Evaluation of four growth media for membrane filtration counting of *Clostridium perfringens* in water

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Abstract

Four agar growth media, tryptose-sulphite-cycloserine-egg yolk (TSC), TSC without egg yolk (TSC-EY), a polymyxin-containing medium (MCP), and Wilson and Blair glucose sulphite iron medium (WB), were compared for utilization in the membrane filtration counting of *Clostridium perfringens* in water. Comparative tests were done on tapwater seeded with preparations of *C. perfringens* spores or vegetative cells, as well as samples of naturally polluted water. In the latter case isolates were identified for evaluation of selectivity. WB was eliminated from detailed studies because of low counts and limited selectivity. MCP, TSC and TSC-EY were equally efficient for the recovery of *C. perfringens* spores, but the latter two media yielded significantly higher counts of vegetative *C. perfringens* cells as well as presumptive *C. perfringens* in samples of naturally polluted water. The selectivity of MCP for *C. perfringens* was 94 % compared with 26 % of TSC and 24 % of TSC-EY, but the recovery of *C. perfringens* was more efficient on the latter two media, which yielded the highest counts when membranes were overlaid with an agar top layer and plates incubated in an anaerobic jar. In view of the limited recovery efficiency, high cost and complexity of MCP, its use is justified only when highly selective recovery of *C. perfringens* is required. Membrane filtration using TSC-EY as growth medium proved a relatively economical, practical and reliable method for the recovery of presumptive *C. perfringens* from water.

Introduction

Clostridium perfringens has various attractive features as an indicator of water quality. The incidence of this bacterium in aquatic environments is almost exclusively ascribed to faecal pollution (Cabelli, 1977) and its spores are exceptionally resistant to unfavourable environmental conditions and water treatment processes (Grabow *et al.*, 1978; Bisson and Cabelli, 1980). Counts of *C. perfringens* in wastewater and polluted water environments generally outnumber those of enteric viruses or pathogenic bacteria, and the spores are more resistant to water treatment and disinfection processes than any of these organisms tested so far, which implies that the absence of *C. perfringens* in treated water supplies is a reliable indication of the absence of enteric viruses and bacterial pathogens (Bisson and Cabelli, 1980; IAWPRC Study Group on Water Virology, 1983). The exceptional longevity of the spores implies that the presence of *C. perfringens* in water environments in the absence of other indicators such as *Escherichia coli*, is a useful indication of remote faecal pollution (Cabelli, 1977). The absence of *C. perfringens* from water intended for human consumption is also desirable because it is an opportunistic pathogen (Cabelli, 1977; Borriello *et al.*, 1984).

Although *C. perfringens* meets all the requirements of an indicator of faecal pollution or the efficiency of water treatment processes (IAWPRC Study Group on Water Virology, 1983), it is not generally used for this purpose, largely because of a lack of convenient and practical test methods, and the failure to get consensus of opinion with regard to the variety of test methods described (Grabow and Isaäcson, 1978; Bisson and Cabelli, 1979, 1980). Hauschild and Hilsheimer (1974) compared certain growth media for the recovery of *C. perfringens* from naturally contaminated foods and concluded that tryptose-sulphite-cycloserine agar without egg yolk (TSC-EY) yielded the best results. Hirn and Raevuori (1978) preferred a membrane filtration method using tryptose-sulphite-cycloserine agar with egg yolk (TSC) to a tube colony count method for the enumeration of *C. perfringens* in water. Bisson and Cabelli (1979) described an agar medium containing polymyxin B (MCP) for membrane filtration counting in which 93 % of typical colonies were *C. perfringens*. Taylor and Burman (1964) described the Wilson and Blair glucose sulphite iron medium (WB), which was used for membrane filtration tests in various studies, but never compared with other media (Grabow and Isaäcson, 1978; Grabow *et al.*, 1978).

This paper deals with a comparison of TSC, TSC-EY, WB and MCP media for membrane filtration counting of *C. perfringens* in water. The results contribute to information required for the selection of practical and reliable methods to utilize this valuable indicator in water quality evaluation.

Materials and methods

Growth media

TSC and TSC-EY were prepared as specified by the manufacturers using Oxoid Perfringens Agar Base (Code CM587), Oxoid TSC Supplement (SR88) and Oxoid Egg Yolk Emulsion (SR47). MCP and WB were prepared from basic ingredients as described by Bisson and Cabelli (1979) and Taylor and Burman (1964), respectively.

Membrane filtration tests

A conventional membrane filtration method using 47 mm diameter Gelman GN-6 membranes (pore size 0,45 μm) was applied (Grabow, 1981). Membranes on TSC and TSC-EY were overlaid with TSC-EY agar (Hauschild and Hilsheimer, 1974) and those on WB with 3 % (w/v) Difco Nutrient Agar (Taylor and Burman, 1964). Membranes on MCP were not overlaid (Bisson and Cabelli, 1979). In some experiments (Tables 1 and 5) the top layer was also omitted from TSC and TSC-EY plates. When membranes were overlaid with a top layer, they were placed face downward onto the growth medium. Unless otherwise stated, plates were incubated anaerobically in gas jars using Oxoid BR38 anaerobic gas generating kits. Incubation was at 45 °C

TABLE 1
MEMBRANE FILTRATION COUNTS ON DIFFERENT GROWTH MEDIA OBTAINED IN SIX TESTS ON TAPWATER SAMPLES SEEDED WITH *CLOSTRIDIUM PERFRINGENS* SPORE PREPARATIONS

Result	Count/100 mℓ MCP − top layer	TSC + top layer	TSC − top layer	TSC-EY + top layer	TSC-EY − top layer
Average count	14×10^7	15×10^7	4×10^7	15×10^7	9×10^7
Median count	11×10^7	14×10^7	5×10^7	14×10^7	9×10^7
Range of counts	$(5 - 31) \times 10^7$	$(4 - 29) \times 10^7$	$(1 - 7) \times 10^7$	$(4 - 24) \times 10^7$	$(2 - 19) \times 10^7$
Standard deviation	9×10^7	8×10^7	2×10^7	7×10^7	6×10^7

for 14 h in the case of WB and 20 h in the case of the other media. Tests were done in triplicate and results recorded as average values.

Pure cultures of *C. perfringens*

A typical strain of *C. perfringens* (designated AVS1), derived from a human stool, was used. Vegetative cells were cultured in Robertson's meat medium incubated at 37 °C for 18 h (Holdeman *et al.*, 1977). Spores were derived by growing the strain in a sporulation medium at 37 °C for 24 h as described by Ellner (1956). Microscopic evaluations were done to ensure that harvests contained at least 95 % of vegetative cells or 85 % of spores.

Identification of isolates

Colonies were picked from membranes, purified on the same medium and cultured in Robertson's meat medium for identification using the following criteria (Holdeman *et al.*, 1977; Bisson and Cabelli, 1979): gram-positive rod, obligatory anaerobic, motility, fermentation of lactose, mannose, sucrose, aesculin, starch, cellobiose, mannitol, glucose and arabinose, stormy fermentation of milk, production of lecithinase and gelatinase, nitrate reduction, catalase activity, and indole production. Metabolic end-products in biochemical tests were evaluated by means of gas chromatography (Holdeman *et al.*, 1977).

Water samples

Samples for counting naturally occurring clostridia were collected from a series of experimental maturation ponds, settled biological filter effluent (humus tank effluent), and sand filtered river water at the Daspoort experimental site in Pretoria, maturation pond effluent in Windhoek, a borehole in Windhoek and various boreholes in Natal. Samples not analysed within 4 h were kept below 10 °C and processed within 24 h.

Statistical evaluation of results

Counts were considered to differ statistically significantly when a 95 % confidence limit was exceeded in assessment by Student's t-test.

Results

The results of comparative counts on tapwater samples seeded with preparations of *C. perfringens* spores (Table 1) show almost identical counts on MCP, and TSC and TSC-EY with top layer. However, omission of the top layer from TSC and TSC-EY plates yielded lower counts, with the difference smallest in the case of TSC-EY. The difference in counts obtained on the various media was statistically not significant, except in the case of TSC without top layer where counts were significantly lower than on TSC with top layer.

In tests on tapwater samples seeded with preparations of vegetative cells of *C. perfringens*, counts were slightly higher on TSC-EY than on TSC (statistically insignificant), but much higher than on MCP (statistically significant) (Table 2).

TABLE 2
MEMBRANE FILTRATION COUNTS ON DIFFERENT GROWTH MEDIA OBTAINED IN SIX TESTS ON TAPWATER SAMPLES SEEDED WITH A PREPARATION OF VEGETATIVE *CLOSTRIDIUM PERFRINGENS* CELLS

Result	Count/100 ℓ MCP	TSC	TSC-EY
Average count	40×10^4	29×10^6	31×10^6
Median count	16×10^4	27×10^6	30×10^6
Range of counts	$(4 - 150) \times 10^4$	$(13 - 55) \times 10^6$	$(13 - 51) \times 10^6$
Standard deviation	57×10^4	15×10^6	16×10^6

The results of tests on various water samples from the environment aimed at evaluating the recovery of naturally occurring clostridia, show that counts were generally slightly higher on TSC than on TSC-EY, but considerably higher than on MCP (Table 3). Due to the wide variation in counts, the difference in results for the Windhoek maturation pond effluent was statistically insignificant. Identification of 50 colonies from each medium revealed that all typical colonies were members of the genus *Clostridium*, but the selectivity for species differed. In the case of MCP 94 % of identified colonies were *C. perfringens* and the remaining 6 % were *C. perenne*. On TSC medium 26 % were *C. perfringens*, 60 % *C. ghoni* and 14 % *C. perenne*. Colonies on TSC-EY comprised 24 % *C. perfringens*, 66 % *C. ghoni*, 6 % *C. perenne*, 2 % *C. sphenoides* and 2 % *C. novyi* type A. These identification data permit an assessment of the potential recovery of *C. perfringens* on the different media since they indicate that 94 %, 26 % and 24 % of colonies on MCP, TSC and TSC-EY, respectively, can be expected to be *C. perfringens*. This implies that since the average count of presumptive *C. perfringens* in the Windhoek maturation pond effluent was 262 on MCP, 1358 on TSC and 1243 on TSC-EY (Table 3), the expected recovery of *C. perfringens* was 246 on MCP, 353 on TSC and 298 on TSC-EY. In

TABLE 3
MEMBRANE FILTRATION COUNTS OF PRESUMPTIVE *CLOSTRIDIUM PERFRINGENS* ON DIFFERENT GROWTH MEDIA OBTAINED IN TESTS ON VARIOUS ENVIRONMENTAL WATER SAMPLES

Sample	Count/100 mℓ MCP	TSC	TSC-EY
Windhoek maturation pond effluent			
4 Samples tested:			
Average	262	1358	1243
Median	179	1100	1085
Range	30-660	400-2830	370-2430
Standard deviation	291,9	1035,6	878,2
Pretoria settled biofilter effluent	5×10^4	26×10^4	18×10^4
Windhoek borehole	0	2	1
Natal boreholes			
No. T70920	2	80	85
No. T70921	0	1	1
No. T70923	0	1	0
No. T70924	0	0	1
No. T70925B	0	1	0

the case of Pretoria settled biofilter effluent with presumptive *C. perfringens* counts of 50 x 10^3 on MCP, 260 x 10^3 on TSC and 180 x 10^3 on TSC-EY (Table 3), the expected recovery of *C. perfringens* was 47 x 10^3 on MCP, 68 x 10^3 on TSC and 43 x 10^3 on TSC-EY. The Natal borehole no. T70920 with presumptive counts of 2 on MCP, 80 on TSC and 85 on TSC-EY (Table 3), would have expected *C. perfringens* recoveries of 2 on MCP, 21 on TSC and 20 on TSC-EY.

TABLE 4
MEMBRANE FILTRATION COUNTS OF PRESUMPTIVE *CLOSTRIDIUM PERFRINGENS* ON DIFFERENT GROWTH MEDIA OBTAINED IN TESTS ON SAMPLES FROM EXPERIMENTAL MATURATION PONDS

Sample	Count/100 mℓ MCP	WB
Station 1		
Sample 1	64×10^2	25×10^2
Sample 2	62×10^2	54×10^2
Station 2		
Sample 1	10×10^2	8×10^2
Sample 2	14×10^2	10×10^2
Station 3		
Sample 1	237	150
Sample 2	167	160

In a number of comparative tests on samples from water at various stages in a series of experimental maturation ponds, counts were consistently higher on MCP than on WB (Table 4).

The results of tests aimed at evaluating methods for establishing anaerobic conditions, show that the highest counts were obtained when membranes were covered with a top agar layer and plates incubated in an anaerobic jar (Table 5). Anaerobic conditions established only by a top layer yielded the lowest counts, while plates incubated without a top layer in an anaerobic jar yielded intermediate counts. Plates incubated aerobically (without top layer and not in an anaerobic jar) yielded no counts at all. The results in Table 5 do not show a statistically significant difference, but this is due to the wide variation in the range of counts.

Discussion

The results of this study clearly outline the advantages and disadvantages of the growth media concerned for the membrane filtration counting of *C. perfringens* in water. There was no statistically significant difference (Student's t-test with 95 % confidence limit) in the efficiency of MCP, TSC and TSC-EY (except TSC without top layer) for counting *C. perfringens* spores (Table 1). However, in the case of vegetative cells, MCP was statistically less efficient than TSC or TSC-EY, while the difference between TSC and TSC-EY was negligible (Table 2). Another important disadvantage of MCP for counting vegetative *C. perfringens* cells was the relatively high standard deviation in counts compared with that of TSC or TSC-EY (Table 2). The results of comparative tests on samples of naturally polluted water show that counts of presumptive *C. perfringens* were consistently lower on MCP than on TSC or TSC-EY (Table 3). As in the case of tests on vegetative cells of *C. perfringens* (Table 2), the standard deviation of counts of presumptive *C. perfringens* in Windhoek maturation pond effluent was higher than the average count of MCP, but not on TSC or TSC-EY (Table 3). The low counts on WB compared with those on MCP in comparative tests on samples of naturally polluted water (Table 4), in addition to findings which indicate limited selectivity on WB (Grabow and Isaäcson, 1978), imply that WB does not warrant further consideration as a growth medium for membrane filtration counting of clostridia in water.

The 94 % selectivity of MCP for *C. perfringens*, compared with 26 % of TSC and 24 % of TSC-EY, represents an important advantage of MCP. The high selectivity of MCP is in agreement with findings of Bisson and Cabelli (1979) according to which 93 % of all isolates were *C. perfringens*. The higher efficiency of

TABLE 5
MEMBRANE FILTRATION COUNTS OF PRESUMPTIVE *CLOSTRIDIUM PERFRINGENS* OBTAINED BY USING DIFFERENT GROWTH MEDIA AND DIFFERENT METHODS OF ESTABLISHING ANAEROBIC CONDITIONS IN SIX TESTS ON SAND FILTERED RIVER WATER

Result	Count/100 mℓ In anaerobic jar				Not in anaerobic jar	
	TSC		TSC-EY		TSC	TSC-EY
	+ top layer	− top layer	+ top layer	− top layer	+ top layer	+ top layer
Average count	260	108	232	154	70	74
Median count	160	90	180	135	53	60
Range of counts	37-800	40-200	47-670	27-350	10-200	10-200
Standard deviation	277,8	69,2	223,5	122,2	66,0	65,4

TSC and TSC-EY for the recovery of vegetative *C. perfringens* cells (Table 2), indicates that the higher counts of presumptive *C. perfringens* and estimated *C. perfringens* on the latter two media in tests on samples of naturally polluted waters (Table 3), were due to the presence of considerable numbers of vegetative cells in these waters.

Aspects other than the efficiency of recovery which should be considered in the evaluation of media include cost, labour, time and the availability of materials. The current cost of MCP is about R1,20 per plate compared with about R0,20 for TSC or TSC-EY. In addition, MCP is labour intensive and time consuming because it has to be prepared from an extensive list of basic ingredients, some of which may not always be readily available. TSC and TSC-EY, on the other hand, are easily prepared from commercial products which generally are freely available.

In view of the various advantages and disadvantages of the media tested, it would appear that the utilization of MCP is justified only when highly selective recovery of *C. perfringens* is required. TSC or TSC-EY proved to be the media of choice for the general recovery of *C. perfringens* and other species of the genus, particularly when present in the form of vegetative cells. TSC-EY has the advantage of being cheaper than TSC, and it is also less time consuming and labour intensive to use, while counts differ negligibly from those on TSC (Tables 1 to 3 and 5).

Anaerobic conditions during incubation proved to play an important role in the recovery of clostridia from water. The results in Table 5 show that membranes should be covered with an agar top layer and plates incubated in an anaerobic jar. The elimination of either the top layer or the use of an anaerobic jar as was done in some earlier studies (Taylor and Burman, 1964; Hirn and Raevuori, 1978; Bisson and Cabelli, 1979), yielded considerably lower counts. The membranes incubated upside down can easily be turned over together with the top layer by means of a forceps, if recovery of colonies for further identification is required.

Membrane filtration using TSC-EY growth medium, membranes covered with a top agar layer, and plates incubated in an anaerobic jar for 20 h at 45 °C, should prove useful for the general screening of drinking-water supplies, particularly untreated supplies where the possibility of remote faecal pollution must be taken into consideration, as in the case of many ground- and some surface waters. Results of these tests should generally be negative (Table 3; Cabelli, 1977; Grabow and Isaäcson, 1978; Grabow *et al.*, 1978). In the case of positive results, isolates should be identified because although drinking-water should be free of all clostridia (Cabelli, 1977; Grabow and Isaäcson, 1978; Grabow *et al.*, 1978), only the presence of *C. perfringens* can be considered as conclusive evidence of faecal pollution.

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