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COMPARATIVE STUDIES OF BACILLUS CEREUS STRAINS ISOLATED FROM VARIOUS FOODS AND FOOD POISONING OUTBREAKS

By

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RAEVUORI, M., T. KIUTAMO and A. NISKANEN: *Comparative studies of Bacillus cereus strains isolated from various foods and food poisoning outbreaks.* Acta vet. scand. 1977, *18*, 397—407. — Certain properties of 22 Bacillus cereus strains isolated from different foods and food poisoning episodes were investigated in order to evaluate possible differences between strains isolated from diarrhoeal and vomiting type food poisoning outbreaks.

None of the strains isolated from vomiting type episodes produced acid from salicin and mannose, whereas 80 and 40 % of the strains from diarrhoeal type outbreaks were positive, respectively. No association between the antibiotic sensitivity pattern or the fatty acid composition and the source of a strain could be found, although some strains differed from the general pattern of B. cereus in some instances. No significant differences in the production of the skin factor between strains isolated from the two types of outbreaks were found either. The findings of this study support the observation that the food environment itself essentially affects the enterotoxin formation of B. cereus.

bacillus cereus; food poisoning; enterotoxin; antibiotic sensitivity; fatty acid composition; skin test.

Bacillus cereus is known to be the causative agent of two distinct types of food poisoning. The older and therefore better known type is characterized by diarrhoea, abdominal pain and an incubation time of from 8 to 16 hrs. The first report of an outbreak with diarrhoeal type syndromes evidently caused by B. cereus was published by Lubenau in Germany (*Lubenau* 1906). The first detailed description of this type of food poisoning, however, was published by Hauge in Norway no earlier than 1950 (*Hauge* 1950). Different foods have been implicated in the outbreaks reported from several countries particularly in Europe.

In 1972 the first report of another type of B. cereus food poisoning, characterized by nausea and vomiting and with an incubation period of only 1 to 5 hrs., was published (*Public Health Laboratory Service* 1972). Since then several outbreaks of this type have occurred in different countries, including Finland, all associated with the consumption of boiled or fried rice.

Goepfert et al. (1972) and more recently *Gilbert & Taylor* (1976) have reviewed the role of B. cereus as a food poisoning organism. Some recent reports of outbreaks have not yet been available for review: An episode due to ingestion of vegetable sprouts in the USA (*Portnoy et al.* 1976), and the rice outbreaks in Canada (*Mathias et al.* 1976), Netherlands (*Beckers* 1976), and Finland (*Raevuori et al.* 1976).

B. cereus is known to produce at least three distinct extracellular toxins: haemolysin, lethal toxin and phospholipase C (*Johnson & Bonventre* 1967). Using the rabbit ligated ileal loop technique, the fact that haemolysin and phospholipase C do not cause fluid accumulation, as an enterotoxic substance produced by B. cereus does, has been established (*Goepfert et al.* 1973). This enterotoxic substance, which is protein in nature, is synthesized and secreted during the logarithmic phase of bacterial growth. The enterotoxin has recently been purified both in the USA (*Spira & Goepfert* 1975) and the USSR (*Ezepchuk & Fluer* 1971, *Gorina et al.* 1975), but practical serological detection methods are not yet available.

The purpose of this study was to evaluate the possible differences in certain bacteriological characteristics between B. cereus strains isolated from diarrhoeal and vomiting type food poisoning outbreaks as well as other sources. The following criteria were used in the comparison: certain biochemical tests, antibiotic resistance, fatty acid composition of the bacterium, and the formation of the enterotoxic substance, evaluated using the rabbit skin vascular permeability test.

MATERIALS AND METHODS

Strains

The bacterial strains used in the study are shown in Table 1. Stock cultures of the organisms on porcelain beads were prepared according to the method of *Hunt et al.* (1958).

Table 1. Origins of the 22 B. cereus strains studied.

Group	Strain number	Source	Country
1 Isolated from diarrhoeal type outbreaks	F 4370	Barbecued chicken	Canada
	F 4433	Meat loaf	USA
	B-4ac	?	USA
	VTT 346	Barbecue oil	Finland
	VTT 358	Jellied meat	Finland
2 Isolated from vomiting type outbreaks	F 3502	Fried rice	Great Britain
	F 3605	Boiled rice	Great Britain
	F 4431	Indonesian rice dish	Netherlands
	F 4810	Vomit	Great Britain
	VTT 352	Boiled rice	Finland
3 Isolated from different sources	ATCC 14579	? (neotype)	USA
	ATCC 9139	?	USA
	2006	?	USA
	01552	?	USA
	A-1	?	USA
	VTT 305	?	Finland
	VTT 329	Caseinate	Finland
	VTT 331	Smoked fish	Finland
	VTT 332	?	Norway
	VTT 354	Rice	Finland
	VTT 355	Garlic salt	Finland
	VTT 356	White pepper	Finland

Bacteriological methods

Generally accepted methods and growth media were used. The media were prepared in the State Veterinary Medical Institute. Commercial agar bases were used. The incubation temperature was 37°C and the incubation times as follows: blood agar, 24 hrs; lecithinase agar, 24 hrs; indole formation, 48 hrs; nitrate reduction, 48 hrs; production of acetylmethylcarbinol, 48 hrs; citrate utilization, 48 hrs; production of urease, 24 hrs; motility, 24 hrs; and sugar fermentation media, 4 days.

Antibiotic sensitivity

The sensitivity patterns of the 22 strains for the eight antibiotics shown in Table 3 were analyzed using diagnostic sensitivity test agar plates (Oxoid) and Neo-sensitabs (Rasco, Taa-

strup, Denmark) disks. The optic densities of the bacterial suspensions used were measured by a spectrophotometer (Unicam SP-600, Cambridge, England) and were found to be between 79.5 and 94.5 at 650 nm (red filter). These figures corresponded to plate counts 5.8×10^7 and 1.8×10^7, respectively, determined by plating on blood agar. The cultivated disks were incubated for 24 hrs. at 37°C. The length of a single inhibition zone was used as the indicator of antibiotic sensitivity.

Fatty acid composition

Gas chromatographic analyses of the bacterial fatty acid composition were made using the method of *Niskanen et al.* (1975).

Enterotoxin production and the skin test

A method described by *Spira & Goepfert* (1975) was used. Brain heart infusion broth (Difco) supplemented with 0.1 % glucose was used as the growth medium. Protein was quantitatively recovered after the ammonium sulphate saturation of the culture filtrate and centrifugation (Sorvall RC2-B, 30 min. at $12840 \times g$) in a sodium bicarbonate buffer, pH 7.9, in a volume 1/100 that of the original volume of culture filtrate. Assays for the permeability-affecting activity of culture fluids and culture fluid concentrates were made according to a method by *Glatz et al.* (1974). The New Zealand white rabbits used in the tests weighed 3 to 4 kg. Physiological saline, the growth medium and the buffer solution were used as controls. The mean length of two perpendicular measurements of the radius of an individual skin reaction area was used as the basis for comparative calculations.

RESULTS

Certain biochemical characteristics of the B. cereus strains studied are shown in Table 2. The sensitivity patterns for the eight antibiotics used in the study of all the 22 strains as well as for the strains originating from diarrhoeal and vomiting types of food poisoning outbreaks are shown in Table 3. Four strains had statistically significant differences ($P < 0.05$) for at least one antibiotic. The length of the inhibitory zone for the individual strains differed from the mean inhibitory zone of all the strains by more than two standard errors in the following cases:

F 3502, very sensitive to ampicillin and penicillin; F 4370 and VTT 332, very resistant to neomycin; and VTT 356, very resistant to tetracycline.

T a b l e 2. Certain biochemical characteristics of the B. cereus strains studied.

Test	Percentage of positive reactions		
	all strains studied	strains isolated	strains isolated
	(groups 1, 2 and 3, Table 1, n = 22)	from diarrhoeal type outbreaks (group 1, Table 1, n = 5)	from vomiting type outbreaks (group 2, Table 1, n = 5)
Haemolysis of calf blood	100	100	100
Production of lecithinase	100	100	100
Indole formation	0	0	0
Nitrate reduction	86	100	80
Production of acetyl-methylcarbinol	100	100	100
Citrate utilization	100	100	100
Production of urease	9	0	0
Motility	100	100	100
Acid formation from:			
Adonitol	0	0	0
Arabinose	0	0	0
Dulcitol	0	0	0
Fructose	91	80	100
Galactose	0	0	0
Glucose	100	100	100
Glycerol	100	100	100
Inositol	0	0	0
Inuline	0	0	0
Lactose	0	0	0
Maltose	5	0	0
Mannitol	0	0	0
Mannose	27	40	0
Raffinose	0	0	0
Rhamnose	0	0	0
Saccharose	64	60	80
Salicin	55	80	0
Sorbitol	0	0	0
Trehalose	100	100	100
Xylose	0	0	0

The mean fatty acid compositions in percentages of the strains isolated from diarrhoeal and vomiting types of outbreaks are shown in Table 4. Strain F 4431 differed in its fatty acid com-

T a b l e 3. Patterns of sensitivity to certain antibiotics of B. cereus
strains from different sources.

Antibiotic tested	Lengths of inhibitory zones (mm) (mean ± s)		
	all strains studied (groups 1, 2 and 3, Table 1, n = 22)	strains isolated from diarrhoeal type outbreaks (group 1, Table 1, n = 5)	strains isolated from vomiting type outbreaks (group 2, Table 1, n = 5)
Ampicillin	2.11 ± 2.85	1.12 ± 1.23	5.41 ± 4.31
Chloramphenicol	10.74 ± 1.00	10.56 ± 1.41	11.20 ± 0.84
Erythromycin	9.75 ± 3.08	8.92 ± 4.45	10.29 ± 1.61
Neomycin	8.99 ± 0.46	9.35 ± 0.23	9.02 ± 0.33
Penicillin	0.43 ± 2.04	0.00 ± 0.00	1.91 ± 4.27
Polymyxin	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Streptomycin	8.92 ± 2.56	7.05 ± 3.95	9.66 ± 1.15
Tetracyclin	8.62 ± 1.69	8.90 ± 0.80	10.07 ± 0.57

T a b l e 4. Mean fatty acid composition (± s) of B. cereus strains
isolated from different types of food poisoning.

Fatty acid	Percentage of fatty acid (mean ± s). Strains isolated from	
	diarrhoeal type outbreaks (group 1, Table 1, n = 5)	vomiting type outbreaks (group 2, Table 1, n = 5)
br C 13:0	15.60 ± 2.16	15.46 ± 3.89
br C 14:0	3.30 ± 1.07	2.68 ± 0.43
n C 14:0	2.36 ± 0.66	3.16 ± 2.39
br C 15:0	22.70 ± 4.16	20.80 ± 4.38
n C 15:0	0.20 ± 0.00	0.20 ± 0.00
br C 16:0	2.82 ± 1.12	2.80 ± 0.77
n C 16:0	26.04 ± 5.88	29.96 ± 9.78
br C 17:0	15.62 ± 3.01	16.70 ± 4.45
n C 17:0	0.20 ± 0.00	0.20 ± 0.00
br C 18:0	0.00 ± 0.00	0.98 ± 0.90
n C 18:0	5.32 ± 3.08	4.08 ± 0.86
n C 18:1	5.92 ± 2.40	2.98 ± 2.85

position from the other nine strains analysed. The individual
fatty acid composition differed by more than two standard
errors from the mean of the 10 strains in the case of four fatty
acids.

The mean radii of the areas of vascular permeability due to
intracutaneous injection of B. cereus culture filtrate or culture

T a b l e 5. Vascular permeability of rabbit skin after intracutaneous injection of B. cereus culture filtrate and culture filtrate concentrate from strains of different origin.

	Mean radius (in mm $\pm$ s) of the blueing area of the rabbit skin	
	experiment 1	experiment 2
Strains from diarrhoeal type outbreaks (group 1, Table 1, n = 5)		
— Culture filtrates	1.48 ± 0.29	0.95 ± 0.62
— Culture filtrate concentrates	4.20 ± 1.48	4.20 ± 1.72
Strains from vomiting type outbreaks (group 2, Table 1, n = 5)		
— Culture filtrates	1.45 ± 0.57	0.70 ± 0.27
— Culture filtrate concentrates	4.15 ± 1.42	3.50 ± 1.00

filtrate concentrates prepared from strains originating from diarrhoeal and vomiting type food poisoning outbreaks are presented in Table 5. Every culture filtrate caused a blueing reaction in the skin, the area of which ranged from 0.8 to 12.6 mm². The respective figures for the culture concentrates were 19.6 and 132.7 mm². No significant differences were found between the reactions caused by the different strains studied.

DISCUSSION

The results of the biochemical tests (Table 2) follow the patterns that are considered typical for B. cereus (*Goepfert et al.* 1972, *Gibson & Gordon* 1974, *Gilbert & Taylor* 1976). The tendency for strains from vomiting type outbreaks to produce acid from salicin, reported by *Gilberg & Taylor*, was found in this study, too. Four of the five isolates from vomiting type food poisoning outbreaks were obtained from Dr. R. J. Gilbert and obviously had been used in his former studies.

. No association between an antibiotic sensitivity pattern and the source of a strain could be found in our studies. All the strains tested were resistant to polymyxin, and only one strain (F 3502) was susceptible to penicillin.

Gas chromatographic fatty acid analysis of a B. cereus cell has been used as a diagnostic tool (*Niskanen et al.* 1975) as well as in epidemiological investigations (*Raevuori et al.* 1976). With the exception of one strain the gas chromatograms of the 10

strains analysed in this study (Table 4) followed the general pattern for B. cereus (*Niskanen et al.*). Strain F 4431 differed considerably from this typical fatty acid composition. According to the results the gas chromatograms cannot be used for distinguishing between B. cereus strains isolated from diarrhoeal and vomiting type outbreaks.

Spira & Goepfert (1972) used the ligated rabbit ileal loop test for the detection of the enterotoxic substance produced by B. cereus. *Glatz & Goepfert* (1973) found a good correlation between the results obtained from the loop test and the guinea-pig skin assay method. The skin test was further developed by *Glatz et al.* (1974), using rabbit as the test animal. This test correlated very well with the loop test and was more sensitive and also more convenient to carry out. In our study the methodology of *Spira & Goepfert* (1975) was followed in the production and concentration of the enterotoxic substance and its detection by the rabbit skin test.

According to the results of this study, no significant differences in the production of the permeability factor between the strains from diarrhoeal and vomiting type outbreaks can be found. Thus the rabbit skin permeability test cannot be used to distinguish between these two aetiologically differing strains. There are differences between the two experiments made for each strain (Table 5). This can be explained to a certain extent by a difference in test animals: an older and heavier animal was used in the second experiment. The difference in the levels of response does not affect the comparative conclusions because only one rabbit was used per experiment.

Melling et al. (1976) were able, by means of the rabbit ileal loop test carried out according to *De & Chatterje* (1953), to distinguish between strains isolated from vomiting and diarrhoeal type episodes. According to our findings both of the strains they used (F 4810 and F 4433) were skin-positive, although F 4433 gave a considerably stronger reaction. The skin test is significantly more sensitive than the ileal loop test (*Glatz & Goepfert* 1973). Thus it is possible that the strains isolated from vomiting type outbreaks produce amounts of the enterotoxic substance which are too small to be detected by the ileal loop technique, but large enough to give a positive skin response.

The small number of replications (two) in the skin test experiments does not allow for statistically sound conclusions.

The results, however, indicate that B. cereus strains which cause vomiting type food poisoning outbreaks also produce the skin permeability factor. Variations in toxin formation and destruction in different foods and laboratory growth media have been demonstrated for many food poisoning bacteria, including B. cereus (*Baird-Parker* 1971, *Ivers & Potter* 1977). For instance B. cereus produces haemolysin and phospholipase C in pea but only lethal toxin in banana (*Ivers & Potter*). *Spira & Goepfert* (1972) noticed in their studies of the ileal loop factor that the nature of the bacterial growth medium was important for the toxic response. Certain nutrients are obviously required for the formation of the toxin. Enzyme induction, caused by certain chemicals in the environment, could also explain differences in toxin formation observed in various media. Recently, evidence has accumulated that the vomiting and diarrhoeal type food poisoning are due to the production of two distinct toxins (*Turnbull* 1976, *Melling et al.*).

Taylor & Gilbert (1975) have developed a serotyping scheme for B. cereus. According to studies by *Gilbert & Parry* (1977) the selection of strains of particular serotype occurs during the preparation of cooked and fried rice, due evidently to differences in heat resistance or growth rate. Our study did not involve these parameters.

According to the results of this study, B. cereus strains isolated from the vomiting and diarrhoeal types of food poisoning outbreaks do not differ significantly in their biochemical characteristics, antibiotic resistance, fatty acid composition or formation of skin permeability factor in laboratory medium. There are obvious differences in toxin production of B. cereus strains isolated from different types of food poisoning outbreaks. In order to study this variation in different foods, immunological methods of detection of the enterotoxic substances must be developed.

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SAMMANFATTNING

Jämförande studier över stammar av Bacillus cereus isolerade från olika livsmedel och fall av matförgiftningar.

I detta arbete undersöktes vissa egenskaper hos 22 stammar av Bacillus cereus, isolerade från olika livsmedel och fall av matförgiftning. Meningen var att försöka kartlägga eventuella skillnader mellan stammarna isolerade från matförgiftningar av diarré- och uppkastningstyp.

Alla stammar isolerade från fall av uppkastningstyp producerade inte syra från salicin och mannos, medan 80 och respektive 40 % av stammarna från fall av diarrétyp var positiva. Inga samband mellan antibiotikasensibilitet eller fettsyrasammansättning och källan för en stam kunde påvisas, fastän några stammar i vissa fall avvek från det allmänna mönstret for B. aureus. Någon signifikant skillnad i framkallandet av den s.k. hudfaktorn mellan stammar isolerade från de två olika typerna av matförgiftning kunde inte heller påvisas. Resultaten stöder uppfattningen att miljön i livsmedlen i sig själv i väsentlig grad påverkar bildningen av enterotoxin hos B. cereus.