CLOSTRIDIA ISOLATED FROM FAECES

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CANCER of the colon may result from the action of carcinogens produced from steroids in the intestine by gut bacteria (Aries *et al.*, 1969; Hill *et al.*, 1971). Clostridia are among the most metabolically active of intestinal bacteria. Several species have been shown to degrade steroids (Norman and Grubb, 1955; Capek, Hanc and Tadra, 1966), and study of a strain of *Clostridium paraputrificum* showed that this organism produced a 3-oxo-5 β steroid Δ^4 -dehydrogenase (Aries, Goddard and Hill, 1971) which performs a key reaction in the production of the aromatic steroids postulated as possibly related to the causation of cancer of the colon. Further studies demonstrated that this enzyme is possessed by many strains of *C. paraputrificum* and related organisms (Goddard *et al.*, 1975).

In the course of a study of faeces from groups of people in various parts of the world, in which the incidence of cancer of the colon varies widely, we isolated numerous clostridia. In the present paper we report on the identity of these isolates.

MATERIALS AND METHODS

Samples of faeces were obtained from groups of normal healthy adult men and women consuming a mixed western diet in England, Scotland and the USA, and from those consuming a diet rich in carbohydrate in Japan and Uganda (Hill *et al.*, 1971; Peach *et al.*, 1974). Samples from the USA came from both black and white persons.

Samples of faeces were also obtained from normal healthy adults, consuming the local diet, in Hong Kong. Additional samples of faeces from normal healthy adults aged between 45 and 75 years consuming a mixed western diet, in Wales, were obtained as part of a prospective study on the role of clostridia and steroids in the development of cancer of the colon.

Transport and storage of specimens. Fresh samples of faeces were diluted 1 in 10 in 10% (v/v) glycerol broth and immediately frozen in solid carbon dioxide or in liquid nitrogen for transport to London (Crowther, 1971). The samples were stored at -50°C .

Isolation of clostridia

Ten-fold dilutions of the specimens were prepared in quarter-strength Ringer's solution containing peptone 0.5% (w/v); 0.1-ml samples of appropriate dilutions were spread on the surface of plates of egg-yolk lactose agar containing 100 μg neomycin per ml (modified

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medium of Willis and Hobbs, 1958). The remainder of each dilution was heated at 70°C for 10 min. and 0.1-ml samples of appropriate dilutions were spread on the surface of egg-yolk lactose agar.

Seeded plates were packed into anaerobic jars which were then evacuated and filled with hydrogen. After a few minutes, the jars were re-evacuated and filled with a gas mixture containing 30% CO₂ and 70% hydrogen. They were incubated at 37°C for 2 days. Cold "D" catalyst was used in the anaerobic jars (Englehard Industries, Cinderford, Gloucestershire).

From each of the plates spread with unheated material, two colonies with an opalescent halo were picked, and from the isolation plates spread with heated material 10 colonies without an opalescent halo were picked. The colonies were streaked on egg-yolk lactose agar plates without neomycin for purification. Sporing aerobic bacilli and faecal streptococci that survived heat treatment were discarded. Pure strains were stored at 4°C in Robertson's meat medium.

Biochemical tests

Liquid media for biochemical tests were distributed in 5-ml screw-capped bottles and seeded with two drops of actively growing culture from Robertson's cooked meat medium. The tops of the bottles were loosened before incubation in an anaerobic jar containing an atmosphere of 30% CO₂ and 70% H₂.

Fermentation tests. The fermentation tests were modifications of those described by Cato *et al.* (1970); 0.5 g of aesculin or 1.0 g of other test carbohydrate was added to a 100-ml volume of basal medium containing peptone 2.0 g, yeast extract 1.0 g, cysteine hydrochloride 0.05 g, sodium formaldehyde sulfoxylate 0.03 g, CaCl₂ 0.001 g, MgSO₄ 0.001 g, KH₂PO₄ 0.005 g, NaHCO₃ 0.05 g, and NaCl 0.01 g. The pH was adjusted to 7.1±0.1 and the media were dispensed in volumes of 4.5 ml before autoclaving at 115°C for 20 min. Cultures were incubated anaerobically at 37°C for 3 days. The final pH of media was estimated by the addition of two drops of bromocresol purple indicator (BDH). Fermentation of aesculin, fructose, glucose, lactose, maltose, mannose, salicin, starch, sucrose and xylose was tested. The reaction of the strains in litmus milk was also examined.

Microfermentation tests were used for the study of the clostridia isolated from specimens collected in Wales. Sterile 2% (w/v) solutions of glucose, lactose, maltose, mannitol, salicin, sucrose and urea were dispensed aseptically in 100-μl amounts into sterile multi-well plastic trays with a Canalco Autotiter IV multi-channel dispensing and diluting apparatus (Fisons Scientific Apparatus, Leicester). Cultures of bacteria were added to sugar solutions from a 100-μl automatic pipette (Finnpipette, Jencons Ltd). The basal medium contained gelatin 0.3 g, agar 0.1 g, tryptone 1.0 g, yeast extract 0.3 g, cysteine hydrochloride 0.05 g, sodium formaldehyde sulfoxylate 0.03 g, CaCl₂ 0.001 g, MgSO₄ 0.001 g, biotin solution (1.25 mg per 100 ml) 0.4 ml, haemin-menadione solution (haemin 50 mg and menadione 5 mg per 100 ml) 1.0 ml, rumen fluid 20 ml and distilled water 80 ml. The trays of inoculated sugars were incubated for 3 days at 37°C in an anaerobic jar containing an atmosphere of 30% CO₂ and 70% H₂. The final pH was estimated with bromocresol purple indicator. Preliminary results showed that these micro-fermentation tests gave results comparable to those of conventional fermentation tests. Similar micro-tests have been used for the identification of enterobacteria and streptococci (Huhtanen, Naghski and Dellamonica, 1972; Jayne-Williams, 1975).

Test for starch hydrolysis. Two or three drops of Lugol's iodine were added to a 3-day culture in the starch medium. A negative test was denoted by the development of a blue colour.

Test for aesculin hydrolysis. Two or three drops of a 1% (w/v) solution of ferric chloride were added to a 3-day culture in aesculin medium. A positive test was denoted by the development of a black colour.

Test for gelatin liquefaction. The test cultures were seeded into Robertson's cooked meat medium with added charcoal gelatin disks (Oxoid). The appearance of free charcoal particles in the medium after incubation for 14 days at 37°C indicated liquefaction of the gelatin.

Test for nitrate reduction and indole production. Nitrate, nitrite and indole were detected by conventional procedures (Cowan and Steel, 1965) in cultures grown anaerobically for 72 h in indole-nitrate medium (Baltimore Biological Laboratories, USA).

Nagler's reaction. On one-half of an egg-yolk-agar plate two or three drops of *C. welchii* (*C. perfringens*) antitoxin (Burroughs Wellcome Ltd) were spread and allowed to dry. The plate was then streak-inoculated with cultures of clostridia. Plates were examined after anaerobic incubation for 3 days. Inhibition by antiserum of the production of an opalescent halo was a positive reaction.

Test for toxin production. Cultures were seeded into Robertson's cooked-meat broth previously enriched with 1 ml of a sterile glucose solution (10% w/v) and 0.5 ml of a sterile solution of calcium chloride (25% w/v) and incubated overnight. CBA-strain mice were challenged intraperitoneally with 0.5 ml of the supernate. The identity of the toxin was determined by neutralisation tests involving the incubation of 1.3-ml volumes of the culture supernate with 0.2-ml volumes of specific antisera (Burroughs Wellcome Ltd) before injection into the mice. Only strains that could be ascribed to toxigenic species on biochemical grounds were tested.

Gas-liquid chromatography

Preparation of the sample. A 4-ml volume of a 3-day culture in 1% glucose broth (Cato *et al.*, 1970) was acidified with 0.5 ml of 9M H₂SO₄ and extracted with 4 ml diethyl ether. After centrifugation to break the emulsion, the ether layer was pipetted off and dried over magnesium sulphate. Reference solutions were prepared as described by Cato *et al.* (1970). Controls were of uninoculated glucose broth. Pyruvic, lactic and succinic acids are unstable at the operating temperature of the gas chromatograph. These acids were detected by prior preparation of their methyl esters, which are stable and readily separate on the column. Freshly prepared diazomethane (de Boer and Backer, 1954) was used as the methylating agent, two to three drops being added to a portion of the extract.

Chromatographic conditions. Analyses were performed on a Pye 104 chromatograph fitted with a flame-ionisation detector and a 150-cm glass column packed with Diatomite C that had been treated with phosphoric acid and impregnated with 10% polyethylene glycol (prepared column no. 12440/2/10 PMP/01F78 available from Pye-Unicam Ltd, Cambridge). The operating temperature was 125°C and the carrier gas was oxygen-free N₂ passing through at 30 ml per min. A 1- μ l portion of the sample for analysis was injected on to the column.

Head-space analysis. For some strains, analyses of cultures for the presence of volatile acids were performed on a Perkin Elmer F.40 automatic head-space analyser.

A 4-ml volume of a glucose-broth culture was pipetted into the vial provided and 0.1 ml of 9M H₂SO₄ was added. The vial was closed with a 20-mm butyl rubber serum stopper and the stopper sealed in position with an aluminium ring. The sealed vials were placed in the water bath at 90°C and left for 30 min. to equilibrate. The machine automatically sampled and analysed the vapour phase of each vial.

The chromatograph was fitted with a flame-ionisation detector and a 2-metre stainless steel column packed with 15% Carbowax 20M and 2% phosphoric acid Chromosorb W 80-100 mesh (prepared column no. B.S.13865, part no. 498-1222 available from Perkin Elmer Ltd, Beaconsfield). The carrier gas was oxygen-free N₂ passing at 30 ml per min. The column-oven temperature was 130°C and the detector oven was at 200°C. A 5-second injection period was used.

Identification of the isolates

Biochemical tests were performed and volatile acid end-products analysed on all the isolates. When necessary, tests for the production of toxins and non-volatile acid end-products were also performed. Isolates were assigned to species as defined by Buchanan and Gibbons (1974), with reference also to Smith and Holdeman (1968), Willis (1969) and Holdeman and Moore (1972).

In view of our interest in the metabolic significance of clostridia in the intestine, the isolates were assigned to three main groups on the basis of analysis of the end-products of glucose

TABLE I
The number of clostridia in faeces from various sources

Country of origin	Number of subjects	Log ₁₀ number of viable clostridia per gram of faeces: mean (and range*)	
		Clostridia producing lecithinase	Clostridia not producing lecithinase†
England	36	4.2 (D-7.4)	5.7 (D-6.7)
Scotland	11	4.1 (D-7.0)	5.6 (3.2-6.9)
Wales	65	4.2 (D-7.7)	4.7 (D-7.2)
USA	8	3.7 (D-6.2)	5.4 (D-6.5)
Uganda	19	4.0 (D-7.0)	5.2 (D-7.2)
Japan	11	4.7 (2.4-7.6)	5.5 (3.8-6.7)
Hong Kong	25	4.7 (D-7.1)	5.5 (3.2-7.2)

* D = less than 100 organisms per gram of faeces.

† Counted after heating the specimen at 70°C for 10 min.

TABLE II
The source and percentage of specimens yielding one, two, three, or more species of clostridia

Country of origin	Percentage number of specimens yielding the stated number of species per sample			
	1	2	3	4 or more
England	19	33	19	28
Scotland	36	27	9	27
Wales	26	29	23	21
USA	37.5	37.5	25	...
Uganda	32	47	21	...
Japan	45.5	27	18	9
Hong Kong	16	32	28	24

... = none detected.

metabolism. The first group comprised clostridia that produced acetic acid as their major metabolic product, the second organisms that produce butyric acid alone or with acetic acid as their major products, and the third group clostridia that produce a complex mixture of acids. Most of the frankly proteolytic clostridia are found in this third group.

RESULTS

The number of clostridia detected was similar in all the groups of specimens studied (table I); clostridia were detected in all save one of the specimens. Only one species of clostridium was isolated from 26% of the samples, and 20% yielded four species or more (table II).

Most of the clostridia isolated from specimens from England, Scotland, Wales and the USA, were assigned to species; only 4% of these strains were

TABLE III
The identity of the major groups of isolates

Identity of isolate	Number of strains isolated from						
	England	Scotland	Wales	USA	Uganda	Japan	Hong Kong
Group I							
<i>Clostridium indolis</i>	3	4	...	...	4	2	...
<i>C. putrefaciens</i>	3	...	34	1	...	...	...
<i>C. ramosum</i>	10	...	29	4	...	8	4
Others	6	1	1	2	2	1	4
Unidentified	23	...	4	...	4	1	7
Group II							
<i>C. felsineum</i>	8	8	...	...	7	...	1
<i>C. paraputrificum</i>	49	33	105	9	6	7	8*
<i>C. perfringens</i>	31	9	67	10	21	11	3†
<i>C. rectum</i>	4	...	11	...	2	...	6
<i>C. sporosphaeroides</i>	5	...	31	...	...	...	...
Others	29	16	20	4	7	8	15
Unidentified	...	...	4	...	15	...	19
Group III							
<i>C. bifermentans</i>	9	20	12	3	5	3	2†
<i>C. cadaveris</i>	1	7	...	...	3	...	...
<i>C. sporogenes</i>	7	8	3	1	...	1	...†
Others	3	...	1	1	2	...	...
Unidentified	...	...	...	...	...	4	9

... = none detected.

* Only one of these strains was able to dehydrogenate the steroid nucleus.

† Lecithinase-producing colonies were subcultured from three specimens.

unidentified. In contrast, 29% of the strains from Uganda, Japan and Hong Kong remain unidentified.

C. perfringens, *C. bifermentans* and *C. paraputrificum* were isolated from all the groups of specimen examined (table III); *C. perfringens* was equally common in all groups, whereas *C. bifermentans* was most common in specimens from Scotland. *C. paraputrificum* was common in specimens from England, Scotland, Wales and the USA but occurred less frequently in specimens from Uganda, Japan and Hong Kong (table IV). Of the eight strains of *C. paraputrificum* obtained from specimens originating in Hong Kong, seven were atypical in that they were unable to dehydrogenate the steroid nucleus and did not hydrolyse aesculin.

Many other species of clostridia were detected during this study; however, most were isolated so infrequently that no comment can be made as to their distribution (table V).

DISCUSSION

The organisms isolated in this study represent the most numerous clostridia present in the specimens examined that (1) were lecithinase positive or (2) produced spores. Attebury, Sutter and Finegold (1974) and Moore and

TABLE IV
The percentage number of specimens yielding the major groups of isolates

Identity of isolate	Percentage number of specimens from						
	England	Scotland	Wales	USA	Uganda	Japan	Hong Kong
Group I							
<i>C. indolis</i>	6	36	...	...	16	9	...
<i>C. putrefaciens</i>	6	...	21.5	12.5	...	...	...
<i>C. ramosum</i>	17	...	12	37.5	...	27	16
Others	14	9	1.5	25	10.5	9	12
Unidentified	33	...	5	...	16	9	28
Group II							
<i>C. felsineum</i>	11	45.5	...	...	10.5	...	4
<i>C. paraputrificum</i>	61	91	43	75	26	36	12*
<i>C. perfringens</i>	53	54.5	60	75	53	64	12†
<i>C. rectum</i>	11	...	14	...	10.5	...	20
<i>C. sporosphaeroides</i>	11	...	21.5	...	...	...	...
Others	39	64	21.5	25	16	27	56
Unidentified	...	...	5	...	47	...	44
Group III							
<i>C. bifermentans</i>	19	73	17	25	21	18	8†
<i>C. cadaveris</i>	3	54.5	...	...	10.5	...	...
<i>C. sporogenes</i>	17	45.5	3	12.5	...	9	...†
Others	8	...	1.5	12.5	10.5	...	...
Unidentified	...	...	...	...	...	27	16

... = none detected.

* Only one of these strains was able to dehydrogenate the steroid nucleus.

† Lecithinase-producing colonies were subcultured from only three specimens.

Holdeman (1974) isolated *C. aminovalericum*, *C. oroticum*, *C. ramosum*, *C. innocuum*, *C. paraputrificum*, *C. perfringens*, and *C. sporosphaeroides* from faeces by use of selective media and by subculture of large numbers of colonies from non-selective media. Clostridia designated as groups "A" and "D" were detected and described by Moore and Holdeman (1974) during their study of Japanese-Hawaiians. The organisms detected in the latter studies may well have been present as vegetative organisms, and indeed the high bacterial counts reported by these investigators, compared with counts in this study, support this contention. The use of heat-treated specimens enabled us to detect a wider range of bacterial species than did these other workers, but at the expense of a reduction in bacterial numbers.

The use of serial dilutions inevitably leads to the masking of organisms present in small numbers by those in larger numbers and it is likely that clostridial flora of faeces is even more diverse than appears in the present study. Further, the isolation rate of any particular species is affected. Thus, the lower-than-expected rates for the isolation of *C. perfringens* may in part be accounted for by the masking of these organisms in mixed populations. If suitable enrichment procedures were employed it is likely that a much higher

TABLE V
The identity of the minor groups of isolates

Species	Number of subjects yielding the stated species	Countries of origin
Group I		
<i>C. aminovalericum</i>	2	England
<i>C. limosum</i>	3	USA, Japan, Hong Kong
<i>C. oroticum</i>	4	England, USA, Uganda
<i>C. sphenoides</i>	7	England, Scotland, Wales, Hong Kong
Group II		
<i>C. acetobutylicum</i>	1	England, Wales
<i>C. aurantibutyricum</i>	1	Hong Kong
<i>C. bejerinkii</i>	2	Hong Kong
<i>C. butyricum</i>	2	Wales, USA
<i>C. chauvoei</i>	7	England, Scotland, Uganda and Japan
<i>C. innocuum</i>	8	England, Hong Kong
<i>C. malenominatum</i>	3	USA, Japan
<i>C. pasteurianum</i>	3	Scotland, Uganda
<i>C. plagram</i>	1	Scotland
<i>C. pseudotetanicum</i>	3	England
<i>C. sartagoformum</i>	9	England, Wales
Group A*	6	England, Wales, Hong Kong
Group D*	3	England, Hong Kong
Group III		
<i>C. difficile</i>	1	USA
<i>C. glycolicum</i>	1	England
<i>C. irregularis</i>	1	Uganda
<i>C. putrificum</i>	3	England, Hong Kong
<i>C. sticklandii</i>	1	Uganda

* Un-named groups described by Moore and Holdeman (1974). These organisms may constitute new species.

rate of isolation would be achieved both for *C. perfringens* and other prominent clostridia.

Clostridia that produce lecithinase are of particular interest because of the importance of *C. perfringens*. The analysis of the acid end-products of glucose metabolism is an additional aid to their identification. *C. limosum*, *C. perfringens* and *C. bifermentans* all produce lecithinase detectable on media containing egg-yolk, but these species differ widely with respect to their end-products. *C. sordellii* differs from the other organisms in this group in its ability to hydrolyse urea and can also be distinguished from *C. perfringens* and *C. bifermentans* on the basis of end-product analysis (Holdeman and Moore, 1972). *C. barati*, an organism resembling *C. perfringens*, was isolated from faeces by Attebery *et al.* (1974), but was not detected in this study.

C. paraputrificum was isolated more often from samples from England, Scotland, Wales and the USA than from samples from other areas. The relationship of these isolation rates to incidence of cancer of the colon is

FIGURE.—The relation of the rates of isolation of *C. paraputrificum* to the incidence of cancer of the colon (incidence data of Doll, 1969).

represented in the figure. This relationship is consistent with our hypothesis that *C. paraputrificum*, because of its ability to dehydrogenate the steroid nucleus, is implicated in the causation of cancer of the colon. The results from Hong Kong need explanation; the incidence data quoted relate to the whole population, whereas incidence data for the sub-population examined by us are not yet available. In a retrospective study of patients with cancer of the large bowel, *C. paraputrificum* was isolated more often from the faeces of cancer patients than of other patients (Hill *et al.*, 1975). The results of the present study, when considered in conjunction with those of our studies on cancer patients, provide a *prima facie* case for the involvement of *C. paraputrificum* in the aetiology of cancer of the colon and underline the need for more information about the factors allowing the persistence of particular clostridia in the gut.

SUMMARY

Clostridia were isolated from human faecal samples from England, Scotland, Wales, USA, Hong Kong, Uganda and Japan. Strains were identified on the

basis of various biochemical reactions and on the type of fatty acid produced from glucose. Analysis of these acids was by gas-liquid chromatography.

Clostridium paraputrificum was common in stool samples from England, Scotland, Wales and USA, but rare in samples from Hong Kong, Uganda and Japan.

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