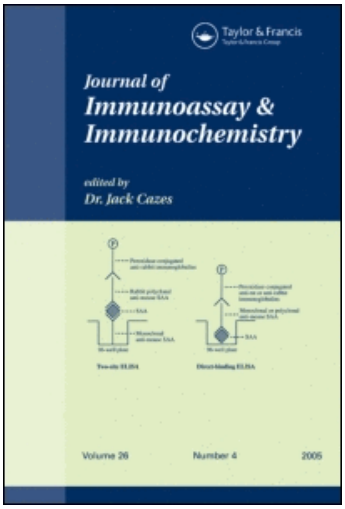

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Effects of Chlorine Injury, Starvation, and Colitag™ Enrichment on Sandwich Enzyme-Linked Immunosorbent Assay (sELISA) Detection of *Escherichia coli* O157:H7

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Effects of Chlorine Injury, Starvation, and Colitag™ Enrichment on Sandwich Enzyme-Linked Immunosorbent Assay (sELISA) Detection of *Escherichia coli* O157:H7

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Abstract: The validity of ELISA for detection of *E. coli* O157:H7 under many conditions is not proven. In this work, sELISA was able to detect bacteria after sub-lethal chlorine exposure and after seven days of starvation with little to no change in limit of detection and fluorescence signal as long as chlorine was not present in the sample or was neutralized by sodium thiosulfate. After Colitag™ enrichment, sELISA detected ~3 colony forming units/ml of originally added *E. coli* O157:H7. Thus, the present sELISA is valid for detection of *E. coli* O157:H7 in water sources, although sample matrices may interfere with assay.

Keywords: *E. coli* O157:H7, Immunoassay, Water, Chlorine disinfectant, Starvation, Nonculterable bacteria

INTRODUCTION

Escherichia coli O157:H7 causes serious illness, including bloody diarrhea and hemolytic uremic syndrome, and occasionally death.[1,2] Approximately 73,000

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cases occur annually[3] and most outbreaks are associated with contaminated food and drink.[2,3] A significant number of outbreaks have been associated with recreational and drinking water including well and spring water, residual faucet water, and municipal water supplies.[3–6] In some cases, water treated with chlorine was linked to outbreaks due to improper chlorination.[7] Thus, there is a need for methods that rapidly, quantitatively, and sensitively detect this pathogen in recreational and potable waters, especially as its presence does not always correlate with the typical water indictor organisms.[8]

Enzyme-linked immunosorbent assays, especially when coupled with biosensor platforms, are capable of providing rapid detection of bacteria.[9] Biosensor immunoassays can rapidly detect low levels of *E. coli* O157:H7 in ground beef[10,11] and apple juice[12] and, thus, are a promising method for direct monitoring of *E. coli* O157:H7 in recreational and potable water supplies. However, before conventional or biosensor-based immunoassays can be widely used, the effects of chlorine-exposure and starvation on bacterial properties pertinent to immunoassay detection need to be determined. Accuracy of immunological methods for bacterial detection is dependent upon the fidelity of antibody-antigen binding, which can be affected by several factors, including sample matrix[13] and bacterial stress such as acid stress.[14,15] The type of enrichment medium, as well as stress (acid, cold, heat and salt), affected the detection of *Listeria monocytogenes*, due to the differential expression of surface proteins.[16] Temperature, salt concentration, and oxidative stress have also been shown to affect detection of *E. coli* O157:H7.[15] Most importantly, a loss of the *E. coli* O157:H7 O antigenicity has been reported during long-term starvation.[17] *E. coli* O157:H7 becomes non-culturable during prolonged storage in water and after exposure to low doses of chlorine,[8] which may influence antibody-binding to these bacteria. Although there is evidence that the non-culturable *E. coli* O157:H7 may lose virulence and the ability to divide,[8] it is still possible that these stressed bacteria may be a risk to human health.

Given the possibility of a reduced ability to detect *E. coli* O157:H7 exposed to chlorine or starvation in water sources when using immunoassay, the present work determined whether chlorine and/or starvation stress alter the efficacy of antibody-based detection of *E. coli* O157:H7. Secondly, the efficacy of enrichment using the EPA-approved Colitag™ test kit coupled with sELISA for *E. coli* O157:H7 detection was determined. The results support the use of the present sELISA for detection of *E. coli* O157:H7 in chlorinated and non-chlorinated water sources and after Colitag™ enrichment.

EXPERIMENTAL

Bacterial Isolates and Incubations

E. coli O157:H7 isolates used in this study were the Odwalla apple juice outbreak (CDC 1996) strain, obtained from Dr. Harvey George (Massachusetts

Department of Public Health State Laboratory Institute, Jamaica Plain, MA), and ATCC strain #43894 (American Type Culture Collection (Manassas, VA). All bacterial incubations were performed at 37°C at 250 RPM, unless specified. Bacteria, maintained on tryptic soy agar (TSA, BD Diagnostic Systems, Sparks, MD) plates at 4°C, were revived for the experiments described below by overnight incubation of an isolated colony in tryptic soy broth (BD Diagnostic Systems, Sparks, MD).

Chlorinated water was prepared by adding aliquots of a standardized chlorine solution (Hach, Loveland, CO) to molecular grade water followed by filtration to remove any bacterial contamination. Total chlorine was measured in the final water samples with a kit (Hach, Loveland, CO). Bacterial pellets, obtained by centrifugation at 3,000 × g for 10 min at room temperature, were resuspended in molecular grade, filtered water to a standard optical density at 600 nm. One mL aliquots of bacterial suspensions were added to 24 mL of chlorinated water for a final concentration of approximately 10^8 cells/mL. Bacterial suspensions were incubated in tubes that allowed for gas exchange at room temperature for 0 h, 1 h, 72 h, and 7 days. The tubes were gently shaken twice each day for the 72 h and 7 day incubations. Bacteria were harvested by centrifugation at 3,000 × g for 10 min at room temperature, washed 2X with phosphate-buffered saline (PBS), and resuspended at a specified optical density in PBS for sELISA, viable counts, and microscopic cell counts.

For Colitag™ enrichment, overnight cultures were resuspended in autoclaved molecular grade or tap water, serial-diluted, and allowed to incubate without shaking for 20 min, at which time aliquots were added to the enrichment medium or were enumerated by plating on TSA plates. Each packet of enrichment medium was made up to 50 mL using autoclaved molecular grade water and then 5 mL of this mixture was added to 5 mL of each serial diluted bacterial sample. Enriched samples were pre-incubated at 35.0°C ± 0.5°C for 4 h to allow injured cells to recover. Samples were then incubated at 44.5°C ± 0.2°C for 20 h. At the end of incubation, samples were examined for a yellow color indicating the presence of fecal coliforms. The presence of MUG-negative *E. coli* was determined by adding approximately 1–2 drops of Kovac's reagent to a 1 mL aliquot of each sample. A reddish-purple color forming on the surface of the solution indicates the presence of *E. coli*. Aliquots of the incubated bacteria were used for sELISA.

Sandwich ELISA

Aliquots of a goat anti-*E. coli* O157:H7 polyclonal antibody (KPL; Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 2 μg/ml in PBS were incubated overnight at 4°C in 96-well NUNC Maxisorp® microplates (Nalge Nunc International, Rochester, NY). Subsequent steps were

performed at room temperature unless specified. Wells were washed once with PBS, incubated for 1 h with PBS containing 0.2% casein hydrosylate and 0.2% IgG-free bovine serum albumin, and then washed once with PBS. Each bacterial sample (100 μL) was added in triplicate for incubation at 37°C for 1 h. The wells were washed 1X with PBS, and 100 μL of the goat anti-*E. coli* O157:H7 polyclonal antibody conjugated with horse radish peroxidase (1 μg/mL) was added. After 30 min, the wells were washed 3X with PBS containing 0.05% Tween and the QuantaBlu® substrate (Pierce, Rockford, IL) with stabilized peroxide was added to the wells and incubated for 45 min. Reactions were stopped by addition of the QuantaBlu® stop solution. Fluorescence intensity was measured using a FL_x800 Bio-Tek microplate reader (Winooski, VT). Optimal capture and detection antibody concentrations were determined by checkerboard titration. Net fluorescence intensity was calculated by subtracting the background values (wells without antigen) from the total intensity. Assay precision was determined by calculating the coefficient of variation. Only those assays with a coefficient of variation $<10\%$ were included in the results.

To determine if chlorinated samples could be used directly in sELISA, a 5 mg/L chlorinated water solution was prepared using a chlorine standard solution (Hach, Loveland, CO), and its concentration was verified using the Hach procedure described above. The 5 mg/L water solution was serial diluted and an aliquot of bacterial suspension was then added to each tube for a final concentration of 10^6 cells/mL. The concentration of the suspension used for inoculation was determined by $OD_{600\,nm}$ and verified by plate counts on TSA. For chlorine-neutralized solutions, sodium thiosulfate (0.8 mM final concentration) was added prior to the addition of the bacteria. Samples were immediately subjected to sELISA.

Cell Enumeration

Bacterial numbers were determined by viable counts (colony forming units) on TSA plates and by microscopic cell counts. For viable counts, serial dilutions of bacterial samples in PBS were added to triplicate agar plates for overnight incubation at 37°C. For microscopic counts, total bacterial number and percent live bacteria were determined using a LIVE/DEAD *Bac*light™ kit (Molecular Probes, Eugene OR) with a method modified from Braux et al.[18] Aliquots (100 μL) of the serial diluted cells were incubated in the stain solution in the dark for 15 min. Stained bacteria were captured on 0.2 μm black filters, which were mounted on glass slides and observed at 1,000X using an oil immersion lens with a Zeiss epifluorescence microscope. Live (green fluorescent) and dead (red fluorescent) bacteria were viewed with fluorescein and Texas Red bandpass filter sets, respectively. Cell counts were performed on 20 randomly selected fields.

Statistical Analysis

Values are reported as means $\pm$ standard error of the mean for $n = 6$ measurements. Statistical significance was determined by Analysis of Variance followed by Dunnett Multiple Comparison tests using GraphPad Instat™ software.

RESULTS AND DISCUSSION

The effect of chlorinated water as a sample matrix on sELISA performance is shown in Figure 1. Chlorinated (0–5 mg/L chlorine) water samples, with and without sodium thiosulfate, were spiked with approximately 10^6 cells/mL immediately prior to being added to sELISA plates. Fluorescence intensity was reduced 86% by 5 mg/L chlorine ($p < 0.001$), but not significantly by 0.01, 1, and 2 mg/L chlorine. Sodium thiosulfate reversed the negative effect of 5 mg/L chlorine. Chlorine's adverse effect on sELISA was, in part, due to increased background fluorescence levels (0 mg/L: 1239 $\pm$ 63 and 5 mg/L: 11305 $\pm$ 1031, $p < 0.001$). Sodium thiosulfate negated this effect (1234 $\pm$ 93). Since the presence of chlorine can interfere with assay performance, further experiments were done with bacteria that had been harvested and washed with PBS prior to sELISA.

The effects of bacterial incubation in chlorinated water on sELISA performance were studied using bacteria resuspended in PBS. Net fluorescence intensity versus total (live + dead) cell concentration for the MA isolate, as a function of a 1 h incubation in chlorinated water, is plotted in Figure 2. Incubation in 0.1 or 2 mg/L chlorine had no effect on the linear

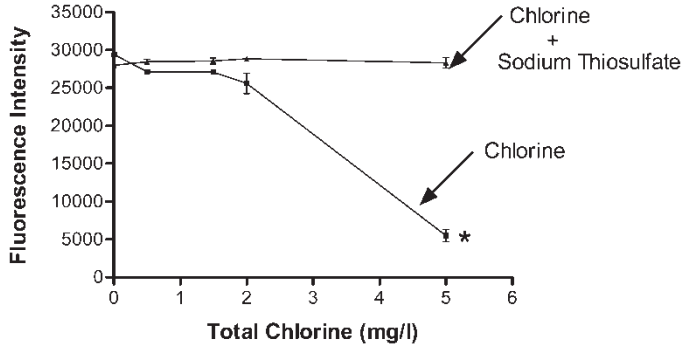

Figure 1. Fluorescence intensity of *E. coli* O157:H7 (MA isolate; 10^6 colony forming units/ml), determined by sandwich ELISA in presence of various concentrations of chlorine with and without sodium thiosulfate. Values are mean $\pm$ standard deviation of triplicate readings. $*$ indicates $p < 0.001$ chlorine versus chlorine with sodium thiosulfate.

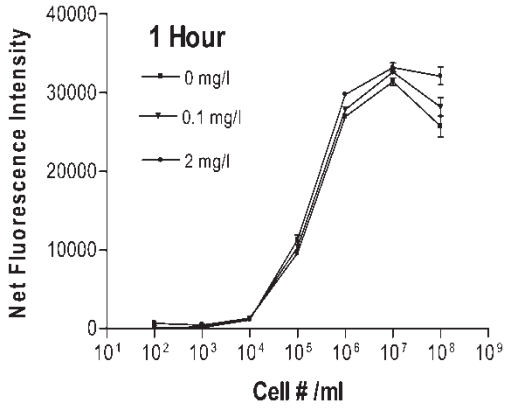

Figure 2. Net fluorescence intensity, determined by sandwich ELISA, after a 1 h incubation of *E. coli* O157:H7 (MA isolate) in 0–2 mg/L free chlorine. Cell number per ml was determined by microscopic cell count. After incubation, cells were harvested, washed and serially diluted in buffer prior to sELISA and cell counts. Net fluorescence intensity was calculated by subtracting the fluorescence intensity of samples without bacteria from samples containing bacteria. Values are expressed as means $\pm$ standard error of the mean, n = 6 replicates.

range of detection (10^4-10^7 total cells/mL). The net fluorescence intensity for 10^5 total cells/mL was elevated by the 2 mg/L chlorine treatment ($p < 0.01$, $n = 6$). Net florescence intensity values were 9629 ± 267 (0 mg/L), 10363 ± 101 (0.1 mg/L) and 11919 ± 132 (2 mg/L). Similar results were found for the ATCC isolate (Table 1).

The effects of 72 h and 7 day incubations in autoclaved, molecular grade water with 0–2 mg/L chlorine on sELISA detection of *E. coli* O157:H7 were investigated using the ATCC isolate. The sELISA linear range of detection (10^4-10^7 total cells/mL) was not altered by the 72 h chlorine treatment (Figure 3a), but the net florescence intensity for the 10^5 total cells/mL was increased approximately 10% by the 2 mg/L chlorine (Table 1). The 72 h

Table 1. Effect of bacterial incubation in chlorinated water on sandwich ELISA detection of *E. coli* O157:H7 (ATCC #43894)

	Net fluorescence intensity at 10^5 cells/ml		
Incubation time	0 mg/L chlorine	0.1 mg/L chlorine	2 mg/L chlorine
1 hour	26,725 $\pm$141	27,801 $\pm$ 320	29,748 $\pm$ 385[b]
72 hours –	26,405 $\pm$ 215	29,411 $\pm$ 283[b]	29,300 $\pm$ 499[b]
7 day	24,828 $\pm$ 200[#]	25,909 $\pm$ 228[#]	26,761 $\pm$ 278[a,#]

[a]$p < 0.01$ versus 0 mg/L; [b]$p < 0.001$ versus 0 mg/L; [#]$p < 0.01$ 1 h versus 7 days.

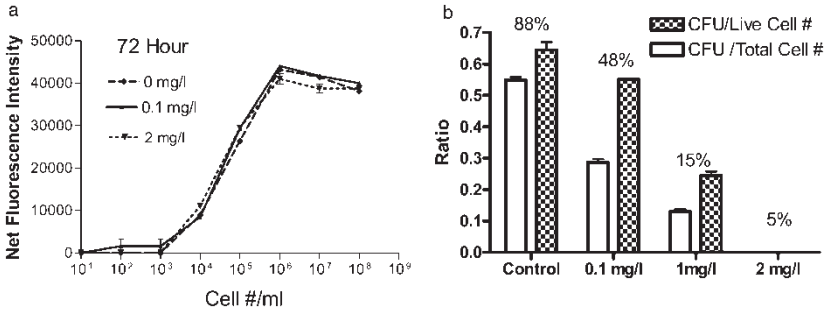

Figure 3. a) Net fluorescence intensity, determined by sandwich ELISA as a function of bacterial concentration, after incubation of the ATCC 43894 isolate for 72 hours in 0, 0.1, or 2 mg/mL chlorine in filter-sterilized, molecular grade water. Cell number per mL was determined by microscopic cell count. After incubation, cells were harvested, washed, and serially diluted in buffer prior to sELISA and cell counts. Net fluorescence intensity was calculated by subtracting the fluorescence intensity of samples without bacteria from samples containing bacteria. Values are expressed as means $\pm$ standard error of the mean, n = 6 replicates. b) Ratio of colony forming units to microscopic cell number after a 72 hour incubation in 0–2 mg/L chlorinated water. Total cell number and percent live cells were determined by the microscopic method outlined in "Methods" and colony forming units were determined by plating on tryptic soy agar. Values are expressed as means $\pm$ standard error of the mean, n = 6 replicates. Statistical significance is noted by $^*p < 0.001$ versus 0 mg/L chlorine.

incubation in chlorinated water reduced the ratio of colony forming units per number of live and total cells as well as the percent live cells (Figure 3b). For example, 1 mg/L chlorine reduced the percent live cells from 88% to 15% ($p < 0.001$) and the ratio of CFU/live cells from 0.65 ± 0.02 to 0.28 ± 0.008 ($p < 0.001$). At 2 mg/L chlorine, there were no detectable CFU/mL and only 5% of the cells were determined to be viable by the microscopic technique. It would appear that chlorinated samples contained culturable, viable but non-culturable, and dead cells. Since the net fluorescence intensity per total cell concentration was not reduced by chlorine incubation, the present sELISA is able to detect injured/dead cells as well as those that are culturable. Incubation for 7 days in sterile water, with and without chlorine, did not alter the linear range of detection (10^4–10^7 total cells/mL). Moreover, the net fluorescence intensity for 10^5 total cells/mL was 8% higher for the chlorine-treated versus non-treated cells (Table 1). Interestingly, the net fluorescence intensity for 10^5 total cells/mL was reduced approximately 8% by day 7 for all samples (Table 1).

Experiments were undertaken to determine if *E.* coli O157:H7 sELISA can be used in conjunction with Colitag^{TM} coliform water test system, which can detect fecal coliforms simultaneously with *E. coli* in 24 h. Bacteria were suspended in molecular grade water or tap water prior to

incubation in the enrichment medium for 24 h, after which colorimetric detection was performed and aliquots were removed for sELISA. Background fluorescence intensity was higher for the enrichment medium (6985 $\pm$ 199) compared to PBS (1300 $\pm$ 155 $p < 0.001$, $n = 6$). Even with the increased background, approximately 3 original CFU/mL were detected by sELISA for bacteria suspended in molecular grade water prior to enrichment (Table 2). In contrast, the colorimetric reaction was able to detect 3×10^3 original CFU/mL. Tap water incubation prior to enrichment resulted in a drastic loss of cells with detection of 3×10^6 original CFUs/mL by the colorimetric reaction and 3×10^3 original CFU/ml by sELISA.

Taken together, these results indicate that chlorine exposure does not harm the sELISA detection of *E. coli* O157:H7. Indeed, there was a slight increase in fluorescence intensity per cell number after chlorine exposure, although the linear range of detection was not altered. Interestingly, starvation for 7 days caused a small reduction in fluorescence intensity per cell number, but this 8% change did not affect the linear range of detection, suggesting that starvation would not be an issue for bacterial detection. Importantly, the present sELISA can be dovetailed with a recently EPA-approved fecal coliform and *E. coli* indicator kit. Indeed, very low levels of bacteria could be detected by sELISA after this procedure. The major adverse finding was that sampling directly from chlorinated water reduced fluorescence intensity, in part because of an increased background. However, chlorinated water could be sampled directly if sodium thiosulfate was added. The sELISA in these experiments employs a commercially-available antibody that has been successfully used in many applications,[13,14] including biosensor assays.[10–12]

Our results indicate that the present sELISA is a good method for detecting *E. coli* O157:H7 under starvation and chlorine-stress conditions, where cells may be viable but not culturable. Thus, sELISA may be a necessary addition to conventional methods of enumeration of *E. coli* O157:H7, such as viable counts on selective media, which may not accurately determine the concentration of viable cells following exposure to chlorine because bacteria may become non-culturable after sub-lethal chlorine injury.[8,19–21] In the present research, chlorination, especially at higher doses, reduced the ratio of colony forming units to total cells measured using the *Bac*light™ method, but a subset of cells was found to be viable by this microscopic technique. Chlorination of water supplies is typically effective, but ineffective treatments do occur.[3,5] Moreover, chlorine residual can dissipate under certain conditions,[5] and sub-lethal starvation and chlorine-induced injury is a concern for recreational waters[22] where there is less control over factors such as organic material that can adversely affect chlorine disinfection.[23] Since the 1970s, there have been reports of chlorine injured bacteria but, whether these bacteria are virulent is controversial.[8,19,24] *E. coli* O157:H7 can persist for days to weeks in microcosms during starvation conditions.[24] Starvation and sub-lethal chlorine injury can also be encountered in food processing.[25–27] Moreover, certain

Table 2. Effect of prior incubation in Colitag™ water test system on sandwich ELISA detection of *E. coli* O157:H7 (ATCC #43894) as a function original colony forming unit/ml

	Net fluorescence intensity					
CFU/ml	3.0×10^4	3.0×10^3	3.0×10^2	3.0×10^1	3.0	0
MG water	27235 ± 883^c	27319 ± 415^c	9465 ± 235^b	8119 ± 118^c	7892 ± 152^a	5433 ± 199
Tap water	7848 ± 200^b	7673 ± 398^b	6388 ± 302	5933 ± 224	5693 ± 224	5433 ± 305

[a]$p < 0.05$ compared to background; [b]$p < 0.01$ compared to background; [c]$p < 0.001$ compared to background; MG = molecular grade water.

isolates of *E. coli* O157:H7 have higher resistance to chlorine[23,28] and starvation causes the development of a chlorine resistant phenotype.[24] Under proper conditions of temperature and nutrients, injured organisms may be able to repair sub-lethal injury.[22,29] Until the controversy of whether stressed cells are viable is settled, it is important to utilize a methodology that can detect non-culterable *E. coli* O157:H7 if bacterial enumeration must be conducted after chlorine exposure.

In contrast to the present work with chorine, other investigations indicated differences in immunoassay performance for bacteria grown under various conditions. We reported a loss of assay sensitivity for acid-stressed *E. coli* O157:H7, but not for acid-adapted bacteria[14] or apple juice-exposed bacteria.[13] A loss of O antigenicity, measured by agglutination of *E. coli* O157:H7, was seen when *E. coli* O157:H7 was grown under starvation conditions for months.[17] Most importantly, the lost O antigenicity was not recovered by enrichment. Composition of the enrichment broth has been reported to alter immunomagnetic separation[30] and ELISA sensitivity.[31] However, cold stressing of bacteria in cheese did not alter automated-enzyme-linked florescent immunoassay performance,[32] and freeze-thawing spiked ground beef did not reduce sensitivity of a solid phase fluorescent immunoassay.[33] Selective enrichment media, as well as stress (e.g., acid, cold, heat, and salt), may reduce the detection of *Listeria monocytogenes*, due to the differential expression of surface proteins.[16] The KPL polyclonal antibody used for our studies binds to the O antigen of the lipopolysaccharide layer and not to proteins (unpublished data). Thus, the difference between the various studies may relate to the antibody target with LPS components being more consistently expressed than certain surface proteins. In support of this hypothesis, a study of *E. coli* O157:H7 in water found no major alterations in LPS pattern after starvation although outer membrane proteins did change.[34]

Our results found that chlorinated water as a sample matrix was detrimental to sELISA. Other sample matrices, such as apple juice[13] and raw surface water[30] have a similar effect on antibody-based assays. Additives, such as sodium thiosulfate, used to neutralize chlorinated samples in this paper, and polymers, such as polyvinylpyrrolidone and polyvinylpolypyrrolidone, used to amend apple juice and raw surface water, respectively, in previous studies,[13,30] may be helpful in negating the effects of substances that interfere with immunoassays and immunomagnetic separations. In contrast, there appeared to be few matrix effects with immunological lateral diffusion assays using source and finished water samples.[35]

CONCLUSIONS

Our results show that sELISA can be a useful method to detect stressed *E. coli* O157:H7 in water sources, as long as sample matrices do not interfere with

assay performance. Moreover, enrichment in the EPA-approved Colitag™ medium could be dovetailed with the present sELISA. The results support the conclusion that combined chlorine and starvation injury does not alter the epitopes necessary for antibody detection of this pathogen.

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