ROUTINE TYPING OF *CLOSTRIDIUM WELCHII*

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During the investigation of a number of epidemics of *Clostridium welchii* food poisoning (Hobbs, Smith, Oakley, Warrack & Cruickshank, 1953) the opportunity arose of examining some 500 different strains of *Cl. welchii* for toxigenicity. Work on so large a scale made it essential to develop a routine for this purpose, and after several trials we devised the following sequence of tests, which have proved entirely satisfactory in our hands, and may be of interest to others.

METHODS

Toxin production. Organisms obtained in pure culture are grown for 5 hr. in 1 % glucose broth, previously steamed for ½ hr. and cooled; comparison with other media shows that the one suggested is adequate for estimation of toxigenicity. The cultures are then filtered through Seitz sterilizing pads and the filtrates examined for toxins as soon as possible.

Table 1. *Tests for haemolysins and substances affecting egg-yolk emulsions*

Tube	Content	
1	1 ml. filtrate + 0·5 ml. saline	+ 0·5 ml. borate-buffer saline, pH 8
2	1 ml. filtrate + 0·5 ml. A	
3	1 ml. filtrate + 0·5 ml. saline	
4	1 ml. filtrate + 0·5 ml. A	
5	1 ml. filtrate + 0·5 ml. saline	+ 0·5 ml. M/5-phosphate buffer, pH 6·5 + M/10-sodium thioglycollate
6	1 ml. filtrate + 0·5 ml. A	
7	1 ml. filtrate + 0·5 ml. B	
8	1 ml. filtrate + 0·5 ml. C	
9	1 ml. filtrate + 0·5 ml. C	+ 0·5 ml. borate-buffer saline, pH 8
10	1 ml. filtrate + 0·5 ml. D	

Table 2. *Antitoxin content of sera used in haemolytic and lecithinase tests*

Serum	α-antitoxin (units per 0·5 ml.)	θ-antitoxin (units per 0·5 ml.)	δ-antitoxin (units per 0·5 ml.)
A	5·0	< 0·01	< 0·02
B	< 0·01	5·0	< 0·02
C	5·0	5·0	< 0·02
D	5·0	⩽ 5	1·0

α-antitoxin concentration expressed in international units; θ- and δ-antitoxin units expressed in terms of provisional laboratory standards.

Examination for haemolysins and for substances affecting egg-yolk emulsions. Series of tubes are set up as in Table 1. A control set of ten tubes, in which broth is substituted for filtrate, is always included in the test.

A, B, C and D are horse sera containing antitoxins to various antigens of *Cl. welchii* (Table 2).

https://doi.org/10.1017/S0022172400015527 Published online by Cambridge University Press

The tubes are mixed by inversion over non-absorbent paper and allowed to stand for half an hour. 0·5 ml. of filtered egg-yolk emulsion (L.V.) (Macfarlane, Oakley & Anderson, 1941) is added to tubes 1 and 2, and 0·5 ml. washed sheep red cells in borate-buffer saline to the remaining eight. The tubes are then incubated for 1 hr. in a water-bath at 37° C., examined for immediate haemolysis, and read finally after standing overnight at room temperature. The results are interpreted as in Table 3.

Table 3. *Results of tests for α-, θ- and δ-toxins, and conclusions to be drawn from them*

Tube ...	1	2	3	4	5	6	7	8	9	10	
Content ...	Filtrate (F)	F + anti-α	Filtrate (F)	F + anti-α	Filtrate (F)	F + anti-α	F + anti-θ	F + anti-α, θ	F + anti-α, θ	F + anti-α, θ, δ	Conclusion
	−	−	−	−	−	−	−	−	−	−	α, θ, δ absent
	+	−	+	−	+	−	+	−	−	−	α present
	+	−	+	−	−	−	−	−	−	−	α present
	−	−	+	+	+	+	−	−	−	−	θ present
	−	−	−	−	+	+	−	−	−	−	θ present
	+	−	+	−	+	+	−	−	−	−	α + θ present
	+	−	+	+	+	+	−	−	−	−	α + θ present
	+	−	+	−	+	+	+	−	−	−	α + θ present
	+	−	+	+	+	+	+	−	−	−	α + θ present
	+	−	+	+	−	−	−	−	+	−	α + δ present
	+	−	+	+	+	−	−	−	+	−	α + δ present
	+	−	+	+	+	+	−	−	+	−	α, θ, δ present
	+	−	+	+	+	+	+	−	+	−	α, θ, δ present
Buffer ...	Borate-buffer saline pH 8				Phosphate thioglycollate				B.B.S.		
Indicator...	L.V.				Sheep red cells						

In tubes containing L.V., + = opalescence. In tubes containing red cells, + = haemolysis.

If tube 2 shows opalescence, either high concentrations of α-toxin are present, or some substance other than α-, θ- or δ-toxin is active in the test. If tube 8 or tube 10 shows haemolysis, either high concentrations of α-, θ- or δ-toxin are present, or some haemolysin other than α-, θ- or δ-toxin is active. Haemolysis due to θ-toxin will occur in tube 4 only if the θ-toxin is strong or the filtrate reduced; haemolysis due to α-toxin will occur in tubes 5 and 7 only if the α-toxin is very strong. In such special cases the amount of specific antitoxin added to the tubes may be increased. If large amounts of δ-toxin are present in a filtrate it may be necessary, in order to detect θ-toxin in haemolytic tests, to use horse or rabbit cells, which are not haemolysed by δ.

Examination for collagenases and for substances attacking azocoll (Oakley, Warrack & Warren, 1948). The following screening test may be used for *Cl. welchii* κ and λ antigens:

Four racks each of ten tubes are set up: in each of these serial twofold dilutions

https://doi.org/10.1017/S0022172400015527 Published online by Cambridge University Press

of filtrate are made up in broth-saline in volumes of 1 ml. To each tube of the first rack, 1 ml. of broth-saline is added; to each of the second, 1 ml. of broth-saline containing 5 units of *Cl. welchii* κ-antitoxin and no detectable λ-antitoxin; to each of the third, 1 ml. broth-saline containing 20 units λ-antitoxin and 0·0025 unit κ-antitoxin; and to each of the fourth, 1 ml. broth-saline containing 5 units κ-antitoxin and 20 units λ-antitoxin. The tubes are then mixed by inversion over non-absorbent paper and allowed to stand at room temperature for half an hour; 1 ml. azocoll suspension (Oakley, Warrack & van Heyningen, 1946) is added to each tube, and the whole incubated overnight in a water-bath at 37° C. A typical set of results, using well-marked release of colour as the end-point, may be interpreted as in Table 4.

Table 4. *Results of tests for κ- and λ-toxins and the conclusions to be drawn from them*

Rack	Mixture	Titre of mixture (azocoll tests)		
1	Filtrate + broth-saline	1 in 8	1 in 128	1 in 256
2	Filtrate + κ-antitoxin	.	1 in 128	1 in 64
3	Filtrate + λ-antitoxin	1 in 8	1 in 2	1 in 256
4	Filtrate + κ- + λ-antitoxins	.	1 in 2	1 in 2
	Toxins detected	κ	λ	$\kappa+\lambda$

In addition, 1, 0·5 and 0·1 ml. volumes of filtrate are incubated overnight, each with a single piece of collagen paper (Delaunay, Guillaumie & Delaunay, 1949), 3 mm. square and about 40μ thick. Disintegration of the collagen paper is taken as confirmation of the presence of κ-toxin.

Examination for hyaluronidase. Serial twofold dilutions of filtrates are made up in borate-buffer saline, pH 8, in volumes of 1 ml. and tested for hyaluronidase by the A.C.R.A. test (Oakley & Warrack, 1951) using horse synovial fluid as substrate.

The specificity of the hyaluronidase may be confirmed by showing that 5 units *Cl. welchii* μ-antitoxin neutralize about 500 standard indicating doses of the hyaluronidase.

Examination for deoxyribonuclease. Serial twofold dilutions of filtrates in broth-saline may be tested for deoxyribonuclease by the fixed rabbit leucocyte test (Warrack, Bidwell & Oakley, 1951), or better by the A.C.R.A. test with sodium deoxyribonucleate as substrate and 1 in 4 protein hydrolysate in water plus 0·074 % $MgSO_4 . 7\ H_2O$ (0·003 M) as diluent.

Examination for necrotizing and lethal toxins. 0·2 ml. of each filtrate examined is injected intracutaneously into depilated albino guinea-pigs; 0·2 ml. of the same filtrate incubated for half an hour with 5 % trypsin (final concentration) is also injected. If a well-developed necrotic reaction is produced by either injection within 48 hr., mixtures of 1 ml. filtrate (crude or trypsinized) and 0·2 ml. of borate-buffer saline containing 1 unit α-antitoxin are made up, allowed to stand for half an hour and injected intracutaneously into guinea-pigs in volumes of 0·2 ml. It is important to note that α-toxin may survive half an hour's incubation with 5 % trypsin, a process adequate to activate ϵ- and ι-prototoxins. Necrotic reactions produced by trypsinized filtrates are not, therefore, necessarily due to ϵ- or ι-toxins, and may be due to α-toxin. The point may easily be settled by tests with α-antitoxin.

If the necrotizing effects are not neutralized by α-antitoxin, the tests in Table 5 should be carried out. The antitoxin contents of sera F, G and H are given in Table 6; E is the diluent (borate-buffer saline, pH 8). All the mixtures are allowed to stand for half an hour at room temperature, and are then injected intracutaneously into guinea-pigs in volumes of 0·2 ml. The results, and their interpretation are given in Table 5. As the necrotizing effects of filtrates from food-poisoning strains of *Cl. welchii* were invariably inhibited by *Cl. welchii* α-antitoxin, it is unnecessary to test the filtrates for other necrotizing toxins.

If all the mixtures evoke a necrotic reaction, mixtures of 1 ml. filtrate (diluted if necessary) plus 1 unit α-antitoxin and 5 units ι-antitoxin should be injected. The whole test for necrotizing toxins is then performed on trypsinized filtrates, in which ϵ- and ι-toxins are activated.

Table 5. *Tests for necrotizing toxins of* Clostridium welchii

Mixture	Results		
To 1 ml. filtrate add 0·6 ml. E	+	+	+
To 1 ml. filtrate add 0·2 ml. F and 0·4 ml. E	+	+	+
To 1 ml. filtrate add 0·2 ml. G, 0·2 ml. F and 0·2 ml. E	−	+	+
To 1 ml. filtrate add 0·2 ml. H, 0·2 ml. F and 0·2 ml. E	+	−	+
To 1 ml. filtrate add 0·2 ml. H, 0·2 ml. G and 0·2 ml. F	−	−	−
Toxins detected	β	ϵ	$\beta+\epsilon$

Dilute filtrate if necessary.

Table 6. *Antitoxin content of test sera F, G and H*

Serum	*Cl. welchii* α-antitoxin (units per 0·2 ml.)	*Cl. welchii* β-antitoxin (units per 0·2 ml.)	*Cl. welchii* ϵ-antitoxin (units per 0·2 ml.)
F	5	$<0{\cdot}01$	$<0{\cdot}01$
G	0·2	20	$<0{\cdot}01$
H	0·2	$<0{\cdot}01$	10

β- and ϵ-antitoxin units in terms of laboratory standards.

A little practice soon makes one familiar with the characteristic necrotic reactions produced by *Cl. welchii* α-, β-, ϵ- and ι-toxins, and by the mixtures of them that occur naturally. It may be worth while noting that the β-reactions produced by type B filtrates are, owing to the presence of hyaluronidase in the filtrates, very irregular in shape, while the β-reactions of types C and F filtrates, which are free from hyaluronidase, are smaller and much more nearly circular.

Intravenous tests for lethal toxins. We have seldom found these necessary, as the known toxins detected by them, except η and γ, are more conveniently identified in intracutaneous tests in guinea-pigs. Neither η- nor γ-toxin can readily be demonstrated without extensive immunological investigation, and neither is of much importance for typing. If, however, intracutaneous tests are impracticable or inconvenient, intravenous tests may be planned in the same way as intracutaneous tests; 0·5 ml. of each mixture is injected intravenously into mice.

In several instances filtrates were submitted to a thorough serological investigation of the type described by Oakley (1943); these extensive tests invariably confirmed the results obtained by simple methods.

With these routine tests the investigation of toxin production by 500 strains was quickly and economically carried out. Judging by the accuracy of typing obtained with the numerous known strains put in as controls, the methods are reliable as well as reasonably simple.

Table 7 gives the properties and distribution among the types of the known soluble antigens of *Cl. welchii*.

Table 7. *The soluble antigens so far detected in* Clostridium welchii *culture filtrates*

Designation	Activity	Occurrence in filtrates of *Cl. welchii* types A	B	C	D	E	F
α	Lethal, necrotizing, haemolytic lecithinase C	+ + +	+	+	+	+	+
β	Lethal, necrotizing	−	+ + +	+ + +	−	−	+
γ	Lethal	−	+	+	−	−	+
δ	Haemolytic, lethal	−	+	+ +	−	−	−
ϵ	Lethal, necrotizing (activated by trypsin)	−	+ +	−	+ + +	−	−
η	Lethal (validity doubtful)	(+)	−	−	−	−	−
θ	Haemolytic (oxygen labile, ? lethal)	+ + −	+	+	+	+	−
ι	Necrotizing, lethal (activated by trypsin)	−	−	−	−	+ +	−
κ	Collagenase (lethal, necrotizing gelatinase)	+ +	−	+	+ −	+	−
λ	Proteinase, disintegrates azocoll and hide powder but not collagen; gelatinase	−	+ + +	−	+ −	+	−
μ	Hyaluronidase	+ −	+	−	+ −	−	−
ν	Deoxyribonuclease	+	+	+	+	+	+

+ + − or + − = present in some strains, (+) = limited to very few strains.

Degraded strains. Some strains of *Cl. welchii* lose their capacity to produce particular antigens. Thus type B strains have lost their capacity to produce ϵ-antitoxin (Dalling & Ross, 1938); type C strains have failed to produce β-toxin (Taylor, 1940); and it is claimed that type D strains have ceased to produce ϵ-toxin (Borthwick, 1937). Degradation of strains may lead to incorrect typing. Some assistance may then be obtained by testing for the minor antigens κ, λ and μ. A strain producing β, λ and μ, or β and μ, is far more likely to be a degraded type B strain than a type C strain; a strain producing no β, but producing δ, κ and no μ, is probably a degraded type C strain.

We should like to express our very grateful thanks to Dr Betty C. Hobbs and Miss Muriel E. Smith for providing the filtrates.

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(*MS. received for publication* 23. x. 52.)