Comparison of methods for the enumeration of *Clostridium perfringens* spores in water

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ABSTRACT

Four methods for enumerating *Clostridium perfringens* spores in water were evaluated: (1) the IMM (Iron Milk Medium) method (MPN); (2) the LS (Lactose Sulfite Broth) method (MPN); (3) the m-CP (membrane filtration *Clostridium perfringens* Agar) method (membrane filtration); and (4) the TSC (Tryptose Sulfite Cycloserine Agar) method (membrane filtration). The performance of these methods was compared with that of the DRCM (Differential Reinforced *Clostridium* Medium) method (MPN) as adopted by CETESB (Brazil's Environmental Sanitation Technology Company) for the analysis of *C. perfringens* spores in water. Statistical analysis was performed according to ISO 17994:2004 (Water Quality – Criteria for Establishing Equivalence between Microbiological Methods). The LS, m-CP, and TSC methods were considered not equivalent to the DRCM method, as they gave significantly lower results. The IMM showed inconclusive results and, according to ISO 17994:2004, analysis of a greater number of samples is needed to draw definitive conclusions comparing IMM and DRCM.

Key words | analytical methods, *Clostridium perfringens*, spore count, water

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INTRODUCTION

Clostridium perfringens is a sulfite-reducing anaerobic spore-forming bacterium widely found in nature and is generally regarded as a part of the natural microbiota of the human and animal intestinal tract (Labbe 2001; Juneja *et al.* 2003). The conditions prevailing in the intestinal tract are optimal to induce sporulation of vegetative cells, as opposed to what is observed in culture media (Labbe 1980). Spores are excreted from the body with the feces and subsequently reach water bodies. In aquatic environments, spores exhibit exceptional longevity as a result of their great resistance to adverse environmental conditions. For that reason, their use as indicators of water quality has been the subject of several studies. *Clostridium perfringens* spores are useful for the detection of remote fecal contamination in situations in which other, less resistant, indicators, such as *Escherichia coli* and fecal streptococci would not have survived (Payment & Franco 1993; Medema *et al.* 1997; Payment 1999; Lanao *et al.* 2010). Several methods have been used to enumerate *C. perfringens* in water.

The DRCM method was proposed by Gibbs & Freame (1965) and specified by ISO 6461-1 (1986) as a method for the detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) in water. The DRCM method is a multiple tube fermentation technique which estimates the bacterial density or most probable number of bacteria in a sample. The sample is inoculated into DRCM broth (Differential Reinforced *Clostridium* Medium), a selective differential enrichment medium for sulfite-reducing clostridia. The differential characteristics are conferred by the sodium sulfite and ferric citrate. Reduction of the sulfite results in sulfide, which reacts with the iron salt forming iron sulfide (darkening or blackening of the culture medium). In Brazil, it was adopted by the Environmental Company of São Paulo State – CETESB (1993) for the analysis of *C. perfringens* in water with a confirmation step in Litmus Milk, a medium in which *C. perfringens* causes 'stormy fermentation' – a typical characteristic of the species. Fermentation of the milk results in the coagulation of the proteins and the production of an abundance of gas that tears the acid clot apart. In addition, the acid produced during fermentation also causes the color of the pH indicator litmus to turn from blue to pink. The DRCM method

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is simple in that it requires the use of two culture media that are both readily commercially available. On the other hand, the method is relatively slow as in the presumptive test, DRCM is incubated at 35 °C for 48 h and, in the confirmation test, litmus milk is further incubated at 35 °C for another 48 h. In addition to the DRCM, there are other methods for the enumeration of *C. perfringens* in water.

The IMM method was proposed by St. John *et al.* (1982) for the enumeration of *C. perfringens* in soil, sludge and water. It is a multiple tube fermentation method for determining the most probable number. Samples are inoculated into IMM (Iron Milk Medium), a culture medium composed of milk supplemented with iron as reducing agent. After incubation at 45 °C/16–18 h, the tubes exhibiting stormy fermentation of the milk are considered confirmed. The IMM Method is, at the same time, extremely simple, fast and inexpensive, as it does not require any confirmatory testing and the culture medium consists of only milk and elemental iron powder. It was adopted by the AOAC International in 1993 (AOAC Official method 993.10) for the MPN count of *C. perfringens* in shellfish, but included a confirmatory step of acid phosphatase detection, as described in the m-CP method below: from each IMM tube exhibiting stormy fermentation, a single line is streaked on a membrane filter placed on m-CP agar plates. After incubation at 45 °C/24 h in an anaerobic jar, the plates are exposed to ammonium hydroxide vapor, to check for acid phosphatase reaction. The results of the interlaboratory study is reported by AOAC: Oysters, 10^3 *C. perfringens* cells/g: Log average, 3.76, $s_r = 0.18$, $s_R = 0.46$, $RSD_r = 4.9\%$, $RSD_R = 12.2\%$. Oysters, 10^4 *C. perfringens* cells/g: Log average, 4.61, $s_r = 0.30$, $s_R = 0.33$, $RSD_r = 6.62\%$, $RSD_R = 7.2\%$. Oysters, 10^6 *C. perfringens* cells/g: Log average, 5.59, $s_r = 0.29$, $s_R = 0.31$, $RSD_r = 5.2\%$, $RSD_R = 5.5\%$.

The LS method was proposed by Beerens *et al.* (1984) for the enumeration of *C. perfringens* in food and feces and was further adapted and used by Bezirtzoglou *et al.* (1994) for analyzing water samples from different aquatic environments. The LS method is a multiple tube fermentation method for determining the most probable number. Samples are inoculated into LS Broth (Lactose Sulfite Broth), a selective differential enrichment medium for sulfite-reducing clostridia. The differential characteristics are conferred by sodium metabisulfite, iron citrate and lactose. Reduction of the sulfite produces sulfide, which in turn reacts with iron, forming iron sulfide (black) and causing blackening or darkening of the culture medium. Fermentation of the lactose results in the production of gas, which is collected in Durham tubes. Incubation is performed at 46 °C for 24 h and the tubes exhibiting darkening of the culture medium along with abundant gas production are considered confirmed positive for *C. perfringens*. In the opinion of the authors, there is no need for additional tests to confirm the identity of the cultures isolated, however, the comparative studies available to date were conducted with feces and foods only and, consequently, there are no comparative results for the analysis of water. Although LS Broth is not commercially available, the method is very simple (no confirmatory tests needed) and fast (requiring only 24 h of incubation).

The m-CP method was described by Bisson & Cabelli (1979) and modified by Armon & Payment (1988) for the enumeration of *C. perfringens* in surface water and water intended for human consumption. It is a membrane filtration technique that uses m-CP Agar (membrane filtration *Clostridium perfringens* Agar) onto which the samples are plated. M-CP agar is a selective differential medium that allows us to verify three typical characteristics of *C. perfringens*: (1) the fermentation of saccharose, evidenced by the change in color of the indicator bromocresol from purple to yellow; (2) lack of β-glucosidase enzyme activity, as *C. perfringens* does not cleave or hydrolyze the chromogenindoxyl-β-D-glucoside added to the medium and, consequently, maintains the characteristic opaque yellow color that the colonies acquire upon acidification; (3) acid phosphatase activity, through the addition of phenolphthalein diphosphate which is hydrolyzed or cleaved by acid phosphatase, releasing phenolphthalein. When the dishes are exposed to ammonium hydroxide vapor, the *C. perfringens* colonies turn red or dark pink as the phenolphthalein is cleaved by the acid phosphatase.

The TSC method is widely used in food analysis (Byrne *et al.* 2008). Its use for the examination of water, in conjunction with the membrane filtration technique, was proposed and evaluated by Sartory (1986) and Sartory *et al.* (1998). A volume of sample is filtered and the membrane filter placed on the surface of the TSC Agar (Tryptose Sulfite Cycloserine), with the inoculated grid-side facing down. Next, the membrane filter is covered with an overlay of the same medium to enhance the anaerobic conditions, as described by Burman *et al.* (1969) and incubated under anaerobic atmosphere at 45 °C/24–48 h. After incubation, sulfite-reducing clostridia (including *C. perfringens*) typically produce black colonies as a result of the reduction of sulfite to sulfide which reacts with the iron and precipitates in the form of iron sulfide. All black colonies on TSC are counted as presumptive *C. perfringens* and a percentage must be

subjected to the same biochemical confirmation tests used in food analysis.

Although commonly used, there is little information in the literature comparing the performance of these methods. In this context, the main objective of the study was to carry out an initial comparison among the methods, using a limited number of samples. The ISO 17994 statistical protocol was used to analyze the data because it includes a first stage of comparison, which is planned to detect large differences between methods. If the methods happen to differ markedly, a small number of samples might suffice to determine this fact. If large differences are not found (result inconclusive), a second stage should be carried out with more samples, until the system is able to detect difference or equivalence between the methods.

MATERIAL AND METHODS

Samples used in the assays

In this study, a total of 48 water samples were evaluated: 28 samples of treated water, provided by SANASA (Water Supply and Sanitation Company of Campinas, SP – Sociedade de Abastecimento de Água e Saneamento S/A de Campinas, SP) and 20 samples of naturally contaminated raw water collected from the Atibaia and Capivari rivers (Campinas, SP). Each sample was heat treated (70 °C/15 min) to kill vegetative bacteria. The heat treated water samples were individually analyzed for counts of *C. perfringens* spores, with five repetitions, using the five methods in parallel totalizing 25 tests per sample.

Description of the methods for enumerating *C. perfringens* spores investigated

The DRCM method (MPN)

The assay was carried out as described by ISO 6461-1 (1986). For the examination of treated water, ten 10 mL aliquots of each sample were dispensed or inoculated into tubes containing 10 mL of double-strength DRCM Broth (Differential Reinforced *Clostridium* Medium Merck 1.11699). For the analysis of raw water, five 1 mL aliquots, five 0.1 mL aliquots and five 0.01 mL aliquots of each sample were inoculated into tubes containing 10 mL of single-strength DRCM broth. The tubes were incubated at 35 °C/48 h and those showing blackening of the medium were subjected to confirmation as described by CETESB (1993). For that purpose, a small amount of the culture was inoculated into Litmus Milk (BBL 211343) and incubated at 35 °C/48 h. The cultures showing acidification (development of a pink color) and stormy fermentation of the milk were counted as confirmed *C. perfringens*.

The IMM method (MPN)

Following the same procedure described for the DRCM method, sample aliquots were inoculated into tubes containing Iron Milk Medium (IMM) (double strength for 10 mL aliquots and single strength for 1 mL or smaller aliquots). Next, the tubes were incubated at 45 °C for 18 h and the tubes showing stormy fermentation of the milk medium were considered confirmed as *C. perfringens*. The IMM medium was prepared according to Abeyta *et al.* (1985): 10 mL of (commercial) whole milk powder reconstituted at 10% for single strength or 20% for double strength were dispensed in tubes and added with 0.2 g elemental iron powder. The tubes were then sterilized at 116 °C for 10 min. The sterilized medium was used no longer than two hours after its preparation.

The LS method (MPN)

Following the same procedure described for the DRCM method, sample aliquots were inoculated into tubes containing Lactose Sulfite Broth (LS) (double strength for 10 mL aliquots and single strength for 1 mL or smaller aliquots). Next, the tubes were incubated at 46 °C for 24 h and the tubes exhibiting blackening of the culture medium were counted as confirmed *C. perfringens*. The LS broth was prepared according to Beerens *et al.* (1984): the base of the medium – composed of 5 g/L casein peptone (tryptic digest), 2.5 g/L yeast extract, 2.5 g/L sodium chloride, 10 g lactose and 0.3 g/L cysteine chlorhydrate (cysteine HCl) – was adjusted to pH 7.1 ± 0.1, dispensed into tubes (9 mL/tube) containing Durham tubes, sterilized at 121 °C for 15 min and stored under refrigeration. Immediately before use, each tube was added with 0.5 mL each of the following newly prepared aqueous solutions sterilized by membrane filtration: anhydrous sodium metabisulfite 1.2% and ferric ammonium citrate 1%.

The m-CP method (CFU per volume of membrane filtered sample)

For the analysis of treated water, 100 mL of each water sample was passed through a cellulose acetate-based membrane filter, 0.45 μm (Millipore) nominal pore size. For the examination of raw water, 5 mL of each sample was filtered. The membrane filters were transferred to mCP Agar Petri

dishes, prepared as described by Armon & Payment (1988), and subsequently incubated under an anaerobic atmosphere at 45 °C for 24–48 h. Upon completion of incubation, the plates containing yellow colonies were further subjected to confirmation testing. For that purpose, the plates were exposed to ammonium hydroxide vapor and the colonies showing phosphatase activity (changing color to red or dark pink) were submitted to the gelatinase test. For that purpose, the membranes were transferred onto gelatin culture medium plates and those found capable of hydrolyzing gelatin were confirmed as *C. perfringens*.

The TSC-YE method (CFU per volume of membrane filtered sample)

Each sample was filtered as described previously for the mCP method, and the membrane filters were transferred to TSC-YE Agar Petri plates (Tryptose Sulfite Cycloserine Agar Base MERCK 1.11972, supplemented with 0.04% D-cycloserine and 0.5% yeast extract). The membrane filters were carefully rolled onto the medium with the inoculated grid-side facing down. Next, the membrane filters were covered with an overlay of the same culture medium and the plates incubated anaerobically at 45 °C for 24–48 h. Typical *C. perfringens* colonies (black) were subjected to further confirmation testing using the lactose gelatin and the nitrate motility tests described by Labbe (2001): for the lactose fermentation and gelatin liquefaction tests, each culture was inoculated into Lactose–Gelatin Medium by stabbing, incubated at 35 °C/44 h and examined for color change from red to yellow (lactose fermentation positive) and gelatin solidification after chilling at 5 °C/2 h (gelatin not solidified considered positive reaction for gelatin liquefaction). For the motility test and nitrate reduction test, each culture was inoculated into Motility-Nitrate Medium by stabbing, incubated at 35 °C/24, examined for motility (growth only along the line of inoculum and not diffused away from the stab considered negative motility) and for nitrate reduction, adding 0.5 mL each of nitrate test reagent (0.8% sulfanilic acid solution and 0.5% α-naphthol solution) to each culture (an orange color indicates a positive reaction). The cultures testing negative for motility and positive for nitrate reduction, lactose fermentation and gelatin liquefaction were confirmed as *C. perfringens*.

Statistical analysis

Statistical analysis was performed in accordance with ISO Standard 17994 (2004), which defines the criteria for establishing equivalence between methods for microbiological analysis of water.

The DRCM method was used as a reference method and the other four methods as test methods. The MPN based methods (LS an IMM) and the colony count methods (m-CP and TSC-YE) were compared with the MPN DRBC method in the same manner. The ISO 17994:2004 allows the comparison between two quantitative methods based on different principles (MPN or colony counting), varying the number of samples required to achieve a conclusion. But in the first stage of comparison, which was the objective of this study, the number of samples is not a critical parameter.

The results of each method were compared one by one to the results obtained by the reference method. The parameters used for the methods comparison were calculated according to the following guidelines:

Calculation of the relative difference between the counts (RD): each count was transformed to its natural logarithm (Ln) and the value yielded by the reference method was subtracted from the value obtained by the test method and multiplied by 100 to obtain the RD. *Calculation of the mean relative difference* ($\bar{x}$): was calculated by adding the relative differences of all the samples and dividing by the number of samples. *Calculation of the standard deviation (s)*: the standard deviation of the RD values was calculated using Microsoft Excel Excel (desvpad). *Calculation of the standard uncertainty of the mean* ($s\bar{x}$): the standard uncertainty of the mean was calculated by dividing the standard deviation by the square root of the number of samples ($s\bar{x} = s/\sqrt{n}$). *Calculation of the expanded uncertainty (U)*: the expanded uncertainty was calculated by multiplying the standard uncertainty of the mean by a factor $K = 2$. The K value used was two because the distribution of the relative differences was not expected to be normal. *Calculation of the confidence interval of the relative difference at 95%*: the two extremes (x_H and x_L) of the confidence interval were calculated by adding and subtracting the expanded uncertainty to and from the mean relative difference ($x_H = \bar{x} + U$ and $x_L = \bar{x} - U$). *Interpretation of the results and conclusion:* to interpret the results, the ISO 17994 one-sided evaluation was used and the D value was set at 10%, as recommended by ISO 17994 for methods of analysis of drinking water.

RESULTS AND DISCUSSION

None of the 28 treated water samples that were analyzed by the five methods evaluated has a positive test result for *C. perfringens*.

Negative results for treated samples were expected and the ISO 17994:2004 orientation for samples was confirmed to be negative (zero count) by the tested method and the reference method were excluded from the statistical calculations, but were reported in the results nevertheless. Although excluded from the statistical analysis, it was important to show the agreement among the methods for negative results.

Table 1 | Spore counts of *Clostridium perfringens* in the samples analyzed by the five methods investigated in this study

	Counts in Ln (mean of the repetitions)				
Sample	DRCM (MPN/ 100 mL)	IM (MPN/ 100 mL)	LS (MPN/ 100 mL)	TSC-YE (CFU/ 100 mL)	m-CP (CFU/ 100 mL)
1	6,854	ND*	ND*	5,919	4,754
2	6,649	5,869	6,633	ND*	ND*
3	6,588	6,486	5,375	4,997	5,781
4	7,993	7,740	4,754	6,001	ND*
5	7,212	7,090	6,153	6,118	6,377
6	6,816	7,147	3,912	6,328	5,670
7	6,617	7,505	2,996	5,429	ND*
8	5,472	7,170	4,754	6,328	6,370
9	5,268	5,717	4,407	3,689	4,828
10	6,474	7,449	5,656	3,555	4,500
11	ND*	ND*	ND*	5,011	3,750
12	7,313	6,263	6,736	ND*	ND*
13	6,203	5,236	5,903	5,050	5,024
14	6,837	5,961	3,807	4,787	ND*
15	5,663	5,384	3,481	4,317	5,545
16	5,920	4,927	3,689	4,820	2,996
17	5,737	6,474	3,481	4,554	ND*
18	4,820	6,677	5,897	5,924	6,114
19	4,898	5,684	5,159	3,555	4,159
20	5,951	5,775	5,347	3,401	3,555
Média	6,278	6,364	4,897	4,988	4,959

MPN = most probable number, CFU = colony forming unity, *ND = not determined.

The presence of *C. perfringens* was detected in 20 samples of raw or untreated water examined by the five methods evaluated in this study. The spore count results are shown in Table 1. The values for each sample/test method combination are the mean of the repetitions, previously transformed to the Naperian logarithm of the MPN/100 mL. The statistical analysis and conclusions, based on ISO Standard 17994 (2004) are displayed in Table 2.

The DRCM and IMM methods yielded similar counts, which were clearly superior to those obtained by the LS, TSC-YE and m-CP methods (Table 2).

Statistical analysis conducted as described in ISO 17994 (2004) guidance (Table 2) corroborated and confirmed these results: the LS, TSC-YE and m-CP methods were not considered to be equivalent to the DRCM method with the latter having a clearly superior performance.

Recently, Byrne *et al.* (2008) conducted an evaluation study on cultural agar media for *C. perfringens* vegetative cells and spores and also found that Reinforced Clostridial Agar (RCA), similar to DRCM, was a significantly better medium for recovering thermally treated and untreated *C. perfringens* spores compared with TSC and six other agar media.

The IMM method on the other hand, though not rejected by statistical analysis, yielded inconclusive results. This means that a second stage of comparison as recommended by ISO 17994 with a larger number of samples is required in order to make it possible to establish equivalence or not with the DRCM method. For two MPN methods comparison,

Table 2 | Conclusion based on the validation parameters calculated as described by ISO 17994 (2004) for each test method, and compared with the DRCM method

Parameter	DRCM × IM	DRCM × LS	DRCM × TSC-YE	DRCM × m-CP
Number of samples considered in statistical analysis (n)	19	19	18	14
Mean relative difference ($\bar{x}$)	11,640	−135,119	−121,314	−98,468
Standard deviation (s)	87,840	128,672	98,820	117,825
Standard uncertainty of the mean ($s\bar{x} = s/\sqrt{n}$)	20,152	29,519	23,292	31,490
Expanded uncertainty (U)	40,304	59,039	46,584	62,980
Upper limit ($x_H = \bar{x} + U$)	51,944	−76,080	−74,730	−35,489
Lower limit ($x_L = \bar{x} - U$)	−28,663	−194,157	−167,898	−161,448
Conclusion ($D = 10\%$)	Inconclusive	Different methods DRCM better	Different methods DRCM better	Different methods DRCM better

the number (n) of samples depends on the number (m) of parallel tubes according to the equation: $n = 1{,}700/m$. With five parallel tubes per dilution, 340 samples (1700/5) should suffice for the detection of a 10% relative difference.

In a complementary study for the IMM method, it is important to confirm the positive IMM tubes as *C. perfringens*, as the method adopted by the AOAC International for shellfish includes a confirmatory step.

According to St. John *et al.* (1982), the IMM method is very simple and fast as it does not require confirmation. In his study, all the cultures showing stormy fermentation of the milk, under the conditions of the assay, were confirmed as *C. perfringens*. In addition, the IMM method is much less expensive and laborious as the culture medium is plain commercial sterilized milk.

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