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NECROTIZING INFECTIOUS ENTERITIS IN PIGLETS, CAUSED BY CLOSTRIDIUM PERFRINGENS TYPE C

I. BIOCHEMICAL AND TOXIGENIC PROPERTIES OF THE CLOSTRIDIUM*)

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Cl. perfringens is characterized partly by its biochemical reactions, partly by its formation of toxins (i.e. toxic antigens).

The subdivision of Cl. perfringens into types A, B, C, D, and E, which is based on the work of *Wilsdon* (1931, 1933), *Glenny et al.* (1933), and *Bosworth* (1940), rests upon the demonstration of one or more of the major, i.e. lethal antigens α, β, ε, and ι.

Demonstration of minor antigens may be of interest in studies of so-called degraded strains, i.e. strains that have lost one of the determinant major type antigens (*Borthwick* 1937; *Dalling & Ross* 1938; *Taylor* 1940), and for the establishment of subtypes.

Cl. perfringens type C can be divided into a number of subtypes (*Brooks et al.* 1957). One of these is the porcine subtype, which is, among other things, characterized by lacking the δ-antigen and by showing non-αδθ hemolysis on ox-blood and horse-blood agar plates.

In a previous paper (*Høgh* 1965) an account was given of the first two outbreaks of necrotizing infectious enteritis ("enterotoxemia") in piglets in Denmark.

In the present study a series of Danish strains of Cl. perfringens, and a few foreign strains, have been examined with regard to biochemical activity and toxin production.

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MATERIAL AND METHODS

The study included 23 strains, 20 of which originated from fatal cases of necrotizing enteritis among piglets in Danish herds of swine, while 2 were of British[1]) and 1 of Hungarian[2]) origin.

The strains were stored in the freeze-dried state. Just before being submitted to examination they were reconstituted in Robertson's Cooked Meat Medium, and thereafter checked for purity on 5 % blood-agar plates which were incubated at 37°C for 18 hrs. under aerobic and anaerobic conditions (Filde & Mc Intosh's jar).

Liquid media were steamed for 20 min. and cooled to about 30°C just before use.

Biochemical activity

Eighteen-hour-old cultures in the basal fermentation medium (i.e. Bacto Thioglycollate Medium w/o dextrose or indicator) were used as seed cultures.

Fermentation tests were made in Bacto Thioglycollate Medium with 1 % of the respective sugars added from mechanically sterilized stock-solutions. Bromcresol-purple (0.1 ml of a 1.6 % alcoholic solution to 100 ml medium) was used as indicator. Readings were taken after incubation for 24 hrs., whereafter the cultures were observed through 10 days.

Testing for indol formation was done by means of Ehrlich's reagent and, in addition, by the vanillin-violet reaction. For the latter reaction, 5 ml conc. HCl and 1 ml 5 % alcoholic vanillin solution were added to 10 ml culture. Both tests were made on three-day-old cultures in Cooked Meat Medium.

Nitrate-reduction, gelatin-liquefaction, H_2S-formation, and reaction in milk were studied by the technique and in the media indicated by *Spray* (1936). Sulphanilic acid and dimethyl-α-naphthylamine were used for demonstrating nitrite. In cases of negative nitrite reaction examination for unreduced nitrate was made with powdered zinc (*ZoBell* 1932) as indicated by *Reed* (1942).

Testing for ability to reduce sulphite to sulphide was done in tall iron-sulphite agar tubes (*Skovgaard* 1958).

Proteolytic properties were studied on coagulated serum and egg-white, as indicated by *Sterne & van Heyningen* (1958).

Examination for heat resistance

Four-day-old cultures in Bacto Thioglycollate Medium with 1 % glucose were tested at 100°C by the method described by *Brooks et al.* (1957).

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[1]) The British strains, designated CWC 6 and 8, were received from Dr. P. D. Thomson, Central Veterinary Laboratory, Weybridge.

[2]) The Hungarian strain, designated 963-XII-17, was received from Dr. St. Szabo, Vice-Director, Central Veterinary Institute, Budapest.

Examination for hemolytic activity

The ability of strains to hemolyse ox and horse blood in the presence of α, δ, and θ antitoxins (non-αδθ hemolysis) was tested on blood-agar plates composed as follows:

Meat-infusion peptone agar (2 % agar)	15 ml
Ten % suspension of washed calf- or horse-blood cells	0.5 ml
Serum R5604	0.5 ml
Serum R3601	0.4 ml

This would give a content of 125 α, 360 δ, and 240 θ antitoxin units per plate. Each strain was spread on half the surface of a plate, and the plates thereafter incubated anaerobically for 18 hrs.

Examination for toxin formation

Culture medium. VF broth (*Prévot* 1960) adjusted to a pH of 7.5 before being autoclaved (121°C for 15 min.) was used as substrate for toxin formation. The broth was dispensed in 300 ml portions in Erlenmeyer flasks and after steaming enriched with 1 % glucose from a 50 % stock solution which had been sterilized by filtration.

Preparation of toxic filtrates. Each flask with VF broth was inoculated with about 30 ml (corresponding to 1/10 of the volume of broth) of an 18-hour-old culture in Cooked Meat Medium and thereafter incubated at 37°C for 7 to 8 hrs. The resulting cultures were centrifuged and sterilized by filtration. Filtrates were stored at —20°C.

Sera. Toxins were identified by means of Cl. perfringens antitoxins, except for the ν toxin, for which no specific antitoxin was available. This toxin was therefore demonstrated merely by its viscosity-reducing effect on deoxyribonucleic acid.

The antitoxin levels (units/ml) of the sera employed were as follows:

Serum R5604:	220 α
„ R5638:	120 θ
„ C3601:	38 α, 900 δ, 600 θ
„ R1974:	400 ϰ
„ RX5755:	5000 λ
„ CWA, K4902 *)	1050 α
„ CWC, K5266 *)	12 α, 2300 β

Trypsinization. A preparation (Pancreatic Trypsin Novo, Salt-free) containing 6 Anson Units of trypsin/g was used for trypsinization of the sterile filtrates. One mg/ml was added to the filtrates, which were thereafter incubated for 2 hrs. at 37°C.

Demonstration of α, δ, and θ toxins. Examination for α, δ, and θ toxins was made by means of test-sera R5604, R5438, and C3601. The procedure followed was that indicated by *Oakley & Warrack* (1953), and the sera were used in dilutions corresponding to the antitoxin

*) Diagnostic type A and type C antitoxins from Burroughs Wellcome & Co.

The other sera were kindly supplied by Dr. Irene Batty, the Wellcome Research Laboratories.

levels employed by these workers. The effect of the toxins was demonstrated by the lecithovitellin test and by test for hemolysis. For the lecithovitellin test a mechanically sterilized 5 % suspension of egg-yolk in saline was used, for the hemolytic test a 10 % suspension of washed sheep-blood cells. Borate-buffered saline (B.B.S.), pH 8, and phosphate-thiolacetic-acid-buffer ("Phosthio") (*Brooks et al.*) were used as diluents. For θ-negative strains the examination was repeated on centrifuged, unfiltered cultures, grown for 5 hrs. under a seal of liquid paraffin. This re-examination was carried through in a reduced system (*Brooks et al.*) and with sera R5604 and R5438 diluted to an antitoxin level of 22 units/ml.

Demonstration of β, ε, *and* ι *toxins.* Examination for β, ε, and ι toxins was made by lethality-test on mice weighing 18 to 22 g. In view of the fact that trypsinization destroys the β toxin but activates the ε and ι toxins, the following, simplified, procedure was followed:

Non-trypsinized:	1 ml filtrate, 0.1 ml saline
	1 ml filtrate, 0.1 ml serum CWA, K4902
	1 ml filtrate, 0.1 ml serum CWC, K5266
Trypsinized:	1 ml filtrate, 0.1 ml saline

The mixtures were left at room temperature for 30 min., whereafter 0.4 ml of each mixture was inoculated i. v. into each of 2 mice.

Trypsinized filtrates which were found toxic were re-tested, partly with serum CWA, K4902, partly with saline added.

Demonstration of α *and* λ *toxins.* Examination for α and λ toxins was made by the Azocoll method (*Oakley & Warrack* 1953). The Azocoll used was prepared from sifted powder of dessicated skin and coloured with azo dye as indicated by the authors. Neutralization tests with serum R1974 (1:100) and serum RX5755 (1:300) were carried out as described in the above-cited paper.

Demonstration of μ *toxin.* The method used in the examination for μ toxin was *Oakley & Warrack*'s (1951) modification of the ACRA (*A*cid-*C*ongo-*R*ed-*A*lcohol) test (*Burnet* 1948) with bovine synovia as the source of hyaluronic acid. The hyaluronidase (μ toxin) activity was measured against a 1:16 dilution of the synovia in borate-buffered saline (pH 8), which corresponded to 8 indicating doses as determined in the preliminary test.

Demonstration of ν *toxin.* Presence of ν toxin was demonstrated by the ACRA test as described by *Oakley & Warrack* (1951). Deoxyribonucleic acid (highly polymerized, Nutritional Chemicals Co.) was used in a 0.1 % solution in veronal buffer (0.025 M, pH 7.5, with 0.003 M-$MgSO_4$ added). In the preliminary test, stable drops were obtained with dilutions of up to 1:4 of this solution of DNA. Filtrates were diluted with a 1.5 % solution of neopeptone.

RESULTS

No growth was obtained on 5 % blood-agar plates after incubation for 18 hrs. under aerobic conditions. After anaerobic

Table 1. Biochemical features and heat-resistance of the porcine subtype.

		Glucose	Lactose	Saccharose	Salicin	Maltose	Mannitol	Indol	Vanillin violet	Iron milk	Iron gelatin	Lead-acetate agar	Nitrate reduction	Iron-sulphite agar	Coagulated serum	Coagulated egg-white	100°C for 1 hr.
Danish strains	(19)	+	+	+	—	+	—	—	—	st.	+	+	+	+	—	—	n.g.
	(1)	+	+	—/+	—	+	—	—	—	st.	+	+	+	+	—	—	n.g.
British strains CWC 6		+	+	+	—	+	—	—	—	st.	+	+	+	+	—	—	n.g.
CWC 8		+	+	—/+	—/+	+	—	—	—	st.	+	+	+	+	—	—	n.g.
Hungarian strain 963-XII-17		+	+	+	—	+	—	—	—	st.	+	+	+	+	—	—	n.g.

( ): Number of strains.
—/+: Late positive.
st.: Stormy fermentation.
n.g.: No growth.

incubation growth of low, circular colonies, about 2 mm large, with a glistening or finely granular surface and an entire edge was seen. When kept for some time in liquid medium, several strains would develop colonies with a dull, granular or radially striated surface and a dentate edge. The Hungarian strain and one of the Danish strains split off rough variant colonies with a dull, striated surface and an irregularly dentate edge.

Table 1 gives the results of the biochemical tests. The strains studied have been found capable of fermenting all the carbohydrates used, except salicin and mannitol. Salicin was, however, fermented by strain CWC 8 after 10 days. This strain and 1 Danish strain did not ferment saccharose until after 2 or 3 days. Both tests for indol were negative, and in milk all strains showed stormy fermentation without blackening and without digestion of the curd. All strains blackened lead-acetate agar and gave rise to black colonies in iron-sulphite agar. Only 3 strains reduced nitrate to nitrite, and the 20 nitrite-negative strains remained negative after the addition of powdered zinc. None of the strains showed peptonization of coagulated serum or egg-white after incubation for 10 days, while gelatin was liquefied after 3 days. None of the strains survived 100°C for 1 hr.

Non-αδθ hemolysis

On blood-agar plates without addition of α, δ, and θ antitoxins hemolysis appeared as a narrow, colourless zone close to the colony. This area of complete clearance was surrounded by a broader zone in which the blood was darkened, and which cleared up when the plates were left at room temperature.

In the presence of α, δ, and θ antitoxins hemolysis was produced on ox-blood agar plates by 9 of the 15 Danish strains examined, on horse-blood plates by 12 of these strains. The 2 British strains and the Hungarian strain showed distinct hemolysis in both kinds of blood. Eight of the Danish strains were retested with regard to hemolysin production. Identical results of the two testings were obtained with 4 negative and 2 positive strains, while 2 strains were positive when first tested but negative when retested.

(α), β, ε, and ι toxins

The examinations for major antigens by the mouse-lethality test are recorded in Table 2. As will be seen, all non-trypsinized

Table 2. Examination for major antigens by lethality test on mice.

		Non-trypsinized filtrate			Trypsinized filtrate	Trypsinized filtrate		Antigens present
		control	CWA, K4902 +	CWC, K5266 +		control	CWA, K4902 +	
Danish strains	(18)	2+	2+	2—	2—			β
	(1)	2+	2+	2—	1+, 1—	2—	2—	β
	(1)	2+	2+	2—	2+	2+	2—	α, β
British strains	(2)	2+	2+	2—	2+	2+	2—	α, β
Hungarian strain		2+	2+	2—	2—			β

( ): Number of strains.
+: Died.
—: Survived.

filtrates were neutralized by CWC antitoxin, but not by CWA antitoxin. Eighteen of the Danish strains and the Hungarian strain were non-toxic after trypsinization. The 2 British strains and 1 Danish strain showed toxicity in trypsinized filtrates, but this toxicity was neutralized by CWA antitoxin. The major antigens disclosed are indicated in the last column of Table 2.

α, δ, and θ toxins

The examinations for α, δ, and θ toxins are recorded in Table 3. α toxin was produced by all strains, θ toxin by nineteen strains and δ toxin by none.

Table 3. Results of testing for presence of α, δ, and θ antigens by the method of *Oakley & Warrack* (1953).

Tube no.		1	2	3	4	5	6	7	8	9	10	Antigens
Antitoxin		o	α	o	α	o	α	θ	α,θ	α,θ	α,δ,θ	present
Danish strains	(13)	+	—	+	+	+	+	+	—	—	—	α, θ
	(3)	+	—	+	—	+	+	+	—	—	—	α, θ
	(4)	+	—	+	—	+	—	+	—	—	—	α
British strains	(2)	+	—	+	+	+	+	+	—	—	—	α, θ
Hungarian strain		+	—	+	+	+	+	+	—	—	—	α, θ
				BBS				"Phosthio"		BBS		
		L. V.		Hemolysis								

( ): Number of strains.
+: Curd or opalescence in lecithovitellin (L.V.), or hemolysis.
—: Negative.

The 4 Danish strains that were θ-negative when tested by the method of *Oakley & Warrack* (1953) were positive on testing of unfiltered culture supernatant.

κ and λ toxins

Titers of filtrates positive in the Azocoll test are given in Table 4. The reaction was inhibited by κ monospecific antitoxin, but not by λ antitoxin. The titer of strain CWC 8 was considerably higher than that of the other positive strains. The Hungarian and 15 of the Danish strains were negative in the Azocoll test.

Table 4. Results of titration of κ-positive strains by the Azocoll test.

Dilution of filtrate		Undil.	1:2	1:4	1:8	1:16	1:32	Control
Danish strains	(2)	+	+	+	—	—	—	—
	(3)	+	+	—	—	—	—	—
British strains	CWC 6	+	—	—	—	—	—	—
	CWC 8	+	+	+	+	+	—	—

( ): Number of strains.

μ and ν toxins

The μ toxin was produced by none of the strains.

Titers obtained by the ACRA test with DNA as a substrate are shown in Table 5. As will appear, all filtrates showed evidence of deoxyribonuclease (ν-toxin) activity, in that they were capable of reducing the viscosity of DNA. Titers varied from 1:4 to 1:16.

Table 5. Results of titration for ν-toxin by the ACRA test.

Dilution of filtrate		Undil.	1:2	1:4	1:8	1:16	1:32	Control
Danish strains	(5)	+	+	+	—	—	—	—
	(11)	+	+	+	+	—	—	—
	(4)	+	+	+	+	+	—	—
British strains	CWC 6	+	+	+	—	—	—	—
	CWC 8	+	+	+	+	+	—	—
Hungarian strain	963-XII-17	+	+	+	—	—	—	—

( ): Number of strains.

All observations concerning the production of the various toxins, including results of the non-αδθ hemolysis test, are summarized in Table 6. Since the foreign strains constitute but a

Table 6. Antigens produced by Cl. perfringens type C (porcine subtype).

		α	β	ε	ι	θ	δ	κ	λ	μ	ν	Non-αδθ hemolysis: ox blood	Non-αδθ hemolysis: horse blood
Danish strains	(20)	20	20	0	0	20	0	5	0	0	20	9/15*)	12/15
British strains	(2)	2	2	0	0	2	0	2	0	0	2	2	2
Hungarian strain		1	1	0	0	1	0	0	0	0	1	1	1
Brooks et al. (1957) piglet strains	(10)	10	10	0	0	8/8	0	8	0	1	9	10	10

( ): Number of strains.
*) 9/15: 9 positive of 15 strains examined.

small part of the material examined, the results obtained with piglet strains by *Brooks et al.* are added for comparison.

It will appear from this survey that α, β, θ, and ν toxins were produced by all strains examined, ε, ι, δ, λ, and μ toxins by none of them. Further, it will be seen that Danish and foreign strains differ with respect to formation of κ toxin, and also with respect to their hemolytic properties in the presence of α, δ, and θ antitoxins.

DISCUSSION

The biochemical features of the porcine subtype of Cl. perfringens type C do not differ from those of other types of Cl. perfringens (*Spray* 1936; *Niilo & Avery* 1963). Yet, amongst 379 strains, *Niilo & Avery* found 10 which were saccharose-negative after incubation for 18 hrs. One Danish and 1 British strain which had not fermented saccharose after 18 hrs. did so after 2 to 3 days. This late fermentation was not due to an inhibitory effect of the acid-base indicator, for the fermentation was found to proceed at the same rate when no indicator was added.

Only 3 of the strains were found nitrite-positive. With powdered zinc as a reducing agent no nitrate could be traced in the nitrite-negative cultures. It is therefore assumed that the strains in question have reduced the nitrate right down to NH_3.

The Danish as well as the foreign strains are characterized by producing the major antigens α and β, but not ε and ι, and

must therefore be classified as type C (*Wilsdon* 1931, 1933; *Glenny et al.* 1933).

The strains examined differ from the classical type C (the "Struck" type) by producing no δ toxin. By producing θ toxin and by being sensitive to 100°C for 1 hr. they differ from the human subtype, previously designated type F (*Oakley* 1948—49; *Zeissler & Rassfeld-Sternberg* 1948—49).

Four Danish strains showed θ toxin in unfiltered culture supernatants, but not in sterile filtrates, a phenomenon which may be due to the sensitivity to oxygen of this toxin.

While *Brooks et al.* (1957) found that 80 % of the porcine strains produced ϰ toxin, this was the case with only 25 % of the Danish strains.

Brooks et al. found non-αδθ hemolysis in 100 % of strains belonging to the porcine subtype and in 75 % of strains of the human subtype, while the rest of the subtypes examined gave no hemolysis when the α, δ, and θ toxins were blocked.

Testing for non-αδθ hemolysis was included in the present study primarily because it was desirable to have the porcine subtype characterized by the same criteria as used by *Brooks et al.*, but also because it would be a great advantage in epidemiological studies on infections with this subtype if it were possible to use this test for screening of isolates of Cl. perfringens.

It turned out that, in the presence of α, δ, and θ antitoxins, hemolysis was produced on ox-blood agar by only 60 % of the Danish strains, on horse-blood agar by 80 %. The 2 British strains and the Hungarian strain regularly produced hemolysis in the presence of the 3 antitoxins. On this point, the Danish strains have thus proved to deviate considerably from those examined by *Brooks et al.* Not only were some of them repeatedly found to be negative, but a few of them were only intermittently positive. Variable results of the non-αδθ hemolysis test have also been seen in a British material (*Batty* 1965).

The non-αδθ hemolysis test is therefore of no avail either for characterization of the porcine subtype or as a screening test in epidemiological studies on necrotizing infectious enteritis in piglets.

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SUMMARY

Twenty Danish and 3 foreign strains of the porcine subtype of Cl. perfringens type C have been studied with regard to biochemical activity and toxin formation.

The strains examined showed no deviation from the biochemical features characteristic of Cl. perfringens. Two of the strains showed delayed fermentation of saccharose, 1 of them also of salicin. Only 3 of the strains gave a positive nitrite reaction. The rest of the strains (20) presumably reduced nitrate to NH_3 within 3 days.

All of the strains examined produced the major antigens α and β, none of them ε and ι. Of the minor antigens, θ and ν were produced by all the strains, κ by 2 of the foreign strains and 5 of the Danish strains. None of the strains gave reaction for the δ, λ and μ antigens.

Some of the Danish strains were hemolytic in the presence of α, δ, and θ antitoxins, others not, while a few varied. The foreign strains were non-αδθ hemolytic.

ZUSAMMENFASSUNG

Infektiöse nekrotisierende Enteritis bei Saugferkeln verursacht durch Clostridium perfringens Typ C. I. Die biochemischen und toxigenen Eigenschaften des Clostridiums.

Zwanzig dänische und 3 ausländische Stämme von dem porcinen Subtyp C sind mit Hinblick auf die biochemischen Reaktionen und die Toxinproduktion untersucht worden.

Beurteilt auf Grundlage der biochemischen Reaktionen haben die untersuchten Stämme sich nicht von den Reaktionen unterschieden, die für Clostridium perfringens charakteristisch sind. Zwei der Stämme erwiesen sich als spät saccharosevergärend und der eine ebenfalls alt spät salicinvergärend. Nur 3 der untersuchten Stämme zeigten positive Nitrit-Reaktion. Die übrigen 20 Stämme reduzierten Nitrat zu NH_3 nach dreitägiger Inkubation.

Alle die untersuchten Stämme produzierten die Major-Antigene α und β, jedoch nicht ε und ι. Von den Minor-Antigenen wurden θ und ν von allen Stämmen produziert. Zwei von den ausländischen sowie 5 dänische Stämme zeigten positive Reaktion für κ. Keine von den Stämmen zeigten Reaktionen für δ, λ und μ.

Bei dem non-αδθ Hämolysetest wurden im dänischen Material teils hämolytische teils anhämolytische Stämme sowie Stämme mit variabler Hämolyse gefunden. Die ausländischen Stämme erwiesen sich als non-αδθ-hämolytisch.

SAMMENDRAG

Infektiøs nekrotiserende enteritis hos pattegrise forårsaget af Clostridium perfringens type C. I. Clostridiens biokemiske og toksigene egenskaber.

Tyve danske og 3 udenlandske stammer af den porcine subtype C er undersøgt med henblik på biokemiske reaktioner og toksinproduktion.

Bedømt ved de biokemiske reaktioner har de undersøgte stammer ikke adskilt sig fra de reaktioner, der er karakteristiske for Clostridium perfringens. To af stammerne fandtes sent saccharoseforgærende og den ene tillige sent salicinforgærende. Kun 3 af de undersøgte stammer gav positiv nitrit-reaktion. De øvrige 20 stammer reducerede nitrat til NH_3 efter 3 døgns inkubation.

Alle de undersøgte stammer producerede major antigenerne α og β, men ikke ε og ι. Af minor antigenerne blev θ og ν produceret af alle stammer, 2 af de udenlandske samt 5 danske stammer gav positiv reaktion for κ. Ingen af stammerne gav reaktion for δ, λ og μ.

Ved non-αδθ hæmolysetesten fandtes i det danske materiale dels hæmolytiske og dels anhæmolytiske stammer samt stammer med variabel hæmolyse. De udenlandske stammer fandtes non-αδθ hæmolytiske.

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