Isolation and Characterization of a Putrefactive Anaerobe *Clostridium sporogenes* from Fishes and Shell Fishes

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Fishes and shell fishes were analysed for the occurrence of *Clostridium sporogenes*. 21 isolates of *C. sporogenes* were examined for cultural properties and heat resistance. All the strains isolated were found to be poor sporeformers. The spores of the above *C. sporogenes* strains were highly heat resistant.

Clostridium sporogenes, a proteolytic gas former, is widely distributed. It is generally regarded as a harmless saprophyte occurring in the intestine of man and animals. It is a common contaminant of meat and fish and prominently associated with the spoilage of canned foods (Herson & Hulland, 1980).

The occurrence of *C. Sporogenes* has been reported in fish (Shewan, 1962). The majority of clostridia isolated from canned foods such as fish and meat are identified as *C. Sporogenes* (Aschehoung & Jansen, 1950; Kato *et al.*, 1971; Smith, 1975; Crossley, 1941).

There is practically very little information on the occurrence of *C. Sporogenes* in Indian fishes. The role of this mesophilic sporeformer in the spoilage of canned foods is well understood. Hence the present investigation was made to study the incidence and characterization of *C. Sporogenes* in fishes and shell fishes.

Meterials and Methods

Fish and shell fish samples analysed for the isolation of *C. Sporogenes* were *Rastrelliger kanagurta, sardinella longiceps, Lutjanus bohar, epinephelus tauvina and Perna viridis.*

In fish samples, the muscle with skin and the digestive tract were cultured for the enumeration of *C. Sporogenes*. About 10 g of the samples was aseptically removed and inoculated into 90 ml cooked meat medium (CMM) for initial enrichment and incubated at 37°C for 72 h.

For enumeration, tryptone soytone glucose yeast-extract Agar (TSGYA) which is a modification of the one developed by Ferreira *et al.*, (1981) was used. One litre of the medium in distilled water contained glucose 30g, tryptone 50 g, soytone 5, yeast extract 20 g, sodium bicarbonate 1 g, L-cysteine hydrochloride 0.5 g and Agar 20 g. All ingredients except glucose, sodium bicarbonate and L-cysteine hydrochloride were dissolved in distilled water by heating, the pH was adjusted to 7.4 and then autoclaved for 15 min at 121°C. The glucose (50%), sodium bicarbonate (10%) and L-cysteine hydrochloride (1%) solutions were filter sterilized and then added to the tempered medium. The medium was supplemented with 10 ml egg yolk suspension (50% egg yolk in saline). This was overlaid with dithiothreitol agar (DTTA) which consisted of 2% agar with 0.01% filter sterilized dithiothreitol.

A series of 10 fold dilutions of the

enrichment culture was made in peptone saline (PS) solution (0.086 M sodium chloride, 1% Bactopeptone of PH 7.2) and 0.1 ml of appropriate dilutions were spread inoculated on pre-dried TSGYA plates and 10-12 ml of DTTA was poured over the medium. The plates were incubated at 37°C for 48 h anaerobically.

Presumptive *C. Sporogenes* colonies showing lipase reaction on TSGYA plates were isolated and maintained in CMM. 21 isolates were identified as *C. Sporogenes* using cultural and biochemical characteristics (Smith & Hobbs, 1974; Mac Faddin, 1980).

Colonial properties were observed on brain heart infusion agar (BHIA) and egg yolk agar. Strains were examined for their ability to ferment glucose, glycerol, lactose, maltose, salicin and sucrose by the method of Princewill (1978). Cultural and biochemical characteristics like motility, nitrate reduction, gelatin liquefaction, action on milk, hydrogen sulphide production and indole production were studied according to the methods of FDA (1978).

Sporulation frequency of *C. Sporo genes* strains was determined by the following method. 10 ml of BHI broth supplemented with 0.05% L-cysteine hydrochloride was inoculated with 0.5 ml of 24 h CMM culture and incubated at 37°C for 48 h. Serial decimal dilutions of the BHI broth were made and smears were prepared on slides and stained with Ziehl - Neelsen's carbol fuchsin. Spore count was determined by the direct microscopic count (Anon, 1978). After examining at least 7 fields, spore count was calculated according to the definition given by Anon (1978).

The heat resistance of spores of the *C. Sporogenes* isolates was determined qualitatively and quantitatively by the method of Nakamura *et al.*, (1977). In the quantitative test, viable counts were determined using pour plates on TSGYA incubated at 37°C for 48 h anaerobically. The same procedure was used to determine the heat-resistant spore population after heating the culture.

Results and Discussion

The identity of the cultures isolated were confirmed as *C. Sporogenes* as described in the 8th (Smith & Hobb, 1974) edition of Bergey's Manual of Determinative Bacteriology. Morphological and biochemical characteristics studied are given in Table 1.

Table 1. *Criteria for confirmation of C. Sporogenes*

Biochemical characteristics	Result
Aerobic culture	no growth
Gram staining	positive rods
Spores	Oval, subterminal
Motility	positive
Gelatin liquefaction	positive
Action on milk	digestion
Hydrogen sulphide production	positive
Lipase	positive
Lecithinase	negative
Indole production	negative
Nitrate reduction	negative

Maltose	acid, gas
Lactose	negative
Sucrose	acid, gas
Salicin	acid, gas
Glucose	acid, gas
Glycerol	acid, gas
Pathogenic for laboratory animals	negative

The identity characteristics of all the strains isolated fit those of *C. Sporogenes*. Morphologically, the colonies on egg-yolk agar and BHIA were entire and smooth with a raised centre and a very short rhizoid periphery. Occasionally small creamy white colonies with radial ridges and crenated edges or smooth translucent colonies were seen. Nakamura *et al.*, (1977) observed two types of colonies type (I and II) of *C. Sporogenes*. Princewill (1978) also observed that colonies of *C. Sporogenes* were of two types - type A and B which were similar to types I and II colonies of Nakamura *et al.* (1977). The *C. Sporogenes* strains isolated in the present study were similar to colony type II/type B. All the strains fermented glucose, maltose, glycerol, sucrose and salicin but not lactose.

Qualitative studies on the heat-resistance of spores showed that all the strains resisted heating at 100°C for 1 h and all but two of the strains resisted heating at 100°C for 2 h. Quantitative studies on the heat resistance of spores were carried out in five strains. The data are presented in Table 2.

Table 2. *Sporulation and heat resistance of Spores of C. Sporogenes strains*

Bacterial strain	Total viable counts/ml ($X10^8$)	No. of cells/ml resitant to heat 75°C 15 min ($x10^4$)	100°C, 1 h	100°C, 2 h
SI	0.51	0.5	1200	20
SII	1.20	11.0	5200	80
MI5	7.80	10.1	1800	48
MI6	0.20	9.0	800	30
M22	0.60	0.3	30	10

All the *C. Sporogenes* strains isolated had highly heat resistant spores.

Heating at 75°C for 15 min can kill the vegetative cells. Hence the counts obtained after heating at 75°C for 15 min indicated the number of spores present. Sporulation was very poor in all the strains. The percentage of spores in the total number of cells was about 0.01. This observation was consistent with the microscopic count.

Acording to Nakamura *et al.* (1977) colony type II strains of *C. Sporogenes* were non sporulating and some had highly heat resistant cells while others had no heat resistance. Present study shows that though *C. Sporogenes* strains of colony type II are poor spore-formers, in a study with more than millions of cells of colony type II strains, the spores will be present in significant numbers to give a count by the cultural means

after heating at 100°C for 2 h.

Nakamura *et al.*, (1977) have described that colony type II strains of *C. Sporogenes* were homologous to C. botulinum strain type A 190 in DNA - DNA reassociation assays. According to him, it remains to be shown whether or not *C. Sporogenes* strains homologous to *C. botulinum* are non - toxigenic strains of *C. botulinum*. Bulloch *et al.* (1919) and Prevot et al. (1967) reported that strains similar to the colony type II of *C. Sporogenes* had been encountered.

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