Evaluation of fluorogenic TSC agar for recovering Clostridium perfringens in groundwater samples

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Abstract Clostridium perfringens is widely recognised as a reliable water pollution indicator. Since several media can be employed for the membrane filtration enumeration of this microorganism, the main aim of this work was to investigate the ability of fluorocult-supplemented TSC-agar (Merck) for recovering Cl. perfringens from public springs used for direct human consumption. Cl. perfringens recovery was also performed on mCP agar (Cultimed) according to Directive 98/83 as well as on TSC-Agar (Merck), TSN-Agar (Merck) and SPS-Agar (BBL) media. Variance analysis of data obtained showed no statistically significant differences in the counts obtained among all media employed in this work. However, the Cl. perfringens recovery efficiencies with TSC and fluorogenic TSC agars were significantly greater (P = < 0.05) than the corresponding values of mCP and TSN media. On the other hand, the identification of typical and atypical colonies isolated from all media demonstrated that fluorogenic TSC agar was the most specific medium for Cl. perfringens recovery in groundwater samples (85.3% of typical colonies and 82.8% of atypical colonies confirmed). In summary, the membrane filtration technique with fluorogenic TSC agar showed the best performance characteristics of all the media tested as judged by their recovery efficiency and specificity in these water samples.

Keywords Clostridium perfringens, membrane filtration, fluorogenic medium, water

Introduction Sulphite-reducing clostridia have been used as indicators of faecal pollution for many years. This group shows the important advantage that their spores are more resistant to conditions in water environments, as well as to treatment and disinfection processes than most pathogens including viruses (Grabow, 1990; Payment and Franco, 1993). One of the members of the group, Clostridium perfringens, is highly specific for faecal pollution (Grabow, 1996), and, according to Payment and Franco (1993), is the most reliable indicator for viruses and protozoan (oo)cysts in treated drinking water. Also, it has been reported that Cl. perfringens could be a suitable indicator for the presence of pathogens of faecal origin in surface waters (Sorensen et al., 1989; Payment and Franco, 1993). Current Spanish legislation for monitoring the quality of water for human consumption requires the determination of sulphite-reducing clostridia spores instead of Cl. perfringens. Nevertheless, the new European Guideline 98/83 (EU, 1998) for this kind of water (not transcribed to the Spanish legislation as yet) has adopted this microorganism as the one to be determined in this kind of water. Furthermore, the European Directive stipulates mCP agar as the reference method for this microbiological parameter. This is a complex and expensive medium, the ability of which to recover Cl. perfringens from some water types is still under discussion (Burger et al., 1984; Sartory, 1986; Sartory et al., 1998). Fluorocult-supplemented TSC-agar (Merck) is a TSC agar base containing D-cycloserine and 4-methylumbelliferylphosphate (MUP) disodium salt. MUP is a fluorogenic substrate for acid phosphatase being a highly specific indicator for Cl. perfringens (Schallehn and Brandis, 1973). In view of the increasing legal relevance of this microorganism and, overall, the discrepancies that still persistent in the literature about the best procedure for its enumeration, a comparison of the analytical
performance of different media, including this fluorogenic agar, for *Cl. perfringens* enumeration was carried out.

**Materials and methods**

**Media**

The following media were used: mCP agar (mCP) prepared as specified in European Directive 98/83 (Cultimed, Spain); TSC was prepared from Tryptose Sulfite Cycloserine Agar Base (Merck, Germany) and D-cycloserine (Fluka Chemika, USA); TSCF was prepared from Tryptose Sulfite Cycloserine Agar Base (Merck) and fluorocult TSC-Agar supplement (Merck); TSN was prepared from TSN Agar Perfringens Selective Agar acc. to Marshall (Merck); SPS was prepared from SPS (Sulfite Polymyxin Sulfadiazine) Agar (BBL, USA).

**Analysis of samples**

Groundwater samples (5 l) from public springs used for direct human consumption in rural and urban area of Santiago de Compostela (Spain) were collected in glass bottles, refrigerated and analysed within 24 h. Aliquots (100 mL) were filtered through 47 mm Whatman WCN 0.45 µm pore size membrane filters. Duplicates of these aliquots were placed onto mCP, TSC and TSCF, TSN and SPS media. All plates were incubated anaerobically at 44°C/18–24 h (EU, 1998) in a WA 6200 anaerobic cabinet (Heraeus, Germany). Yellow colonies on mCP which turned any shade of pink or red following a 30 s exposure to ammonia fumes and all black colonies on TSC, TSN and SPS were counted as presumptive *Cl. perfringens*. All black colonies on TSCF emitting light blue fluorescent after exposure to a UV lamp (Wood’s lamp) were also counted as presumptive *Cl. perfringens*. TSC agar base (Merck) without cycloserine was used as a non-selective reference medium. Typical and non-typical colonies of each plate were picked off for confirmation as *Cl. perfringens* based on the following tests: sulphite reduction and lactose fermentation in lactose sulphite broth (Neut et al., 1985), Gram stain, “stormy fermentation”, motility, gelatin liquefaction and nitrate reduction (FDA, 1995).

**Statistical analysis**

Results were analysed by linear regression, analysis of variance and Wilcoxon sign ranks test using Statgraphics software. In order to compare the different media and procedures, the criteria proposed by Levin and Cabelli and by El Shaarawi and Pipes were considered (see Catalao Dionisio and Borrego, 1995).

**Results**

38/51 samples tested gave presumptive positive results in at least one of the culture media used and most were positive in the five media assayed (Table 1). Thus, no measurable sensitivity differences could be detected among them. Variance analysis of the counts showed no statistically significant differences among all media employed. However, when this statistical test was applied to the values of the recovery efficiency of the five culture media, the recovery efficiency of the TSC and TSCF agars were significantly greater (P = <0.05) than the corresponding values of mCP and TSN media (Table 1).

Regression analysis gave support to this result (Figure 1) where the results plotted tended to bias to the Y-axis. Furthermore, Wilcoxon sign ranks test analysis demonstrated significant differences (P = <0.05) among results obtained with mCP or TSN agar media and those obtained on TSC, TSCF or SPS agar media. On the other hand, the identification of typical and atypical colonies isolated from all media demonstrated that TSCF agar was the more specific medium for *Cl. perfringens* recovery in groundwater samples (Table 1).
These values were greater than the corresponding ones obtained with the other media, namely with respect to the percentage of confirmed typical colonies.

Discussion

Our results gave further support to those authors who have previously shown that mCP medium is not the best choice for monitoring of *Cl. perfringens* in water (Sartory *et al.*, 1998). Leaving apart the discrepancies found in this work with results previously published (Burger *et al.*, 1984; Sartory, 1986; Sartory *et al.*, 1998), which certainly deserve further clarification, this work clearly showed that, with the water samples tested, there was no substantial reason for using mCP as a reference method (with legal implications!) all over the EU. At best, mCP produced the same results that could be obtained with other culture media already commercially available. For instance, our results showed that the *Cl. perfringens* recovery efficiency of TSCF was significantly greater (*P* = <0.05) than the...
corresponding values of mCP and TSN media. In addition, the TSCF specificity found in this study was superior to the other media most probably due to the incorporation of a fluorogenic substrate for acid phosphatase, a highly specific indicator for *C. perfringens* (Schallehn and Brandis, 1973). This improvement makes TSCF the medium of choice for recovering *C. perfringens* in groundwater, not only because of its excellent performance but also, because of the simplicity of use and price. So, having in mind the mCP complexity, price and cumbersome methodology of use (not to mention the hazards associated with the use of ammonia fumes), we wonder what were the actual reasons taken into consideration when this culture medium was selected as the reference. When progressively simpler, faster, more reliable and straightforward methodology is expected to be adopted in modern countries as the standard, we are forced to face the adoption of what can be only considered a backwards step. We wonder whether the time to review a Directive still not fully adopted by the member states, has already come.

**Conclusions**

Our results clearly show that there is no substantial reason for using mCP as the reference method for recovering *C. perfringens* from water samples as specified in European Directive 98/83. In this regard, a review of this Directive, not yet fully adopted by all member states, is proposed. This work also indicated that TSCF could be the medium of choice for recovering *C. perfringens* in groundwater, not only because of its excellent performance but also, because of the simplicity of use and cost especially when compared with mCP agar.

**References**


