RECOGNITION BY ELECTROPHORETIC LOCALISATION OF THE EXTRACELLULAR PRODUCTS OF SELECTED STRAINS OF STAPHYLOCOCCI

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PLATE XVII

THE aim of this study was to detect a number of haemolytic and non-haemolytic extracellular products formed by selected strains of *Staphylococcus aureus*. For this purpose we used the electrophoretic localisation technique (Haque, 1967) by means of which the various components of the crude culture filtrates are first separated by electrophoresis in agar gel and then recognised by their biological activities. This technique has the advantages that interactions among the various products are minimised, and that the products can be identified directly by their electrophoretic mobility and biological activities. We anticipated that the use of these criteria of identification would not only help to define the number and type of extracellular products but would aid us in selecting suitable cultures for future studies of the purification of these substances.

MATERIALS AND METHODS

Of the 13 cultures of staphylococci used, the Wood 46-white, Wood 46-clear, Foggie, 502A, 146P, and 681C strains of *S. aureus* have been previously described (Haque, 1967; Murphy and Haque, 1967; Baman and Haque, 1970). *S. aureus* strains 233, T19, E-delta, and N-delta were supplied by Dr S. K. Maheswaran, Department of Veterinary Bacteriology Public Health, University of Minnesota, St Paul, Minnesota; strain Wood 46M was supplied by Dr A. W. Bernheimer, Department of Microbiology, New York University School of Medicine, New York, NY; and strain PG114 was supplied by Dr F. Kapral, The Ohio State University, Columbus, Ohio. The M3 strain of *S. epidermidis*, a glucose-fermenting, coagulase-negative staphylococcus, was isolated in our laboratory from a marmoset.

All cultures were maintained on Trypticase Soy Agar (Difco) slants. Before use they were checked for haemolytic activity by streaking for isolation on rabbit, sheep, human, and horse blood agar plates.

Production of cell-free filtrates. Each culture was grown on dialysis membranes placed over Brain Liver Heart Agar (Difco) plates at 37°C for 20 hours in an atmosphere containing 10–15% CO₂ in air. Growth from 25 such membranes was harvested in 12.5 ml of phosphate-buffered saline (PBS), pH 7.3, centrifuged at 3000 g for 15 min. and the supernate filtered through a sterile 0.22-µm membrane-filter.

Measurement of biological activities. The haemolytic activities of the filtrates were measured by the tube-titration and the substrate-agar-plate methods (Baman and Haque, 1970). Other biological activities were measured by the substrate-agar-plate method.

Preparation of substrate agar. The following substrates were separately added to a sterile 1.5% (w/v) solution of Bacto Agar (Difco) in PBS, pH 7.3, that had been cooled to

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45°C. For haemolysins, 3% (v/v) of washed erythrocytes of rabbit, sheep, human, and horse; for lipase and esterase, respectively 3% (v/v) of Bacto Lipase Reagent (Difco) and 1% (w/v) of α -naphthyl acetate and in both cases 0.015% (w/v) of aniline blue solution (Allied Chemicals) as indicator; for egg-yolk factor, 5% (v/v) of sterile egg-yolk emulsion (Oxoid); for coagulase, 20% (v/v) of sterile human plasma (BioQuest) (Bentley *et al.*, 1967); and for phosphatase, 1% (w/v) of phenolphthalein diphosphate (Baman and Haque, 1970). For staphylokinase, 20% (v/v) of human plasma was added to sterile PBS agar at 80°C (Christie and Wilson, 1941).

The following substrates were added to PBS agar and then sterilised by autoclaving: for DNAase, 0.2% (w/v) of sodium deoxyribonucleate (Nutritional Biochemical Corp.); for protease activities, 0.5% (w/v) of gelatin and 0.5% (w/v) of casein respectively (Bentley *et al.*, 1967); and for lysozyme, 0.3% (w/v) of lysozyme substrate (Difco) (Jay, 1966).

Detection of activities. DNAase and protease activities were detected by flooding the plates with acidified mercuric chloride (15 g HgCl₂, 20 ml HCl, 100 ml water). Phosphatase activity was detected by exposing the plates to ammonia vapour. All other reactions were directly evident. The plates were read after 12, 24, and 48 hours. The reactions that required the addition of an indicator system after incubation were read at 48 hours.

Electrophoretic analysis of filtrates. The electrophoretic localisation technique (Haque, 1967) was employed. Unless mentioned otherwise, electrophoresis was performed for 1½ hours at 5 mA per cm at room temperature. After electrophoresis, four strips of agar parallel to the line of migration and 0.25 mm from the sample wells were removed, and the resulting trenches were separately filled with different substrates contained in agar. The plates were then refrigerated for 12–48 hours when the location of various biochemical reactions was recorded. To do this, the distance in mm between the peak of the zone of activity and the origin was measured and was used to define the electrophoretic mobility of the product. If a particular activity showed tailing, the extent of tailing and its direction were also recorded.

RESULTS

Measurement and characterisation of various biological activities by the tube-titration and substrate-agar-plate methods

Most of the 13 culture filtrates lysed RBC of all four animal species (table I). Interpretation of these zones as to the type of haemolysins contained in the filtrate was difficult. Filtrates of strains 233, Foggie, T19 and 681C, however, were considered to contain β -haemolysin because they produced dark zones of discolouration on sheep-blood agar plates and these cleared considerably after refrigeration. The zones of clear lysis on blood agar plates were due either to α - or δ -haemolysin or to both.

The results of titration against RBC of the four species of erythrocytes (table II) revealed that the preparations had considerable activity against each. The hot-cold lysis of sheep cells by the filtrates of strains 233, Foggie, T19 and 681C indicated that these contained β -haemolysin. The titres of haemolysis of human, horse, and rabbit RBC were interpreted as evidence that most of the filtrates contained α - and δ -haemolysins. Table I also shows the size (in mm) of the zones that the 13 filtrates produced on various substrate-agar plates.

Characterisation of haemolytic activities by the electrophoretic localisation technique

Fig. 1 shows a typical separation of haemolytic activities when a culture filtrate was tested by electrophoretic localisation. The haemolysin remaining

TABLE I
Measurement by the substrate-agar-plate method of the biological activities of culture filtrates
of strains of *staphylococci**

Strain	Size (mm) and nature† of zone of haemolysis of blood of				Size (mm) of zones of enzymatic activities							
	rabbit	sheep	man	horse	DNAase	Gelatinase	Lipase	Staphylokinase	Egg-yolk‡	Lysozyme	Caseinase	Phosphatase
Wood 46-white	16	6	6	5	10	4	12	0	5	11	0	3
Wood 46M	2	—	3	2	3	8	8	8	2	10	7	7
Wood 46-clear	6	2p	—	—	4	3	3	2	2	10	3	5
502A	12	6	7	4	6	13	8	5	5	11	8	8
Foggie	12	10d	7	4	10	6	5	2	4	9	0	6
146P	10	4	8	4	8	11	11	3	8	10	3§	8
681C	8	8d	2	2	7	4	3	0	2	6	0	5
N-delta	5	—	5	—	4	14	4	7	2	7	3	9
E-delta	11	8	13	10	12	6	11	0	Trace	2	10	0
233	14	3d	3	8	8	3	10	9	2	5	0	13
T19	11	9d	4	—	7	4	6	0	4	8	0	6
M3	12	6	10	7	6	3	6	5	6	0	Trace	0
PG114	16	10d	8	6	8	6	8	2	4	10	0	4

* All were *Staphylococcus aureus* with the exception of strain M3, which was identified as *S. epidermidis*.

† p=Partial haemolysis; d=zone of discoloration; the rest=clear haemolysis.

‡ Production of opacity in egg-yolk agar.

§ Diffuse as opposed to clear zone.

at the origin and lysing mainly rabbit, human, and horse RBC is designated as the δ -haemolysin. The haemolysin migrating approximately 18 mm towards the cathode and lysing rabbit, sheep, and horse cells is designated as the

TABLE II
Haemolysis of RBC by cell-free filtrates of staphylococcal cultures

Strain	Titre of haemolysis of RBC of				
	man*	horse*	rabbit*	sheep†	
				at 37°C	after refrigeration
Wood 46-clear	2560	2560	640	2560	2560
Wood 46-white	640	160	640	160	160
Wood 46M	80	40	160	20	40
502A	80	40	640	320	640
E-delta	1280	640	640	80	160
233	160	80	2560	160	1280
146P	1280	160	1280	80	80
Foggie	160	80	320	40	320
681C	80	40	640	40	320
N-delta	320	160	160	10	10
T19	160	80	640	20	160
M3	2560	160	1280	320	640
PG114	5120	160	2560	40	640

* After incubation for 1 hour at 37°C and then refrigeration for 3 hours.

† Read first after 1 hour at 37°C and again after refrigeration for 3 hours.

TABLE III
Characteristics of haemolysins detected by the electrophoretic localisation technique

Haemolysin	Distance migrated (mm) towards		Lysed erythrocytes of*
	cathode	anode	
α	15-18	...	R, S, H.
α_1	15-18	...	R
cationic β	34-36	...	S
anionic β	27-30	...	S
δ	...	0 with tailing up to 7-11 mm	R, H, M
δ_1	...	10-12	R, S, M
ϵ	...	14-15	R, H, M

* R=rabbit; S=sheep; H=horse; M=man.

α -haemolysin, and the third haemolysin migrating approximately 36 mm toward the cathode and lysing only sheep cells is designated as the cationic β -haemolysin.

The electrophoretic mobility and the range of activity of haemolysins found in the culture filtrates of various cultures is shown in table III. In all, seven haemolytic factors were recognised: the α -, α_1 -, cationic β -, anionic β -, δ -, δ_1 - and ϵ -haemolysins. Five of these, namely, the α -, α_1 -, cationic β -, δ - and ϵ -haemolysins have been previously identified by this technique (Haque, 1967;

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FIG. 1.—Separation of haemolysins from the filtrates of *Staphylococcus aureus* strain PG114, δ -haemolysin remaining at the origin ($\downarrow$) and lysing human, horse and rabbit RBC, α -haemolysin migrating 15–18 mm towards the cathode and lysing horse, rabbit and sheep cells and cationic β -haemolysin migrating 36 mm toward the cathode and lysing only sheep cells; + = anode, – = cathode.

Murphy and Haque, 1971; Ali and Haque, 1972). The α - and the α_1 -haemolysins had identical electrophoretic mobility but differed in the range of their haemolytic activity. The former lysed rabbit, sheep, and horse RBC while the latter lysed only rabbit cells. The anionic β -haemolysin migrated more slowly than the cationic β -haemolysin but like the cationic β -haemolysin lysed only sheep cells. Where a culture produced both the cationic and the anionic β -haemolysins the latter appeared as a tailing of the former. The lytic patterns of δ - and ϵ -haemolysins were identical, but the latter migrated 14–15 mm towards the anode while the former remained at the origin, though it occasionally yielded

TABLE IV
*Haemolysins produced by selected strains of staphylococci,
as identified by the electrophoretic localisation technique*

Strain	Haemolysins
Wood 46-white	α , cationic β , δ
Wood 46M	α , δ
Wood 46-clear	α_1 , cationic β
502A	α , cationic β , anionic β
Foggie	α , cationic β , anionic β , δ
146P	α , δ
681C	α_1 , cationic β , anionic β
N-delta	α_1
E-delta	α , δ_1
233	α , cationic β , anionic β
T19	α , cationic β , anionic β , δ
M3	α , ϵ
PG114	α , cationic β , anionic β , δ

a tail that extended from 7–11 mm towards the anode. The δ_1 -haemolysin migrated 10 mm toward the anode and lysed rabbit, sheep, and human RBC. Its electrophoretic mobility and its lytic pattern were therefore different from those of the δ - and the ϵ -haemolysins.

The distribution of various haemolysins among the strains tested is shown in table IV. All cultures with the exception of strain N-delta produced multiple haemolysins. Some, such as the Foggie, T19, and PG114 strains produced at least four haemolysins each, viz., α -, cationic β -, anionic β - and δ -haemolysins.

Miscellaneous enzymes

The distribution of the various enzymes among different strains is shown in table V. Two lipases—designated 1 and 2—could be separated electrophoretically; they migrated respectively 3 and 18 mm towards the anode. Each was active against the Difco lipase reagent, tributyrin, and α -naphthyl acetate. Lipase 1 did not affect the egg-yolk emulsion but lipase 2 did. Strains 502A, Wood 46-white and Wood 46M produced both of these lipases and the remaining cultures produced either lipase 1 or lipase 2.

Only one protease was recognised; it migrated 30–32 mm towards the

anode and hydrolysed both gelatin and casein. The filtrates of strain 502A contained two electrophoretically distinct DNAases; these migrated 20 mm

TABLE V
Products other than haemolysins formed by selected strains of staphylococci and detected by the electrophoretic localisation technique

Strain	Production of					
	Phosphatase	DNAase number		lipase number		gelatinase
		1	2	1	2	
Wood 46-white	+	+	—	+	+	+
Wood 46M	—	+	—	+	+	+
Wood 46-clear	—	+	—	+	—	—
502A	+	+	+	+	+	+
Foggie	+	+	—	+	—	+
146P	+	+	—	+	—	+
681C	+	+	—	+	—	+
N-delta	—	—	+	+	—	+
E-delta	—	+	—	+	—	+
233	+	+	—	—	+	+
T19	+	+	—	—	+	+
M3	+	+	—	—	+	—
PG114	+	+	—	+	—	+

+ = Detected; — = not detected.

FIG. 2.—Composite view of electrophoretic localisation pattern of extracellular products of staphylococci.

and 30–32 mm towards the anode and were designated respectively DNAases 1 and 2. Other cultures contained either DNAase 1 or 2 (table V).

Only 10 of the 13 cultures showed phosphatase activity; it remained at the origin. Lysozyme, coagulase and staphylokinase could not be detected by the electrophoretic localisation technique. A composite view of the various

haemolysins and miscellaneous enzymes found in the 13 culture filtrates is shown in fig. 2.

DISCUSSION

In recent years the filtrates of staphylococcal cultures have been analysed by several different methods; as a consequence, newer products not previously known and multiple molecular forms of certain well known products have been described (Kreger *et al.*, 1971; Maheswaren and Lindorfer, 1970; Kayser and Raynaud, 1965; Haque, 1967; Vesterberg *et al.*, 1967). The purpose of this study was two-fold: to find out (1) how many of the different products could be detected by electrophoretic localisation, and (2) which of them were produced by some of the well known strains of staphylococci.

Our findings reveal that as many as seven different haemolytic products could be differentiated on the basis of their electrophoretic mobility or range of haemolytic activity or both. Most of them had a distinct electrophoretic mobility and thus were easy to define; the α - and α_1 -haemolysins, however, had identical mobilities but differed in their range of haemolytic activity. The latter lysed, even upon concentration, only rabbit RBC while the former lysed rabbit, sheep and horse RBC, and, in higher concentrations, also human RBC. This differentiation, however, is regarded as tentative until the α - and α_1 -haemolysins are purified and compared. The other haemolysins, namely cationic β , anionic β , ϵ , δ , and δ_1 , showed sufficiently different electrophoretic mobilities for them to be regarded as distinct.

The study also revealed two lipases, two DNAases, one phosphatase and one gelatinase, but coagulase, staphylokinase, and lysozyme activities could not be detected by our method. This was disappointing, especially because five isozymes of coagulase have been separated electrophoretically (Miale *et al.*, 1963). Our failure to detect these enzymes after electrophoresis may have been due to the low sensitivity of the test substrate; an effort is therefore being made to improve the sensitivity of the respective tests.

The composition of the filtrates of most of the cultures presented a complex picture. Some cultures, *e.g.*, of the PG114, 233 and Foggie strains, produced four different haemolysins and most of the others produced three; only one culture, of strain N-delta, produced a single haemolysin. This emphasises the need for careful selection of the cultures to be employed in studies aimed at the purification of any particular product and the importance of the methods used for identification and characterisation of any activity or product. The use of methods that reveal the full complexity of the culture filtrate is to be encouraged; at present, three simple and direct methods that can accomplish this are (1) electrophoretic localisation technique (Haque, 1967), (2) the modification of this technique described by Lochman *et al.* (1969), and (3) enzymophoresis (Abramson, 1967). A similar technique, but one that uses isoelectric focusing as the means of separating the various components of the culture filtrates, was recently reported by Wadstrom *et al.* (1974). Together these newer techniques are expected to provide a clearer analysis of the hitherto elusive constituents of staphylococcal filtrates.

SUMMARY

Extracellular products of 13 selected strains of staphylococci were separated and detected by the electrophoretic localisation technique. Seven haemolysins were recognised, and were designated α , α_1 , cationic β , anionic β , δ , δ_1 and ϵ . These migrated respectively c. 18, 18, 36 and 30 mm towards the cathode and 0, 10, and 14 mm towards the anode. Two lipases migrating 3 and 18 mm towards the anode, one gelatinase migrating 36 mm towards the anode, two nucleases migrating 20 and 32 mm towards the anode and one phosphatase remaining at the origin were also recognised.

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