]	5.6 ± 0.7	6.4 ± 0.5	6.7 ± 0.5
With chlorine[3]	3.5 ± 1.1	ND[4]	ND	6.3 ± 0.1
Legionella pneumophila				
Without chlorine	6.1 ± 0.1	5.4 ± 0.5	5.4 ± 0.8	6.6 ± 0.1
With chlorine	3.9 ± 0.4	ND	ND	6.5 ± 0.1
Mycobacterium avium				
Without chlorine	5.9 ± 0.1	5.8 ± 0.3	6.1 ± 0.3	6.0 ± 0.4
With chlorine	5.9 ± 0.4	5.7 ± 0.3	5.9 ± 0.1	5.9 ± 0.3

[1]Without chlorine: log_{10} counts/ml of viable cells represent the average of all nine samples per bacteria before chlorine treatment.
[2]SD: standard deviation.
[3]With chlorine: log_{10} counts/ml of viable cells represent the average of all nine samples per bacteria after chlorine treatment.
[4]ND - not detected.

cells were approximately 1×10^6 cells/mL for each bacterium (Table 5). After chlorine treatment, viable *E. coli* O157:H7 or *L. pneumophila* cells were not detected by culturing. The concentrations of viable *M. avium* cells were unchanged. The estimates of viable cells of each bacterial species were not statistically different for CTC or plate count assays ($p > 0.60$). However, the estimates of viable *E. coli* and *L. pneumophila* based on ChemChrome V6 analysis differed from the plate estimates by about 1×10^3 cells/mL which was statistically significantly different ($p < 0.001$). The total cell counts based on DAPI analysis remained unchanged after chlorine treatment (Table 5).

DISCUSSION

The Centers for Disease Control and Prevention have placed *E. coli* O157:H7 in Category B of their biological disease agents list, meaning that it poses a significant risk to human health because it causes high rates of lethality (Havelaar *et al.* 2004; Thorpe 2004). Rapid methods for evaluating disinfection are needed to protect the United States water supply from biological agents such as *E. coli* O157:H7. The selection of *L. pneumophila* (positive control) and *M. avium* (negative control) was based on their known sensitivity and resistance to chlorine inactivation (Miyamoto *et al.* 2000).

Selection of the three bacteria was based on their isolation from water and on their susceptibility to chlorine. Our strains of *L. pneumophila*, and *E. coli* O157:H7 were eliminated by the similar *Ct* values of 7 and 10, which made *L. pneumophila* a good candidate for a positive control. Counts from our strain of *M. avium* showed no signs of declining at a *Ct* value of 61, which made it a good candidate for a negative control. Similar results by other researchers showed *Ct* values of 6 eliminated *Legionella* while *Mycobacterium* species were found to survive at *Ct* values of 240 (Miyamoto *et al.* 2000). *M. avium* strains required *Ct* values from 51–204 to inactivate 3 log_{10} of growth (Taylor *et al.* 2000).

The chemical composition of outer cellular layers is likely to be the prime factor in the damaging effects of chlorine. The

gram negative *E. coli* O157:H7 and *L. pneumophila* have outer cell membranes composed of phospholipid and lipopolysaccharide. *M. avium* contains a cell wall of mycolate composed of arabinogalactan and lipid. Although no data are available concerning the exact mechanism of chlorine action, it must cross the outer membrane, which is more difficult in the case of *Mycobacterium* due to the membrane composition. The thick hydrophobic barrier prevents diffusion and lowers permeability. The general modes of action are thought to be damage to the lipids and/or proteins of the semipermeable cytoplasmic membrane, resulting in leakage of cellular materials and denaturing of enzymes and other proteins usually by disrupting the hydrogen and disulfide bonds which block metabolism.

The most reliable test for viability is growth (Kell *et al.* 1998; Barer & Harwood 1999; Barer *et al.* 2000). Therefore alternative tests that most closely match plate count results would appear to be the most useful in rapid evaluations of disinfection. CTC provided the most consistent estimates of viability of *E. coli* O157:H7 compared to the standard plate counts. Boulos *et al.* (1999) obtained similar results for the non-Select Agent *E. coli* isolate, ATCC 11229.

Although there are reports that CTC positives can be obtained by means other than the activity of the target electron transport system from respiration and/or a variety of dehydrogenase enzymes present in active bacterial cells (Barer *et al.* 2000), an active respiratory system is generally believed to be the principal means of CTC positive responses in microorganisms (Rodriguez *et al.* 1992). The consistency between plate estimates and CTC estimates of viability was also observed for both the positive (*L. pneumophila*) and negative controls (*M. avium*). CTC estimates of *M. avium*, which are not inactivated at the concentrations of chlorine used in this study, were the same as the estimates of total cells counted by DAPI and viable plate counts. The maximum concentration of chlorine for *M. avium*, 2.2 mg/L, was far below the 15 mg/L used by others and also found not to affect DAPI fluorescence (Saby *et al.* 1997).

On the other hand, the ChemChrome V6 test estimates of viability differed from the plate counts by about a factor of 1000 for the Select Agent *E. coli* O157:H7. The ChemChrome V6 test also produced inconsistent results for the positive control bacterium, *L. pneumophila*. Thus CTC seems to provide a better estimate of viability than ChemChrome V6. A review of the viable but not culturable hypothesis (VBNC) presents the risks involved in assuming that indirect labeling by viability dyes is a dependable indicator of viability (Bogosan & Bourneuf 2001).

The major component of ChemChrome V6 is FDA, which has no fluorescence and passes through the cell membrane where the diacetate group is cleaved off to produce the fluorescent fluorescein which accumulates in the cytoplasm of cells with intact membranes. This reaction is energy-independent. The time required for esterase to leave the cytoplasm of a dead or dying cell may vary with the microorganism. Even traces of the enzyme would result in a positive response.

In this study the cells were exposed to ChemChrome V6 immediately following inactivation of chlorine. The counter stain, CSE/2, added after ChemChromeV6, may not have been able to mask all the esterase from dead or dying cells in the timeframe allowed. It has been suggested that CTC only stains the most actively respiring cells (Sherr *et al.* 1999). If this is the case, fewer false positives would be reported with this dye compared to ChemChrome V6.

Whether CTC estimates of viability will also be applicable to other Select Agents is not clear. Some reports claim that CTC does not work well for all bacteria (Yamaguchi & Nasu 1997; Sherr *et al.* 1999) or lacks sensitivity (Gasol *et al.* 1995) or can be toxic (Ullrich *et al.* 1996; Choi *et al.* 1999). So other Select Agents will need to be tested individually. However, if successfully tested, the use of CTC for disinfection testing could produce results close to the true viability. Since this is a microscopically based analysis, it could easily be performed under field conditions. Similar tests of CTC for other Select Agents and with different types of disinfection are underway.

CONCLUSIONS

1. CTC counts represented an ongoing metabolic function of respiration which closely matched plate counts for *E. coli* O157:H7.
2. If all the ChemChrome V6 has not been released from a dead or dying cell a false positive could result that might require further validation.
3. A negative ChemChrome V6 response would be an excellent indicator of the effectiveness of chlorine.

ACKNOWLEDGEMENTS

The authors thank Dr Eugene Rice for offering guidance throughout the entire study. We also thank Dr Stephen Vesper for his oversight during the writing of the manuscript. The US Environmental Protection Agency (EPA), through its Office of Research and Development, funded and collaborated in the research described here. It has been subjected to the Agency's peer review and has been approved as an EPA publication. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

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First received 6 October 2004; accepted in revised form 21 April 2005