

Comparison of Media and Methods for the Detection and Enumeration of *Clostridium perfringens* in Seafoods

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Three direct plating methods and two most probable number (MPN) procedures were compared for the enumeration of *Clostridium perfringens* in seafoods: the sulfite-cycloserine (SC) agar, sulfite-polymyxin-sulfadiazine (SPS) agar, tryptone-sulfite-neomycin (TSN) agar, LS medium MPN procedure and iron milk MPN procedure. Isolates were confirmed as *C. perfringens*. The two MPN procedures compared very well with the three plating media tested with stock culture of *C. perfringens* from our laboratory collection and the reference strain NCIB 6125. But in fish samples, the two liquid media were found to be more sensitive and hence the MPN procedure using LS medium for the detection of *C. perfringens* in seafoods is suggested.

Clostridium perfringens is widely distributed in nature, occurring in soil, dust and among the intestinal microflora of warm-blooded animals (Smith & Holdeman, 1968). Consequently the organism is a common contaminant of raw foods and food ingredients.

Several media have been developed for the selective isolation of *C. perfringens*. Most of them contain sulphite and iron salt which turns black when sulphite is reduced. Since many other organisms (i.e. Enterobacteriaceae) have the ability to reduce sulphite, addition of several antibiotics as selective agents for *C. perfringens* has been suggested by many investigators (Mossel, 1959; Angelotti *et al.*, 1962; Marshall *et al.*, 1965; Shahidi & Ferguson, 1971; Harmon *et al.*, 1971; Mossel & Pouw, 1973; Handford, 1974). All these antibiotic containing selective plating media are normally used in public health laboratories to detect large numbers of *C. perfringens* in foods already involved in food poisoning outbreak. Numerous liquid media have been developed for enriching low numbers of *C. perfringens* (Gibbs & Freame, 1965; William *et al.*, 1982; Beerens *et al.*, 1982).

Although many different media and methods have been developed for the selective isolation and enumeration of *C. perfringens* from faeces, food and water, little

information is available on their specific application in the examination of seafoods. The purpose of the study was to evaluate current methods for the enumeration of *C. perfringens* in seafoods.

Materials and Methods

Fish samples, procured from local markets in and around Cochin, were brought to the laboratory and analysed within 2 h. Muscle with skin and intestine of fish samples were analysed separately. About 10 g of the sample was aseptically removed and homogenised with 100 ml of sterile diluent having the following composition: g/l-peptone-1, sodium chloride 5; pH 7.2 (Mead *et al.*, 1982). Decimal dilutions were prepared using 9 ml aliquots of the same diluent.

Enumeration by pour plating

Anaerobic incubation of plates was accomplished in Spray's dishes using alkaline pyrogallol method (Spray, 1930). Spray's dishes were placed in 37°C air incubators for 24 h. The following media were used for pour plating:

- 1) Sulfite-cycloserine (SC) Agar (Hauschild & Hilsheimer, 1974a 1974b; Hauschild *et al.*, 1974). This agar is identical with the egg yolk free TSC agar (Hauschild *et al.*, 1974a).

- 2) Sulfite-polymyxin-sulfadiazine (SPS) agar (Angelotti *et al.*, 1962).
- 3) Tryptone-sulfite-neomycin (TSN) agar (Marshall *et al.*, 1965).

Duplicate plates were prepared for each of the three agar media. 1 ml of appropriate dilutions were added to the plates and 10 ml of each medium were added to each plate. After solidification of the medium, plates were overlaid with an additional 5-7 ml of the medium and incubated anaerobically at 37°C for 24 h after which black colonies formed were counted.

Enumeration by MPN procedure

Most probable number method (3 tubes/dilution) was carried out using LS medium (Beerens *et al.*, 1982) and iron milk (IM) medium (William *et al.*, 1982). In the case of LS medium, appropriate dilutions were added at the rate of 10 ml (using 90 ml of LS broth), 1 ml and 0.1 ml (using 9 ml of LS broth). Tubes of iron milk medium were inoculated at the rate of 1 ml, 0.1 ml and 0.01 ml (using 9 ml milk medium). The tubes were incubated 16-24 h in a $45 \pm 1^\circ\text{C}$ water bath.

In the case of LS medium, there is a tendency for *C. perfringens* to die out rapidly during incubation due to decrease in pH to approximately 5.0 by the fermentation of lactose. Therefore for confirmation of *C. perfringens*, loopful of broth from positive tube was transferred to appropriate selective agar media within 24 h. Regarding iron milk medium, *C. perfringens* produces acid in the milk and the resulting decrease in pH becomes inhibitory to *C. perfringens*. Therefore for confirmation, loopful of broth from positive tube was transferred to appropriate agar media within 24 h.

Biochemical tests

From each sample, 5 presumptive colonies per method were tested. Standard biochemical tests were used to confirm *C. perfringens*. Motility and nitrate reduction were tested by using motility nitrate agar (FDA, 1978). Gelatin liquefaction and lactose fermentation were tested by using a lactose-gelatin medium (Hauschild & Hilsheimer, 1974b;

Hauschild *et al.*, 1977; FDA, 1978). These two media were found to be the most useful (Harmon & Kautter, 1978). Both confirmatory media were stored at 4°C and freshly deaerated at 100°C for 10 min before use.

Strains

3 strains of *C. perfringens* randomly selected among several isolates from fish samples and 1 reference strain of *C. perfringens* National Collection of Industrial Bacteria (NCIB) 6125 was also included in the study.

Data processing

The data were analysed statistically using the ANOVA technique. For the purpose of analysis, the data were converted to their respective logarithm values.

Results and Discussion

Tables 1 and 2 present the results of comparative study of five media for the enumeration of *C. perfringens* in seafoods. In the case of samples from muscle with skin and intestine of fishes, LS and IM were only compared since *C. perfringens* could not be detected using media SC, SPS, and TSN. Statistical analysis of the data are presented in Tables 4 and 5.

Table 4 gives the analysis of variance of counts in samples of muscle with skin of

Table 1. Comparison of *C. perfringens* counts in the muscle with skin of fish samples obtained with the 5 test media

Sample	No. of <i>C. perfringens</i> /g of sample by each media				
	SC	SPS	TSN	LS	IM
Ottolithus argenteus	nd*	nd	nd	12.2	9.5
Rastrelliger kanagurta	nd	nd	nd	8.0	7.9
Sardinella longiceps	nd	nd	nd	0.65	0.0
Mugil parsia	nd	nd	nd	0.45	2.1
Lactarius lactarius	nd	nd	nd	0.90	4.0

*nd — not detected

Table 2. Comparison of *C. perfringens* counts in the intestine of fish samples obtained with the 5 test media

Sample	No. of <i>C. perfringens</i> /g of sample by each media				
	SC	SPS	TSN	LS	IM
Ottolithus argenteus	nd*	nd	nd	35	21.5
Rastrelliger kanagurta	nd	nd	nd	nd	nd
Sardinella longiceps	nd	nd	nd	1.6	3.5
Mugil parsia	nd	nd	nd	5	9.0
Lactarius lactarius	nd	nd	nd	2	4.0

*nd - not detected

Table 3. Enumeration of low numbers of a known culture of *C. perfringens* from 3 replicate samples and 1 type culture by each of the 5 test media

Replicate	Average No. of <i>C. perfringens</i> /ml by each medium				
	SC	SPS	TSN	LS	IM
NCIB 6125	35	24	9	11	46
SL12	85	35	19	35	110
LL-4	91	85	44	13	24
SL-10	13	6	2.6	16	1.5

Table 4. Count in samples of skin with muscle

Source	ss	df	ms	F
Total	1.5183	9		
Between media	0.0184	1	0.0184	1
Between samples	1.3470	4	0.3367	8.81**
Error	0.1529	4	0.0382	

**indicate significance at 1% level

Table 5. (ANOVA) Count in intestine samples

Source	ss	df	ms	F
Total	2.3673	9	0.0228	1.21
Between media	0.0228	1	0.5672	30.01**
Between samples	2.2689	4	0.0189	
Error	0.0756	4	0.0189	

**indicate significance at 1% level

fishes. No significant difference in counts was observed in media LS and IM. However there was a significant difference ($p < 0.01$) between samples. Table 5 depicts the analysis of variance of counts in samples taken from the intestine of fishes. There were no significant differences in counts in LS and IM, however, between samples the counts differ significantly ($p < 0.01$).

To test the relative reproducibility and accuracy of the 5 test media, preliminary tests were made with strains of *C. perfringens* isolated from fishes and also with type culture NCIB 6125. The number of cells was kept low so that the ability of each medium to detect fewer than 100 cells/1 ml sample could be evaluated. *C. perfringens* was detected using the 5 test media (Table 3).

Statistical analysis was performed by taking into account all the five test media (Table 6). Analysis of variance of counts showed that there was no significant difference in counts between media at 5% level. But between replicates, the variation is significant ($p < 0.01$).

Table 6. (ANOVA) Count in reference strain and strains from fish samples

Source	ss	df	ms	F
Total	4.7234	19		
Between media	0.6925	4	0.1731	1.52
Between replicates	2.6667	3	0.8889	7.82**
Error	1.3642	12	0.1137	

**indicate significance at 1% level

Results clearly demonstrate the superiority of the liquid media to agar concomitantly allowing quantitative recovery of *C. perfringens* from fish samples. In the case of samples taken from the muscle with skin and intestine of fishes, *C. perfringens* was not recovered in the agar media SC, SPS and TSN. *C. perfringens* was detected in low numbers in the above samples by the two MPN procedures. Under such circumstances, the highest dilution yielding black colonies on agar plates may be beyond the concentration range of *C. perfringens* present originally, and the latter will remain undetected (Angelotti *et al.*, 1962).

Fluid media have several advantages over the solid media. No special precautions are necessary to obtain anaerobiosis. Low numbers of cells appear to be able to develop more readily in broth than in agar (Gibbs & Freame, 1965). Hence MPN method is preferred for the detection of low numbers of *C. perfringens* present in seafoods. The method also permits the testing of larger volume of inocula than is practicable with direct plating.

In the case of LS medium, the test is specific for *C. perfringens* because the organism grows rapidly at $45 \pm 1^\circ\text{C}$. A positive tube must contain a black precipitate due to reduction of sulphite to sulphide and show gas production due to the fermentation of lactose. Result can be obtained within 24 h. Preparation of medium and proposed methodology are also very simple. It also has good selectivity which allows the detection of low numbers of *C. perfringens* in the presence of large numbers of other sulphite reducing bacteria.

Regarding IM medium the test is specific. A positive tube must contain all components which together make a stormy fermentation, that is, production of acid curd with subsequent disruption of the curd by the large volume of gas produced from fermentation of lactose. Best results are obtained when milk media is incubated for a maximum period of 16-18 h. In our studies with fish samples, unidentified bacterial species produced both curd and digestion on the surface of the milk which was

similar to stormy fermentation and therefore misleading.

The results of the two MPN procedures were comparable to other methods for enumerating pure strains of *C. perfringens*. We recommend MPN procedure using LS medium for the detection of *C. perfringens* in raw seafoods, since in raw seafoods, *C. perfringens* count seems to be very low and only MPN procedures detected the presence of *C. perfringens*.

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