PART II

SCIENTIFIC AND TECHNICAL

BACTERIOLOGICAL INVESTIGATIONS OF PRAWN CANNERIES

II. INCIDENCE OF CLOSTRIDIUM PERFRINGENS.

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Prawn processing factories of the three major fish processing centres of the West Coast of India, viz., Cochin, Mangalore and Calicut were surveyed to determine the occurrence of *Clostridium perfringens* in processing areas, and in processed products. Direct plating on Sulphite-polymyxin-sulphadiazine Agar and enrichment techniques were used. Samples of prawn, prawn guts, frozen prawns, canned prawns, water, ice, swab from utensils and soil from the factory premises were examined. Among a total of 461 samples examined, only 32 (6.9%) gave positive results. The incidence of *C. perfringens* was more in prawn guts (80%), followed by soil (50%), prawn (38%), ice (33.3%), frozen prawns (11%), swab (5.0%) and water (1.1%). No *C. perfringens* was isolated from canned prawns.

INTRODUCTION

Among the anaerobic microorganisms of public health significance, *Clostridium perfringens* and *Clostridium botulinum* are the most important food poisoning organisms. *Clostridium perfringens* has been implicated as the etiological agent in numerous world wide food poisoning incidents in the last 35 years. *C. perfringens* is the principal cause of gas gangrene in man and the specific agent of lamb dysentry. Consumption of food in which certain strains of *C. perfringens* have grown extensively may result in illness characterized by acute abdominal pain and diarrhea, accompanied by little or no nausea and vomiting (Haushild and Thatcher, 1967; Hall *et. al.*, 1963). The illness occurs upon ingestion of large number of viable *C. perfringens* (Dische and Elek, 1957; Hobbs *et. al.*, 1953). The incubation period is usually from 8 to 22 hr., and the illness of short duration (1 day or less).

The works of Knox and MacDonald (1943); Hobbs *et. al.*, (loc. cit.), and Dische (loc. cit.), have indicated that *C. perfringens* is one of the principal causes of food borne illness in the British Isles. Meat dishes were found to be the com-

mon vehicle for this organism. In the United States, McClung (1945) reported the first confirmed outbreak of *C. perfringens* caused by cooked chicken and gravy. Since then, many studies have been reported in the United States on isolation and identification of *C. perfringens* from diverse foods. *C. perfringens* has been recovered from prepared frozen foods (Canada *et. al.*, 1964) fruits, vegetables, home prepared foods, raw meat, poultry and fish (Strong *et al.*, 1963), poultry meat (Ostovar *et. al.* 1971), dehydrated soups, sauces, gravy and spaghtti mixes (Nakamura and Kelly, 1968; and Keoseyan, 1971) and turkey products (Edmund and Busta, 1971). Nikolaeva and Ionova (1967) have conducted an examination of canned fish products for the presence of *C. perfringens*. H. S. Lillard (1971) made a survey of the broiler processing plants in the United States to determine the occurrence of *C. perfringens* in processing areas and in further processed products. So far, no work has been reported in India on the incidence of *C. perfringens* in fish processing centres. The present survey was undertaken to determine the incidence of *C. perfringens* in the three major fish processing centres of the West Coast of India, viz., Cochin, Mangalore and Calicut and to assess whether *C. perfringens* is found in processed fishery products in a frequency high enough to constitute a health hazard.

In the present work Sulphite-polymyxin-sulpha diazine (SPS agar developed by Angelotti *et. al.* 1962) was used as the medium for the isolation of *C. perfringens*. Enrichment techniques for the isolation of *C. perfringens* from foods have been reported by Nakamura and Kelly (*loc. cit.*), Keoscyan (*loc. cit.*) and H. S. Lillard (*loc. cit.*). Thioglycollote medium was used as the enrichment medium in the present study.

Materials And Methods

Media

1). Sulphite-Polymyxin-Sulphadiazine (SPS) agar (Angelotti *et. al.*, loc. cit.) was prepared by dissolving 15g. Bacto Tryptone (Difco), 10 g. Yeast extract (Difco), 5 g. Iron citrate, and 15 g. Bacto agar (Difco) in 1 litre of distilled water. The pH was adjusted to 7.0 ± 0.1 using 0. 1 N NaoH, and the media was sterilised at 121° C for 15 min. Just before use, to each litre of the sterile basal medium cooled to 45° C were added the following Seitz-filtered solutions:-

i). 5. 0 ml of a freshly prepared 10 percent solution of sodium sulphite, Na_2 So_3 $7H_20$.,

ii). 10.0 ml of a 0.1 percent solution of polymyxin B sulphate (Mann Research Laboratories, N. Y.) and

iii). 10.0 ml of a solution of sodium sulphadizine (pfaltz and Bauer) Acetochemical Co. Inc. N. Y., containing 12 mg/ml.

2). Tioglycollate broth contained per litre: Yeast extract (Difco), 5g; Bacto Tryptone (Difco), 15 g; Glucose (Analar), 5 g; NaCl, 2.5 g; L-cystine, 0.75g; sodium thioglycollate, 0.5g. and agar powder (Difco), 0.75 g. The pH was adjusted to 7.0 ± 0.1 and the media sterilized at 121°C for 20 min. Dehydrated Bacto Fluid Thioglycollate medium (Difco) was also used.

Two strains viz. i) NCDC-4992-ATCC, 12912-A typical *Clostridium perfringens* and ii) NCDC-KA3-BP6K- *Clostridium perfringens* supplied by the National Communicable Disease Centre, Atlanta, Georgia, U. S. A., were used for comparison.

These cultures were propagated and maintained in cooked meat medium at room temperature.

Anaerobiosis

For the plating technique Spray anaerobic dish (Fisher Seientific Co. Pittsburg, Pennsylvania) was used (Spray, 1930). All other tubed media were incubated in an anaerobic jar (McIntosh & Fildes Anaerobic Jar). The air inside the jar was expelled by passing carbon dioxide gas under pressure. All tubed media were kept in a boiling water bath for 10 minutes just before use to expel the dissolved oxygen.

Samples for analysis

Samples of raw material (prawns), water, ice, swab, frozen prawns and processed cans were collected from different processing centres at frequent intervals. Wherever possible, guts of prawns were also collected. The method described by Nambiar and Iyer (1971) was followed for the collection of samples. After reaching the laboratory the samples, except the canned products were pour plated with the SPS agar using spray anaerobic dishes. The cans were incubated at 37°C for 10 days and then the brine content and the meat subjected to analysis. The material was macerated with sterile distilled water in a sterile mortar and 1 ml of the suspension was used for plating. Water, ice, swab and brine were directly plated out using 1 ml of the sample. All the platings were done in duplicate. The plates were incubated at RT for upto 72 hr and checked at 18, 24 and 48 hours for the presence of black colonies.

Direct plating on SPS agar gave very low recovery and enrichment techniques were followed. 1 g/ml of the sample was asceptically removed and inoculated into two separate 20 x 150 mm culture tubes containing 15 ml of thioglycollate medium. All tubes were kept in a boiling water bath for 10 min just before use. The tubes were incubated at RT up to 72 hr and checked at 18, 24 and 48 hr for turbidity and gas production, if any. Material from tubes which showed growth were gram stained and further streaked on or pour plated with SPS agar using spray anaearobic dishes. The plates were incubated at RT for 72 hr and checked for the presence of black colonies. Black colonies, if present were picked up and portion was gram stained and the rest transferred to 15 ml of thioglycollate medium. The strains were subcultured at frequent intervals in thioglycollate medium. Gram reaction and other biochemical characteristics of the isolated strains were studied and compared with those of the type cultures.

Motility and nitrate reduction were determined by stab inoculating a portion of the black colony into Motility-Nitrate medium (Angelotti *et. al.*, loc. cit.)

The production of black colonies on SPS agar is itself an indication of the ability of the strains to produce hydrogen sulphide which is precipitated as iron sulphide resulting in black colonies. Indole production, gelatin liquefaction and fermentation of glucose, lactose, sucrose, mannitol and maltose were studied by inoculating the cultures into the respective media prepared in distilled water, preboiled to expel the dissolved oxygen. The tubes were incubated at RT in the anaerobic jar, the oxygen in which was replaced by using carbon dioxide.

Results And Discussion

Table 1 gives the results of the survey carried out in the prawn processing centres in Cochin area. The survey was carried out with particular reference to canning

TABLE I

Frequency of Isolation of *C. perfringens* from prawn processing centers in Cochin.

Source of sample	No. of samples analysed	No. of samples positive	Percentage of positive samples.
Raw material (prawns)	9	0	0
Prawn guts	7	5	71.4
Frozen prawns	2	0	0
Water	155	1	0.6
Swab from utensils	107	0	0
Ice	4	0	0
Soil	4	2	50.0
Canned prawns	92	0	0
Total	380	8	2.10

factories. Peeled and deveined prawns, prawn guts, swab, water and ice samples were collected from the canning factories. A few soil samples from the factory premises were also collected. Processed cans were collected after the final cooling process. Samples of frozen prawns from two freezing plants were also collected. The results indicate that the frequency of isolation of *C. perfringens* is more in the case of prawn guts (71.4%), followed by soil samples (50%). Only one water sample showed the presence of *C. perfringens* and none was observed in the swab, ice, peeled & deveined prawns, frozen prawns and processed cans. The total incidence of *C. perfringens* was very low (2.1%).

The results of the survey carried out in the prawn freezing factories in Mangalore area are given in Table 2. In these factories, prawns were frozen in the peeled and undeveined form. Material was taken just before freezing and also from the frozen blocks, prawn guts, water, ice and swab from different utensils were also analysed. All the five raw material samples and the three prawn gut samples gave positive results. Five samples of frozen prawns were analysed and only one (20%) showed the presence of *C. perfringens*. A few swab, ice and one water sample also showed the presence of *C. perfringens*. The incidence of *C. perfringens* was the highest (36.9%) in the samples from this area.

Table 3 gives the results with regard to the processing centres in Calicut. The survey was carried out in two freezing plants and one canning factory. Here also prawns were frozen in the peeled and undeveined form. The rawmaterial samples included four peeled and undeveined prawn samples from freezing plants, and peeled and deveined prawns, material immediately after blanching and material just before filling the cans from the canning factory. Three out of seven raw material samples showed the presence of *C. perfringens* and all were peeled and undeveined material from the freezing plants. A few swab and ice samples also showed the presence of *C. perfringens*. No *C. perfringens* was isolated from the water, frozen prawns, and canned prawns. The total incidence of *C. perfringens* in the samples from this area was 20%.

TABLE II

Frequency of Isolation of *C. perfringens* from prawn processing centers in Mangalore.

Source of sample.	No. of samples analysed	No. of samples positive	Percentage of positive samples
Raw material (prawns)	5	5	100
Prawn guts	3	3	100
Frozen prawns	5	1	20.0
Water	8	1	12.5
Swab from utensils	20	5	25.0
Ice	5	2	40.0
Total	46	17	36.9

TABLE III

Frequency of Isolation of *C. perfringens* from prawn processing centers in Calicut.

Source of sample.	No. of samples analysed	No. of samples positive	Percentage of positive samples.
Raw material (prawns)	7	3	42.8
Frozen prawns	1	0	0
Water	9	0	0
Swab from utensils	12	2	1.6
Ice	3	2	66.6
Canned prawn	2	0	0
Total	35	7	20.0

461 samples were analysed in total from the three centres and out of this, only 32 (6.9%) showed the presence of *C. perfringens*. The frequency of isolation was the highest in the case of prawn guts (80%), followed by soil (50%,) peeled and undeveined prawns (38%), ice (33.3%), frozen prawns (11%), swab samples (5.0%) and water samples (1.1%). No *C. perfringens* was isolated from canned prawns.

C. perfringens is a common organism found in soil and bottom mud of the sea (Yamagishi *et. al.* 1964.) The higher incidence of *C. perfringens* in the prawn guts may be due to the fact that prawns are bottom feeders. Since, *C. perfringens* found to be present in almost all the prawn gut samples, they can be expected in the peeled and undeveined prawn samples also. All the raw material samples which showed the presence of *C. perfringens* were peeled and undeveined material. When prawns are deveined and washed thoroughly all these organisms may disappear and that may probably be the reason why no *C.*

perfringens was isolated from peeled and deveined material.

Though *C. perfringens* was found to be present in the peeled and undeveined material before freezing, the incidence of this organism in frozen prawns was very low. Hall (1968) has shown that a rapid loss in the viability of *C. perfringens* occurs when food samples are frozen or held under prolonged refrigeration, and according to Canada *et. al.*, (loc. cit.) only 1% of the vegetative cells survive frozen storage for 48 hours.

Since ice blocks are usually dragged on the ground they may get contaminated with the *C. perfringens* present on the floor of the processing centres and thus the incidence of *C. perfringens* in ice samples (33.3%) can be explained. The chances of water getting contaminated with *C. perfringens* are rare since most of the factories depend on municipal water supply which is purified and chlorinated, and hence the low incidence of *C. perfringens* in water samples. Utensils used in processing centres where prawns are frozen in the peeled and undeveined form are likely to get contaminated with the *C. perfringens* from the prawn guts. This may explain the incidence of *C. perfringens* in swab samples from the freezing centres at Mangalore and Calicut. *C. perfringens* can hardly be expected in canned prawns which are subjected to heat treatment to such an extent as to kill almost all the spores and vegetative cells. (Sterilization for 15 min. at 15 lbs steam pressure).

Though all the isolated strains produced black colonies on SPS agar and were strictly anaerobic Gram positive sporeforming rods, variations were observed in some of their biochemical characteristics in a few case. Even the two type cultures differed in some of their biochemical characteristics. Morphological and biochemical characteristics of the isolates and those of the two type cultures are given in Table 4.

Conclusion:

From the results it is observed that the incidence of *C. perfringens* in canned prawns is little and in frozen prawns it is negligible. *C. perfringens* is found mainly in the prawn guts and also to a higher extent in the processing centres where prawns are frozen without removing the veins. It is rarely observed in the water used in the processing centres.

Acknowledgements:

The authors are grateful to Dr. V. K. Pillai, Director of the Institute for his keen interest and helpful suggestions in this investigation. They are also thankful to the managements of the prawn processing factories for their co-operation and to Shri. T. John of this Laboratory for the technical assistance rendered.

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TABLE IV

Characteristics of *C. perfringens* isolated from Prawn Processing Centres.

Serial Number	Source	Morphology	Gram reaction.	Sporulation.	Anaerobiosis (a)	Motility	Nitrate reduca-tion	Indol production	H_2S production	Gelation liquef-action.	Fermentation of (b)				
											Glucose	Lactose	Sucrose	Mannitol.	Maltose.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
1.	Soil	Rods, single & pairs.	+	+	S.A.	−	−	−	+	−	+−	+−	+−	+−	+−
2.	Prawn guts	Rods, short, Single & pairs.	+	+	S.A.	−	−	−	+	−	−−	−−	−−	−−	−−
3.	Prawn guts	Rods, single & pairs.	+	+	S.A.	−	+	−	+	+	++	++	++	−−	++
4.	Well water	Rods, single & pairs.	+	+	S.A.	−	+	−	+	+	++	++	++	−−	++
5.	Prawn guts	Rods, single & pairs.	+	+	S.A.	−	+	−	+	+[c]	++	++	++	−−	++
6.	Prawn guts	Rods, single & pairs.	+	+	S.A.	−	+	−	+	+[c]	++	++	++	−−	++
7.	Prawn guts	Rods, single & pairs.	+	+	S.A.	−	+	−	+	+	++	++	++	−−	++
8.	Soil	Rods, single & pairs	+	+	S.A.	−	−	−	+	−	−−	−−	−−	−−	−−
9.	Swab	Rods, single & pairs	+	+	S.A.	−	+	−	+	+[c]	++	++	++	−−	++
10.	Swab	Rods, single & pairs	+	+	S.A.	−	+	−	+	+[c]	++	++	++	−−	++
11.	Swab	Rods, single & pairs	+	+	S.A.	−	+	−	+	−	++	−−	++	−−	++
12.	Swab	Rods, thin, single & pairs	+	+	S.A.	−	−	−	+	−	−−	−−	−−	−−	−−
13.	Water	Rods, single & pairs	+	+	S.A.	+	−	−	+	+[c]	−−	−−	−−	−−	−−
14.	Ice	Rods, single & pairs	+	+	S.A.	−	+	−	+	+[c]	++	−−	++	−−	++
15.	Prawns (PuD)	Rods, long chains	+	+	S.A.	−	−	−	+	+[c]	−−	−−	−−	−−	−−
16.	Prawn Guts	Rods, single & pairs	+	+	S.A.	−	−	−	+	−	−−	−−	−−	−−	−−
17.	Swab	Rods, single & pairs	+	+	S.A.	−	+	−	+	+	++	++	++	−−	++
18.	Ice	Rods, short, single & pairs	+	+	S.A.	−	+	−	+	+	−−	−−	−−	−−	−−
19.	Prawns (PuD)	Rods, short, single & pairs	+	+	S.A.	−	+	−	+	+	−−	−−	−−	−−	−−
20.	Prawn guts	Rods, short, single & pairs	+	+	S.A.	−	−	−	+	+	−−	−−	−−	−−	−−
21.	Prawns (PuD)	Rods, short, single & pairs	+	+	S.A.	−	−	−	+	+	−−	−−	−−	−−	−−

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
22.	Prawn guts	Rods, single & pairs	+	+	S.A.	—	+	—	+	+	++	++	++	—	++
23.	Prawns (PuD)	Rods, long, single & pairs	+	+	S.A.	—	—	—	+	—	—	—	—	—	—
24.	Frozen prawns	Rods, long, single & pairs	+	+	S.A.	—	—	—	+	—	—	—	—	—	—
25.	Prawns (PuD)	Rods, single & pairs	+	+	S.A.	—	+	—	+	+	++	++	++	—	++
26.	Swab	Rods, single & pairs	+	+	S.A.	—	+	—	+	+	++	++	++	—	++
27.	Ice	Rods. single & pairs	+	+	S.A.	—	+	—	+	+[c]	++	++	++	—	++
28.	Prawns (PuD)	Rods, single & pairs	+	+	S.A.	—	+	—	+	+[c]	++	++	++	—	++
29.	Prawns (PuD)	Rods, long, single & pairs	+	+	S.A.	+	—	—	+	—	—	—	—	—	—
30.	Swab	Rods, long, single & pairs	+	+	S.A.	—	—	—	+	—	—	—	—	—	—
31.	Prawns (PuD)	Rods, long, single & pairs	+	+	S.A.	—	—	—	+	—	—	—	—	—	—
32.	Ice	Rods, single & pairs	+	+	S.A.	—	—	—	+	—	—	—	—	—	—
T-1.	NCDC-4992	Rods, single & pairs	+	+	S.A.	—	—	—	+	+	—	—	—	—	—
T-2.	NCDC-KA3-BP-6K	Rods, single & pairs	+	+	S.A.	—	+	—	+	+[c]	++	++	++	—	++

Prawns (PuD) = Peeled undeveined Prawns ; [a] S.A. = Strictly Anaerobic ;
b++ = Acid & Gas; +— = Acid but no gas; -- = No acid, no gas;
c = Black colour observed in the gelatin media.
\+ = Positive
— = Negative.

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