STUDIES, CHIEFLY IN MICROBIOLOGY.

A COLLECTION OF PUBLISHED AND UNPUBLISHED PAPERS.

presented by

ROBERT CHRISTIE.

for

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INTRODUCTION

The most important section of these works consists of the papers concerning Staphylococci, papers 1 to 14, which were completed in the Research Department of the Commonwealth Serum Laboratories, Melbourne.

The second section, papers 15 to 23, is a miscellaneous group of papers chiefly on microbiological subjects. Most of this work was carried out during the post-war period while I was Clinical Bacteriologist at St. Vincent's Hospital, Prince Henry's Hospital and the Red Cross Blood Transfusion Service, Melbourne.

The papers in both of these sections have been arranged, where possible, in chronological order.

To fulfil the requirements for theses for a Doctorate of Science, these papers can be classified into the following four groups:

A. Author originated idea and carried out most of the laboratory work :
Numbers 2,3,4,5,6,7,10,17,18,21,22.

B. Author originated idea and contributed to the laboratory work:
Numbers 8,13,16,19,20.

C. Author carried out most of the laboratory work but did not originate the idea :
Numbers 1,9,11,12,14,15,23.

D. Author originated the idea and supervised the execution of projects, the laboratory work being carried out by assistants. These papers, numbers 24 to 30, constitute the final section of the thesis.

LIST OF PUBLISHED ARTICLES

1.	Christie, R. and Keogh, E. V.	Physiological and Serological Characteristics of Staphylococci of Human Origin. J. Path. and Bact. 1940 51 p. 189. Pages 1 to 15
2.	Christie, R.	Use of Slide Agglutination to Determine Pathogenicity of Staphylococci. Aust. J. exp. Biol. Med. Sci., 1940 18 p. 397. Pages 16 to 25
3.	Christie, R. and Graydon, J. J.	Observations on Staphylococcal Haemolysis and Staphylococcal Lipase. Aust. J. exp. Biol. Med. Sci., 1941 19 p. 9. Pages 26 to 44
4.	Christie, R. and North, E. A.	Observations on Staphylococcal -toxin Production by Strains Isolated from Staphyloccal Lesions In Man. Aust. J. exp. Biol. Med. Sci., 1941 19 p. 323-327. Pages 45 to 56
5.	Christie, R. and Wilson, H.	Test of Staphylococcal Fibrinolysin. Aust. J. exp. Biol. Med. Sci., 1941 19 p. 329. Pages 57 to 65
6.	Christie, R., Atkins, N. E. and Munch-Petersen, E.	Note on Lytic Phenomena Shown By Group B. Streptococci. Aust. J. exp. Biol. Med. Sci., 1944 22 p. 197. Pages 66 to 75
7.	Christie, R., Graydon, J. J. and Woods, E. F.	Staphylococcal Fibrinolysin. Aust. J. exp. Biol. Med. Sci., 1945 23 p. 127. Pages 76 to 85

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List of Published Articles cont'd...

8.	Munch-Petersen, E., Christie, R., Simmons, R. T. and Beddome, H. A.	Further Notes on the Lytic Phenomenon Shown by Group B Streptococci. Aust. J. exp. Biol. Med. Sci., 1945 23 p. 193.	Pages 86 to 91
9.	North, E. H. and Christie, R.	Observations on the Sensitivity of Staphylococci to Penicillin. Med. J. Aust. 1945 2 p. 24.	Pages 92 to 101
10.	Christie, R., North, E. A. and Parkin, B. J.	Criteria of Pathogenicity in Staphylococci. Aust. J. exp. Biol. Med. Sci., 1946 24 p. 73.	Pages 102 to 117
11.	North, E. A. and Christie, R.	Acquired Resistance of Staphylococci to the Action of Penicillin. Med. J. Aust. 1946 1 p. 176.	Pages 118 to 131
12.	North, E. A., Christie, R. and Rank, B. K.	Penicillin-resistant Staphylococci in Wounds, a Report Based on Study of 59 Cases of Infected War-wounds. Med. J. Aust. 1946 2 p. 41.	Pages 132 to 164
13.	Munch-Petersen, E. and Christie, R.	An Effect of Interaction of Staphylococcal -toxin and Group B Streptococcal Substance On Red Blood Corpuscles and Its Use as a Test for Identification of Str. agalactiae. J. Path. Bact. 1947 59p.367.	Pages 165 to 174
14.	Stratford, B. C., Rubbo, S., Christie, R. and Dixson, S.	Treatment of Nasal Carriers of Staphylococcus Aureus. Lancet 1960 2 p. 1225.	Pages 175 to 185
15.	North, E. A., Keogh, E. V., Christie, R. and Anderson, G.	Experimental Pertussis in the Monkey (Macaca Mulatta). Aust. J. Exp. Biol. Med. Sci., 1940 18 p. 125.	Pages 186 to 194

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List of Published Articles cont'd...

16.	Simmons, R. T. and Christie, R.	Penicillin Sensitivity of Streptococci, Mostly of Groups A, B, C and G. Med. J. Aust. 1946 1 p. 349.	Pages 195 to 208
17.	Newing, W. J. and Christie, R.	Meningitis; Isolation of Organism Resembling N. catarrhalis from C.S.F; Report of a Case. Med. J. Aust. 1947 1 p. 306.	Pages 209 to 213
18.	Christie, R.	Observations on Biochemical and Serological Characteristics of Ps. pyocyanea. Aust. J. exp. Biol. Med. Sci., 1948 26 p. 425.	Pages 214 to 235
19.	Christie, R. and Morton, Mary M.	The Detection of Candida Albicans. Aust. J. Derm., 1953 11 p. 87	Pages 236 to 250
20.	Stratford, B. C., Christie, R. and Dixson, S.	Disinfection of Hospital Blankets. Med. J. Aust. 1960 2 p. 621	Pages 251 to 265
21.	Christie, R., Downing, H. H. and Dumble, Lynette J.	Hypertonic Fragility of Erythrocytes. 1969 - Unpublished.	Pages 266 to 269
22.	Christie, R., Dumble, Lynette J., Downing H. J. and Berah M.	The H1-A Antigen System and The Inclusion of the 7c Antigen in the 4b Antigen. 1969 - Unpublished.	Pages 270 to 272
23.	Dumble, Lynette J., Christie, R. and Downing, H. J.	Incidence of Leucocyte Antibodies Induced by Pregnancy and Their Connection with Abortion. 1969 - Unpublished.	Pages 273 to 276.

LIST OF ARTICLES ON RESEARCH WORK INITIATED AND GUIDED BY THE AUTHOR

24.	Woods, E. F. and Parkin, Barbara J.	The Plate Test for Coagulase Production by Staphylococci. Aust. J. exp. Biol. Med. Sci. 1946 24 p. 33. Pages 277 to 283
25.	Card, Dorothy H.	The Penicillin Resistance of Staphylococci. Med. J. Aust. 1948 1 p. 767. Pages 284 to 287
26.	Keating, Shirley V.	A Note on the Use of Antibiotics In the Treatment of Infections With Some Gram-negative Bacilli. Med. J. Aust. 1951 2 p. 709. Pages 288 to 292
27.	Hore, J. F.	The Staining of Fungi in Skin and Hair. Aust. J. of Med. Tech., Jan. 1955. Page 293 to 294
28.	Morton, Mary M.	A Note on the Detection of Candida Albicans. Aust. J. Derm., 1955 3 p. 13. Pages 295 to 298
29.	Keating, Shirley V.	A Biochemical and Serological Study of the Genus Proteus. Med. J. Aust., 1956 Vol. 43 (2) p. 168. Pages 299 to 315
30.	Dumble, Lynette J.	The Use of Dispersol in Culture Media for the Isolation of Streptococci. Med. Technology in Australia, 1965 7 p. 149. Pages 316 to 323

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PHYSIOLOGICAL AND SEROLOGICAL CHARACTERISTICS OF STAPHYLOCOCCI OF HUMAN ORIGIN

R. CHRISTIE and E. V. KEOGH

From the Commonwealth Serum Laboratories, Melbourne

It is sometimes difficult for the clinical pathologist to decide whether or not a particular staphylococcus is to be regarded as pathogenic. When a typical aureus strain is isolated in pure culture from a definite lesion, such as a boil, the decision is easy. When, however, a strain is isolated in mixed culture from urine or from an infected wound, it may be difficult to determine its aetiological relationship to the disease process. It is customary to regard haemolytic aureus strains as pathogenic and non-haemolytic albus strains as non-pathogenic. Fermentation of mannitol is used by some workers as a criterion of pathogenicity. Most recent writers regard the coagulation of plasma as the most useful single test (Cruickshank, 1937; Cowan, 1938; Fairbrother, 1940). Serological methods have been used for the same purpose, precipitin tests (Julianelle and Wieghard, 1935; Cowan, 1938) and, more recently, slide agglutination (Cowan, 1939).

We have examined by such methods a number of strains isolated in Australia, with results in general conformity with those of other workers. Some additional information of value has been gained during the investigation. The serological classification of the pathogenic strains has been extended, and it will be shown that certain serological types are usually associated with severe lesions.

SOURCES OF STRAINS

Only strains isolated from man have been studied, namely 220 from 165 persons. The majority were isolated in the departments of pathology of the Royal Melbourne Hospital and the Children's Hospital, Melbourne. For these we are very grateful to Dr. Hilda Gardiner and Dr. Reginald Webster. Dr. S. T. Cowan kindly sent us subcultures of his type strains I, II and III and unabsorbed type sera, without which the work could not have been undertaken. For other strains we are indebted to Dr. Lucy Bryce and Miss Cecily Timmins.

METHODS AND RESULTS

Coagulase production

A loopful of culture from a 24-hour agar slope was inoculated into 0.5 c.c. of rabbit plasma obtained by adding blood to an equal volume of 4 per cent. sodium citrate solution, centrifuging, and removing the super-natant fluid. Comparative tests with coagulase+ and coagulase- strains were made in plasma of the rabbit, horse and man. All were equally suitable, provided the plasma had not been kept for over 48 hours. False positive results occurred with older samples even when a large excess of citrate was used. Overnight lysis of a clot which had formed in a few hours was noted on occasion with all types of plasma. Readings were therefore taken at 3, 5 and 24 hours.

One hundred and fifty-six strains were coagulase-positive, 64 were coagulase-negative. No coagulase-negative strain bore a certain aetiological relationship to a pathological condition. All strains so related were coagulase-positive.

Pigmentation

A loopful of a pure culture was inoculated on the surface of an agar plate containing 33 per cent. of milk (Fujita and Yoshioka, 1938) and was incubated for 3 days at 37°C. and for two more days on

the bench. On this medium, aureus, citreus and canary yellow pigments are readily distinguished, while albus colonies are distinctly whiter than the surface of the medium, which has a creamy tint. The intensity of pigmentation varied with different strains of the same colour.

It may be noted that, on milk agar, a translucent zone of clearing round the colony indicates hydrolysis of casein, and an iridescent zone on the surface around the colony is due to the action of staphylococcal lipase on milk fat. This was established by suitable experiments, using plates prepared with fat-free milk and milk fat free of casein.

Of the 156 coagulase+ strains, 136 (from 110 patients) were aureus, 11 (from 6 patients) were canary yellow, 3 (from 1 patient) were citreus and 6 (from 5 patients) were albus. Of the 64 coagulase- strains, 50 (from 38 patients) were albus, 10 (from 9 patients) were aureus, 2 (from one patient) were canary yellow and 2 were citreus.

Haemolysis on blood agar

A loopful of a pure culture was inoculated on a 5 per cent. sheep blood agar plate and incubated overnight at 37°C. in the ordinary atmosphere. The type of haemolysis, α or $\alpha\beta$, was noted (Bryce and Rountree, 1936). If doubt existed as to the presence of β lysis, a second incubation overnight, followed by refrigeration, was used to demonstrate the concentric rings typical of β haemolysis. Sheep blood agar must, of course, be used for demonstration of β (hot-cold) lysis and is, in our experience, superior to horse blood agar for demonstrating α haemolysis. On horse blood agar, some typical α strains showed no lysis until incubated for 48 hours, while other strains which were non-haemolytic on sheep blood agar and which produced no α or β toxin in broth showed lysis on horse blood agar at 48 and occasionally at 24 hours.

Of the 156 coagulase+ strains, 141 were haemolytic on the plates (123 α, 18 β α) and 15 showed doubtful or no haemolysis. Of the 64 coagulase- strains, 46 were non-haemolytic and 18 showed zones of partial haemolysis.

These non-haemolytic and doubtfully haemolytic strains have been tested for the production of lysins in broth. Seven coagulase+ strains produced no α or β haemolysin in broth under increased CO_2 tension. No coagulase- strain produced α or β haemolysin. The discrepancy between these results and those of Cowan (1938) and Gillespie et al. (1939), who found complete correlation between the production of α haemolysin and coagulase, may be due to the fact that our cultures were not tested on first isolation. Briefly, the great majority of coagulase+ strains produce α or α β haemolysis on sheep blood agar, whereas no coagulase- strains of human origin do so. More elaborate methods for demonstrating haemolysin are probably unsuited for use in the clinical laboratory.

Fermentation reactions

Strains were grown in peptone water containing ½ or 1 per cent. of the carbohydrate, brom-cresol-purple being used as indicator. Tests were observed for 21 days.

Ninety-four coagulase+ cultures were tested on a wide range of carbohydrates. All attacked mannitol, lactose, maltose, galactose, trehalose, mannose, glucose, sucrose, fructose, dextrin and glycerol. None attacked arabinose, raffinose, xylose, adonitol, dulcitol, isodulcitol, erythritol, inositol, sorbitol, amygdalin, inulin or starch. Results with salicin were irregular and indefinite. Sixty-three coagulase- strains gave very variable reactions which will not be considered in detail, but only from the point of view of distinguishing coagulase+ and coagulase- strains. Mannitol, trehalose and mannose have a limited value for this purpose (Table 1).

TABLE I

Fermentation reactions of coagulase-negative staphylococci

No. of strains	Mannitol	Trehalose	Mannose
11	+	+	+
5	+	+	-
1	+	-	-
4	+	-	+
1	-	+	+
4	-	+	-
1	-	-	-
36	-	-	+
63	...	...	...

Eleven of the 63 strains attacked all three substances. All produced pigmented colonies, 10 typical aureus, one yellow. No albus strain fermented all three carbohydrates and only four attacked both mannitol and mannose. Any fermentation of these three substances which occurred tended to be delayed with the majority of coagulase-strains (Table II).

TABLE II

Rate of fermentation by staphylococci

Substance	Coagulase	Day on which fermentation occurred							
		1	2	3	4	5	6	7	8
Mannitol	+	53	38	2	0	0	1	0	0
	-	1	4	7	3	2	1	1	2
Trehalose	+	86	2	2	2	0	0	1	1
	-	6	6	2	0	0	0	1	0
Mannose	+	88	5	1	0	0	0	0	0
	-	16	20	1	0	1	0	0	0

Numbers = no. of strains fermenting on day mentioned.

Trehalose is almost as useful as mannitol if a single test substance is used, but both gave positive reactions with a third of the coagulase- strains. This error is approximately halved by the use of the three carbohydrates in conjunction. A strain fermenting mannitol within 48 hours is almost always coagulase+. Slow fermenters are often coagulase-.

Preparation and absorption of agglutinating sera

Rabbits were inoculated according to the scheme of Cowan (1939), using cocci heated for one hour at 60°C. Locally isolated type 1, 2 and 3 strains * were identified by Cowan's sera. Key sera were prepared with the following strains :- MS 73 (Cowan's type I),

*We have adopted Arabic in place of the Roman numerals introduced by Cowan for the reason stated on the following page. (p8)

MS 68 (type 2), MS 120 (type 3), MS 174 (type 4), MS 39 (type 5), MS 69 (type 6), MS 41 (type 7), MS 150 (type 8), and MS 63 (type 9). Several others were made in the course of the work, but were either lacking in specific antibody or identical with one or other of the key sera.

Sera were diluted 1 : 5 in normal saline and 0.5 c.c. of diluted serum was absorbed in each trial with various doses of culture. Types 2 and 7 sera required absorption with the organisms from four agar slopes and an absorbing dose in excess of this did not seriously affect the titre. With the other sera more care had to be exercised in adjusting the absorbing dose, since an excess tended to remove the specific as well as the non-specific agglutinins. The minimal dose varied from one to five slopes. A single absorption with the optimal dose, determined by experiment, was distinctly more satisfactory than repeated absorption with small doses. Type 1 serum was absorbed with S 33 R 4 (Cowan's type III). All others were absorbed with S 11 R 21 (Cowan's type I), except types 2 and 7 sera which were absorbed with MS 48 (type 4-5).

Slide agglutination tests were performed by Cowan's method (Cowan, 1939).

Agglutination tests with unabsorbed sera

One hundred and thirty-one strains were tested for slide agglutination with unabsorbed sera. Eighty-eight coagulase+ strains were agglutinated by one or both of two sera prepared against a type 1 and a type 2 strain respectively. Forty-two coagulase- strains were not agglutinated by either. One strain which was agglutinated by both sera gave an equivocal result in the coagulase test. This strain, on first isolation from a healthy throat, was haemolytic on sheep blood agar but lost this power rapidly on subculture.

Eleven coagulase+ strains were subsequently shown by tests

with absorbed sera to belong to type II (Cowan). Nine of these did not agglutinate with the unabsorbed type 1 serum and two were weakly agglutinated. Of the 67 strains which belonged to other types than 2, four did not agglutinate with the unabsorbed type 2 sera. Three belonged to type 1 and one to type 3.

A combination of unabsorbed sera of types 1 and 2 therefore, agglutinated all coagulase+ and no coagulase- human strains.

Forty-six coagulase+ strains were not agglutinated by absorbed sera of type 1, 2 or 3. Sufficient additional sera were prepared to identify all these strains. Whereas 55 of the 76 strains agglutinated by type 1, 2 or 3 sera were agglutinated by only one serum, 44 of 65 strains agglutinated by sera of types 4-9 were agglutinated by at least two. Three strains were agglutinated by three different sera. It is possible therefore to characterise a strain serologically by stating the serum or sera by which it is agglutinated, and it is usually easy to determine which agglutinates it more strongly. A strain which agglutinates with, for example, type 3 serum only is labelled "type 3". A strain agglutinating with type 4 and type 2 sera, the first more strongly, is labelled "type 4-2". (We have adopted Arabic in place of the Roman numerals introduced by Cowan, since the resulting formula in strains with two or more antigens is less clumsy.) Not much emphasis is placed on the relative importance of the antigens in a strain, and it might be preferable to place the smaller figure first, but the nomenclature adopted brings out some points which are stressed below. The frequency of the various single and composite serological types is shown in Table III.

When several strains of the same type have been isolated on successive occasions from one lesion, or from several sites in one patient, only one has been included in table III.

It will be noted that it is fallacious to estimate the

relative distribution of the various types unless sera are available which agglutinate all the strains. Using the type 1, 2 and 3 sera, 76 strains would have been placed in one of these types, but 21 of them reacted more strongly with other sera. If these three sera only had been used, 63 per cent. of the strains would have been apparently identified, agreeing closely with 70 per cent. identified by Cowan (1939).

TABLE III

Frequency of Occurrence of Serological types of Pathogenic Staphylococci

Major Agglutinogen	Minor Agglutinogen							Total
Type	1	2	3	4	5	6	7	
1	34	0	0	0	0	0	0	34
2	0	10	0	0	0	0	0	10
3	0	0	11	0	0	0	0	11
4	2	3	0	11	16	0	1	33
5	2	1	0	5	9	0	0	17
6	1	0	2	0	0	1	0	4
7	0	0	5	0	0	1	0	6
8	1	1	3	0	0	1	0	6
9	0	0	0	0	0	1	0	1
	40	15	21	16	25	4	1	122

Relation of serological type to pathogenicity

The majority of strains isolated from furuncles, from osteomyelitis, or from the blood belonged to types other than 1, 2 or 3, whereas most of those isolated from the nose, throat or sputum of apparently healthy persons belonged to these types (table IV).

TABLE IV

Correlation Between Source of Strain and Serological Type

Source of Strain	Types 1-3	Types 4-9
Nose, throat, sputum	24	5
Osteomyelitis, blood culture	9	24
Furuncles	8	16

We have somewhat arbitrarily regarded as belonging to types 1-3 only strains which agglutinated with absorbed sera of these types and showed no agglutination with absorbed sera of other types. When strains of types 4-9 were agglutinated by one or more of the absorbed sera 1, 2 and 3, such agglutination was usually of minor degree. Further, it is desired to emphasise the fact that strains from severe lesions are usually agglutinated by more than one serum, whereas those from the respiratory tract are usually agglutinated only by the type sera 1, 2 or 3. These findings are more closely analysed in table V. It will be noted that most of the strains from severe lesions are agglutinated by one or both of type 4 and 5 sera.

TABLE V

Source of Strain and Serological Type

Source of strain	Number of strains showing major agglutination with serum of type									
	1	2	3	4	5	6	7	8	9	?
Nose, throat, sputum	14	4	6	1	3	0	1	0	0	1
Osteomyelitis, blood culture	4	2	3	18	1	1	2	1	1	0
Furuncles	7	1	0	6	8	1	0	0	0	1

The Primary site of infection in osteomyelitis

It was suggested by Williams and Timmins (1938) that the nose was the probable primary site of infection in cases of osteomyelitis, because they frequently found the same kind of staphylococcus in the nose as in the bone lesion. They used the differential reactions with a series of bacteriophages (Burnet and Lush, 1935) as a means of disclosing differences between or identity of strains. We have used serological methods for the same purpose. The results are shown in Table VI.

TABLE VI

Types of Strains From Nose and Throat and From the Lesion In Osteomyelitis

Patient	Type of strain isolated from			
	Bone	Blood	Nose	Throat
Je. . .	1	1	1	...
Ki. . .	1	1	1	1
Wr. . .	2	...	...	2
Dw. . .	4-2	...	4-2	...
McD. . .	4-5	4-5	4-5	...
Si. . .	4-5	4-5	4-5	...
To. . .	4-5	...	4-5	...
Ef. . .	7-6	...	...	7-6
Ti. . .	9-6	...	...	9-6
Cr. . .	4-5	...	1	...
To. . .	4-5	...	2	2
Kl. . .	4-5	...	6-3	...
Th. . .	5-2	...	4-3	...

In 9 of 13 instances, the same strain was isolated from the nose or throat as from the lesion. This degree of correspondence is statistically significant, the probability of its chance occurrence being less than one in a hundred. But as Williams and Timmins remark, although in cases where the same type of staphylococcus was isolated

from the nose or throat as from the lesion, there is little doubt that the strains had the same ultimate origin; this is, at best, only presumptive evidence that the nose or throat is the portal of entry.

DISCUSSION

The most useful single test for identification of pathogenic strains of staphylococci is coagulase production. Negative controls, both of plasma alone and of plasma inoculated with a known coagulase-strain, should always be set up. The necessity for such controls was forcibly impressed on us during attempts to detect coagulase in filtrates of broth cultures. Lack of such controls is evidently responsible for the discrepant reports in the literature regarding the existence of coagulase in such filtrates, which we have consistently failed to demonstrate.

No other test at present available is sufficiently reliable for identification of pathogenic strains in the clinical laboratory, neither haemolysis on blood agar, pigmentation of the colony nor fermentation of mannitol. It is possible that, in the future, slide agglutination with suitable polyvalent sera may be as useful as coagulase production. Stock therapeutic staphylococcal antisera issued by these laboratories have agglutinated, though slowly in some cases, all coagulase+ strains tested, but further investigation will be needed before this method can become generally available.

Elaborate serological classification of staphylococci, apart from its intrinsic interest to the immunologist, finds its justification in the light it throws on the epidemiology and pathogenesis of staphylococcal infections. Such methods have been used (Devenish and Miles, 1939; Gillespie et. al., 1939) in the study of the epidemiology of staphylococcal wound infections in hospital wards with extremely interesting results. Extension of these methods to permit the typing of all strains we have encountered has shown that certain

types predominate in severe infections, while others are common in the nose and throat. This difference in distribution, though striking, is only relative, and strains of all types have been recovered from lesions.

The same phenomenon has been noted in streptococcal infections. Those types of haemolytic streptococci associated with epidemic and endemic scarlet fever are rarely found in the healthy throat or in excised tonsils (Keogh and Kelsey, 1939; Keogh et al., 1939). On the other hand, the types found in some 7 per cent. of normal throats and in 60 per cent. of excised tonsils, are not those responsible for epidemic scarlet fever.

Probably the types of staphylococci commonly inhabiting the nose and throat are strains well adapted to symbiosis with man, causing overt disease only in exceptional circumstances. Presumably also, consequent on their wide distribution, the majority of the population will have acquired a certain amount of immunity towards them. On the other hand, production of a severe lesion implies lack of adaptation of the infecting organism to its host. It is probably, also, that an individual is less likely to be immune to strains rarely found in the community and is therefore more likely to develop an overt infection if infected by such strains. In the present state of knowledge it would be futile to attempt to discuss why apparently weakly pathogenic strains are more widely distributed than strains causing severe infections.

Type-specific immunity, as suggested by Lyons (1937), probably does influence the pathogenesis and course of staphylococcal infections. For example, strains isolated from two furuncles occurring at an interval of two years in one patient were of different and uncommon types. Whether antibacterial immunity is entirely type specific, however, is a matter needing further experimental and clinical investigation.

SUMMARY

1. Two hundred and twenty strains of staphylococci of human origin have been examined by various physiological methods and classified serologically.

2. Coagulase production is the simplest test for pathogenicity at present available. No other test is completely reliable.

3. The serological types of staphylococci recovered from furuncles and severe staphylococcal lesions usually differ from those found in the nose and throat and are more complex antigenically.

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THE USE OF SLIDE AGGLUTINATION TO DETERMINE PATHOGENICITY OF STAPHYLOCOCCI

by R. CHRISTIE

(From the Commonwealth Serum Laboratories, Parkville, N.2, Victoria.)

(Submitted for publication 4th September, 1940.)

The coagulase test is generally regarded as the best single test of pathogenicity in staphylococci (Cruickshank, 1937; Chapman, Berens, Nilson and Curcio, 1938; Fairbrother, 1940).

It was found that unabsorbed sera, prepared in this laboratory from coagulase-positive strains, agglutinated most heterologous coagulase-positive strains but failed to agglutinate coagulase-negative strains (Christie and Keogh, 1940).

It seemed possible, therefore, that agglutination tests with pooled sera prepared with coagulase-positive strains might give results corresponding to those of the coagulase test, and serve as tests for pathogenicity.

An attempt was made to prepare a mixture of sera suitable for this purpose.

MATERIALS AND METHODS

Strains Used.

392 strains of staphylococci were examined; 335 were of human origin and 57 of animal origin. The sources of these organisms

and the results of the coagulase-test are shown in Table 1.

TABLE 1

Origin of the Strains and Results of the Coagulase Tests

Source	Coagulase-positive	Coagulase-negative	Total
Human Boils	45	-	45
Osteomyelitis	37	-	37
Skin infections (acne etc)	24	19	43
C.S.F.	-	2	2
Pleural fluid	1	-	1
Lung (P.M.)	1	1	2
Sputum	10	2	12
Throat swabs	30	1	31
Blood culture	15	1	16
Nasal swabs	26	12	38
Urine	2	17	19
Urethral swabs	2	17	19
Rectal swabs	-	3	3
Post-nasal sinuses	7	22	29
Tooth abscesses	3	1	4
Eye	2	7	9
Ear	2	-	2
Unknown and Uncertain	19	4	23
TOTAL - HUMAN	226	109	335
Bovine Milk (mastitis)	29	6	35
Equine Pus (sinus)	3	3	6
Feline Nasal swabs (? distemper)	0	1	1
Canine Nasal swabs (distemper)	6	4	10
Ovine Udder	5	0	5
TOTAL - ANIMAL	43	14	57
TOTAL - HUMAN AND ANIMAL	269	123	392

Preparation of anti-sera.

Anti-sera were prepared in rabbits by the method of Cowan (1939), using organisms killed at 60°C. After four weeks' treatment, sera were obtained with a final titre of 1:200 to 1:400. They were preserved with merthiolate (1:10,000) and showed no fall in titre after one year's storage at 4°C.

Preparation of Antigen.

The strain was grown for 18-24 hours at 37°C. in 0.05 p.c. glucose broth (Lyons, 1937). The culture was then heated to 100°C., centrifuged, and the organisms re-suspended in physiological saline to give a suspension of 4,000 million organisms per ml.

Where organisms grown on nutrient agar slopes were used, suspension of similar strength was made in saline.

Technique of agglutination test.

Using a capillary pipette a small drop of bacterial suspension was placed on a slide, and a drop of diluted serum added with a 28-gauge platinum loop having a diameter of 1 mm. The drop of suspension was found by weighing to be twenty times the volume of the serum drop. A drop of suspension, without serum, served as control. The slide was then rocked for 60 seconds. Agglutination, when it occurred, was usually complete, or nearly so, in 30 seconds.

Technique of the coagulase test.

Rabbit plasma was generally used, but when more convenient, human or horse plasma. At times all three were used. The blood was withdrawn and added to an equal volume of 4 p.c. sodium citrate; the cells were removed by centrifuging. A loopful of organisms from a 24-hours' agar slope culture was suspended in 0.5 ml. of plasma. Known positive and negative strains were always included as controls. Readings were made after 3, 5 and 24 hours' incubation at 37°C.

RESULTS

(a) Coagulase tests.

269 strains coagulated plasma, in most cases within three hours; 123 strains did not coagulate plasma. Two animal strains, both $\alpha\beta$ toxin-producers, gave anomalous results. One strain, isolated from the nasal discharge of a dog with distemper, did not coagulate human plasma but coagulated horse and rabbit plasma; the other strain, isolated from a sinus of a horse's withers, did not coagulate horse plasma but coagulated human and rabbit plasma. No similar irregularities were detected amongst the human strains.

All definitely haemolytic strains coagulated plasma. One strain, S5, isolated from the pus of osteomyelitis and which coagulated plasma, showed little or no haemolysis on sheep blood-agar and produced no demonstrable haemolysin when cultivated under increased CO_2 tension in "toxin" broth (nutrient broth with 0.1 p.c. agar). 1 ml. of a 24 hours' broth culture injected intravenously into a rabbit killed the animal in nine days. The organisms recovered from the heart blood and abscesses of the kidney, were coagulase-positive and agglutinable with several anti-sera to the same degree as the original organisms, but they were still non-haemolytic on sheep blood-agar.

(b) Agglutination tests with organisms grown in broth.

An unabsorbed serum (prepared from a type I strain received from Dr. S. T. Cowan), diluted 1:10, agglutinated most coagulase-positive strains. Some strains not agglutinated by this serum were agglutinated by a serum prepared from strain S174.

One volume of each of these three sera was pooled and added to seven volumes of saline, so that each serum in the mixture was diluted 1:10. All strains were tested with this pooled diluted serum; the results are shown in Table 2. Of 269 coagulase-positive strains, 257 were agglutinated, 16 of them incompletely;

nine strains were auto-agglutinable. One human strain (S291) and two animal strains were not agglutinated.

Of the 123 coagulase-negative strains, 120 were not agglutinated, one was slightly agglutinated: two were auto-agglutinable.

TABLE 2

Results of slide agglutination tests with pooled sera and staphylococci GROWN IN BROTH

	Coagulase-positive Strains		Coagulase-negative Strains	
	Human	Animal	Human	Animal
Agglutinated	220	37	1	0
Not agglutinated	1	2	106	14
Auto-agglutinated	5	4	2	0
TOTAL	226	43	109	14

The human coagulase-positive organism S291 which was not agglutinated, was further examined. When grown for 24 hours on sheep blood-agar it was weakly haemolytic. In "toxin" broth under increased $C\theta_2$ tension it produced a lysin for rabbit cells which was neutralized by α anti-toxin; no β toxin was detected. It coagulated horse, human and rabbit plasma and fermented mannitol. 1 ml. of a 24 hours' broth culture given intravenously killed a rabbit within 24 hours, and an almost confluent growth of the organisms was obtained on blood-agar inoculated with a drop of the heart blood taken post mortem. An attempt was made to prepare anti-sera with it but, at the end of the usual course of inoculations in rabbits, the sera agglutinated the organisms only in low dilution. Efforts to raise the titre of the sera by giving formalinized and, finally, live organisms were only partly successful. On testing the sera against other pathogens, they were found to

agglutinate some of them in moderately high titre (1:100). Strain S291 was apparently related serologically to the pathogens but was of exceptionally low agglutinability.

The two coagulase-positive animal strains not agglutinated were isolated from dogs. Both produced α and β toxins, and when grown in broth and re-suspended in saline formed coarse suspensions as do many coagulase-negative strains. Cowan (1938) found a serological relationship between certain haemolytic animal strains and saprophytic staphylococci.

(c) Agglutination tests with organisms grown on nutrient agar.

Organisms grown on nutrient agar were less agglutinable than those grown in broth. A serum made from a different strain (S39) from the three used above, was substituted for the type I serum in the pooled serum mixture, and the size of the drop of suspension was reduced to ten times that of the drop of diluted serum. Most coagulase-positive strains were agglutinated by these pooled diluted sera (S39, type II and S174). Three of the first 100 coagulase-negative strains were partly agglutinated. These strains were non-haemolytic on sheep blood-agar, did not produce toxin in broth, did not ferment mannitol, did not coagulate horse, human and rabbit plasma and did not kill rabbits and mice when injected intravenously. Absorption of serum S39 with one of these coagulase-negative strains removed the agglutinins for all three, leaving the agglutinins for the coagulase-positive strains almost unaffected.

Type II and S174 sera were added to this S39 absorbed serum to provide a mixture which would agglutinate all coagulase-positive strains, but partial agglutination of some coagulase-negative strains again occurred. It was found necessary to use the absorbed serum S39 (diluted 1:5) alone and re-test strains not agglutinated by this serum with a mixture of two sera. A type III serum was

found more suitable than serum S174 (see Section (b)), although both sera were equally effective when tested against organisms grown in broth.

As shown in Table 3, the absorbed serum S39 agglutinated 203 (12 incompletely) of 226 coagulase-positive human strains. Using a mixture of type II and S120 sera in which each serum was diluted 1:10, 16 of the remainder were agglutinated; two strains were auto-agglutinable. When grown in broth two of the remaining five were agglutinated by serum S174, and two by type I serum; the other was the apparently inagglutinable strain S291. No coagulase-negative strain was agglutinated.

Of the 43 coagulase-positive animal strains, 38 were agglutinated by serum S39, three by the type II-S120 serum mixture, and two were not agglutinated. No coagulase-negative strain was agglutinated.

TABLE 3.

Results of slide-agglutination tests with staphylococci grown on nutrient agar. Organisms were first tested with absorbed serum S39; if not agglutinated, they were tested with a diluted mixture of Type II and Type III sera.

	Coagulase-positive Strains		Coagulase-negative Strains	
	Human	Animal	Human	Animal
Agglutinated	219	41	0	0
Not agglutinated	5	2	109	14
Auto-agglutinated	2	0	0	0
TOTAL	226	43	109	14

203 of the human strains and 38 of the animal strains were agglutinated by the absorbed serum S39.

The method can be used to test organisms from single colonies. Sufficient material can usually be collected from a colony with a platinum loop to give an adequate suspension in each of two drops of saline, to one of which the serum is then added.

(d) Agglutination of coagulase-negative strains.

Attempts to prepare antisera with coagulase-negative strains were not very successful. Five strains giving different biochemical reactions were injected into rabbits. Even after a prolonged course of injections and, finally, large doses of living organisms, two of the sera could not be raised to a workable titre. The other three were tested against 12 selected coagulase-positive strains and 12 coagulase-negative strains. Nearly all the coagulase-positive strains were agglutinated, some to a slightly higher degree than the homologous strains. Eight of the heterologous coagulase-negative strains were not agglutinated.

DISCUSSION

These results indicate that agglutination with pooled serum and with organisms grown in broth may be used as a laboratory test for pathogenicity. The value of this test is limited by the occurrence of auto-agglutinable strains.

The results also have some theoretical interest in connection with the validity of the coagulase test as a criterion of pathogenicity. Agglutination tests with sera prepared from pathogenic strains divided the staphylococci into two groups. With one exception (S291), the first group included all strains which were pathogenic as judged by their source (45 from boils and 37 from osteomyelitis), together with all strains (258), which were definitely toxigenic. The one characteristic common to all members of this group was ability to coagulate plasma.

The one exception amongst the human strains (S291), although

not agglutinated by the pooled sera made with coagulase-positive strains, was found to be serologically related to the coagulase-positive group.

The second group included no strain which could be judged as pathogenic according to its source and (with the exception of strain S291) no definitely toxigenic strains. Strains of this group did not coagulate plasma.

The result of the pathogenicity test in the rabbit with strain S5 suggests that ability to coagulate plasma or to be agglutinated by a serum prepared with a pathogenic strain may be of greater importance in judging pathogenicity than ability to produce haemolytic toxin in detectable quantity.

The agglutination of most coagulase-positive animal strains by sera made from human pathogenic strains accords with the results of Cowan (1938). Using a precipitin technique he showed a group serological relationship between toxigenic human and most toxigenic animal strains.

The anomalous coagulase reactions (Section (a)) given by the two animal strains confirm Chapman's (1938) conclusion that choice of plasma may affect the result of the coagulase test with strains of animal origin.

Since type I, type II and S174 sera contain together agglutinating antibodies for all pathogenic strains, it seems desirable to include the corresponding strains in polyvalent vaccines.

SUMMARY

335 strains of staphylococci of human origin and 57 strains of animal origin have been examined.

Of 269 coagulase-positive strains, 257 were agglutinated by a mixture of three antisera prepared from coagulase-positive strains; nine strains were auto-agglutinable and could not be tested. One human and two animal strains were not agglutinated; the human strain was serologically related to the coagulase-positive strains in spite of its inagglutinability with the serum mixture. Of 123 coagulase-negative strains, 120 were not agglutinated, two were auto-agglutinable and one was agglutinated slightly.

An attempt to produce agglutinating sera with coagulase-negative strains which would agglutinate coagulase-negative and not coagulase-positive strains was unsuccessful.

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OBSERVATIONS ON STAPHYLOCOCCAL HAEMOLYSINS AND STAPHYLOCOCCAL LIPASE

by R. CHRISTIE and J. J. GRAYDON

(From the Commonwealth Serum Laboratories, Melbourne.)

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The α and β toxins or haemolysins of staphylococci have recently received much attention. They occur in cell-free filtrates, and are antigenically distinct (Glenny and Stevens, 1935; Bryce and Rountree, 1936). The α toxin lyses rabbit and sheep red blood cells at 37°C.; Morgan and Graydon (1936) demonstrated two components, α 1 and α 2, recognizable by their different combining powers with antitoxins containing different proportions of the respective antibodies. The β toxin does not lyse rabbit cells and only lyses sheep cells when incubation at 37°C. is followed by refrigeration. This action of the β toxin on sheep cells is called "hot-cold" lysis.

Bryce and Rountree used "spot-inoculation" of sheep blood agar plates to identify the toxins of staphylococci. After 18-24 hours' incubation at 37°C., colonies of α toxin-producers were surrounded by a zone of complete clearing; β toxin-producers were surrounded by a zone of darkening which became lighter on refrigeration. Strains producing both toxins showed a combination of these two effects, an inner zone of complete clearing surrounded by a darker zone which became lighter on cooling.

Orcutt and Howe (1922) found evidence of lipase-production by staphylococci on blood agar plates containing cream. Colonies of certain staphylococci were surrounded by a zone of clearing although no haemolysis was visible when the organisms were grown on blood agar plates without cream. They showed that staphylococci may form an extracellular thermolabile enzyme which hydrolyses butter-fat, and that the fatty acid (or soap) formed produces the haemolysis on cream blood agar shown by these strains.

Birch-Hirschfeld (1937), using hydrolysis of tributyrin to estimate lipase and sheep cell lysis to estimate haemolysin, examined the relationship between lipase and haemolysin production by 13 strains of staphylococci. The ratio between the amounts of lipase and haemolysin was fairly constant, from which it was deduced that they had a common origin.

Observations made in this laboratory on the lipolytic and haemolytic power of staphylococci are interesting in view of possible interpretations they offer concerning the action of α toxin, β toxin and lipase on sheep red cells.

In the following report the phrase "zone of β haemolysis", unless otherwise indicated by the context, refers to the zone of darkening produced on sheep blood agar by β toxin, although, strictly speaking, this is not a zone of lysis.

EXPERIMENTS AND RESULTS

(a) Detection of Lipase Production by Staphylococci.

Blood agar plates, containing 0.2 p.c. cream, were inoculated with various strains of staphylococci and incubated at 37°C. Control plates without cream were similarly treated. Lipase-production, as indicated by increased haemolysis on the plates containing cream was quite distinct with some strains, less so with others. Incubation for more than 24 hours was usually necessary

before any increase became apparent. Horse and rabbit blood indicated lipase-production earlier than sheep or human blood.

Uncertain results were obtained with strongly haemolytic strains producing only small quantity of lipase.

More direct and less equivocal results were obtained with nutrient agar plates containing 0.1 p.c. washed butter-fat and 0.005 p.c. neutral red. The butter-fat was washed six times by shaking with hot water and centrifuging. On this medium fat hydrolysis was indicated by a zone round each colony (Fig. 1), in which the fat particles were red, whereas those in the rest of the medium were yellow. Twenty-four pathogenic and twenty-four non-pathogenic strains (using ability to coagulate plasma as the criterion of pathogenicity) were cultivated on these plates and examined daily for nine days. With most lipolytic strains red zones appeared within two days, with others not until the fourth day; no appreciable increase occurred after the fifth day. The haemolytic power of the strains was determined from the appearance of their colonies on sheep-blood agar after 24 hours' incubation at 37°C. and 18 hours' refrigeration at 4°C. The results of these tests are shown in Table 1. Lipase is produced independently of haemolysin and appears to have no connection with pathogenicity.

TABLE 1

Results of haemolysin and lipase tests, with number of strains in each group

	Pathogenic strains				Non-pathogenic strains	Total
Type of haemolysis	α	$\alpha\beta$	β	Nil	Nil	
Lipase positive	7	12	-	1	17	37
Lipase negative	1	1	1	1	7	11
TOTAL	8	13	1	2	24	48

Lipolytic activity was much more marked with strain S36 than with any other. Since this strain played a large part in the experiments which followed, its other characteristics were examined.

(b) Particulars of Strain S36.

This organism, though pigmented, was apparently a non-pathogen. It produced little or no haemolysis on sheep-blood agar after 24 hours' incubation at 37°C. No α or β toxin was detected when it was grown under increased CO_2 tension in broth containing 0,1 pc. agar. It did not ferment mannitol, was not agglutinated by anti-sera prepared from serological types of pathogenic strains and did not coagulate plasma. Injected intravenously and intraperitoneally into rabbits and mice in large doses it produced no ill effect. It showed some relationship to the pathogenic staphylococci in its susceptibility to lysis by the staphylococcal bacteriophages, Au I, Au II, Au III and Au IV (Burnet and Lush, 1935).

Its lytic action on red cells in cream blood agar was not due to fermentation of lactose or casein present, since it did not ferment lactose, gave no zone of clearing on fat-free milk agar such as proteolytic strains produce, and did not liquefy gelatine (28 days allowed).

(c) The Effect of Staphylococcal Lipase on Sheep Red Cells Previously Altered By β Toxin.

One strain (S32a, received from Dr. L. M. Bryce) producing β toxin but no α toxin showed no signs of lipolytic activity when tested by the method of Orcutt and Howe or when grown on butter-fat agar. A mixed suspension of this strain and the lipolytic strain S36 was plated out on sheep-blood agar. After 24 hours' incubation typical zones of β haemolysis formed round the colonies of S32a. No haemolysis showed round the colonies of S36 in the parts of

the medium free of β toxin, but when within zones of β haemolysis they were surrounded by an area of clearing.

Fig. 2(a) illustrates such an effect. The growth in the centre is from strain S32a. It is surrounded by two concentric zones of β haemolysis. The inner one was formed within 24 hours, and was partly lightened by the cooling which occurred when the plate was withdrawn from the incubator for examination and spot-inoculation with strain S36. Three colonies of S36 showing clear zones round them can be seen within the area affected by β haemolysin. A fourth colony on the outer edge of the β zone shows clearing only on the segment falling within that zone, with no change in the medium on the other side of the colony. Three colonies of S36, which can be seen on toxin-free parts of the medium in Fig. 2 show no haemolysis. Clearing round colonies of S36 also occurred within the zones of β haemolysis surrounding colonies of αβ strains (Fig.2, b and d).

Fig. 3 shows the effect produced when an otherwise invisible zone of lipase overlapped a zone of β haemolysis. The lipase lysed the cells affected by β toxin but did not lyse normal cells. This plate also shows the lightening in colour of the cells within the zone of β haemolysis which occurred on partial cooling.

Since the combined action on sheep red cells of β toxin and the exudate from the lipolytic non-toxigenic strain gave a result resembling the action of α toxin, the possibility that this exudate converted β toxin into α toxin was examined. Strains S32a and S36 were grown separately and together, for four days, under increased CO_2 tension in broth containing 0.1 p.c. agar. The cultures, after centrifugation, were titrated against rabbit and sheep red cells. The absence of α toxin, was indicated by the absence of rabbit-cell lysin in all the filtrates. The presence of β toxin, indicated by sheep cell lysis after one hour's incubation followed by refrigeration, was demonstrated

in the mixed culture and in the pure culture of strain S32a. The centrifugates of the two pure cultures were then mixed, filtered through a collodion membrane (pore size 380m) and incubated overnight at 37°C. Titration against rabbit and sheep cells showed the presence of β toxin only.

A skin staphylococcus, strain S205, although non-lipolytic, produced clearing in zones of β haemolysis. This suggested that the exudate responsible for the clearing produced by strain S36 was not necessarily lipase. However, comparison of strains S205 and S36 indicated that the clearing caused by the former was due to a different action. The following points of difference were noted: (1) Strain S205 liquefied gelatin whereas S36 did not. (2) The zones of clearing produced by S36 in zones of β haemolysis were faintly opaque and had an indefinite boundary, whilst those produced by S205 were glassy clear and had a sharp boundary. Many air-borne chromogenic bacilli also produce glassy clear, sharply defined zones of clearing in zones of β haemolysis (Fig. 4); two such strains were examined and found to be proteolytic but not lipolytic. (3) Strain S205 and these two air-borne bacilli caused clearing in zones of partial haemolysis round colonies of Cl. welchii on sheep blood agar, whereas strain S36 caused no clearing whatever. Results obtained with lipolytic and non-lipolytic α toxin-producing strains (Section (e)) agreed with the hypothesis that lipase caused clearing in zones of β haemolysis. The conclusion, therefore, was that the clearing produced by S36 was lipolytic in nature, and that produced by S205 was probably due to proteolysis.

(d) Effect of Staphylococcal Lipase on Cells Already Attacked by α-Toxin.

An α toxin-producing strain, S210, non-lipolytic when tested on cream blood agar or butter-fat agar, was spot-inoculated on sheep blood agar. After 24 hours' incubation at 37°C. the growth was

surrounded by a zone of α lysis. This zone was almost colourless but had a slight opacity and an indefinite boundary. Microscopic examination showed that there were no red cells immediately surrounding the colony, and that the number of cells remaining within the zone of α lysis increased with distance from the colony until unaltered medium was reached.

The lipolytic strain, S36, was inoculated on and near this zone of clearing and a further 24 hours' incubation at 37°C. allowed. No increase of clearing was observed (Fig.2c). This indicated that the modification in the cell induced by β toxin was not merely an incomplete stage of that induced by α toxin.

TABLE 2

Results of Haemolysin Titrations Showing the Inhibitory Effect of β Toxin On the Lytic Action of α Toxin on Sheep Red Cells

Series I. Eight drops of each diluted preparation of β toxin were added to the corresponding tube. One drop of 10 p.c. sheep red cell suspension was added to each tube. After one hour's incubation at 37°C., one drop of saline was added to each tube.
Haemolysis after one hour's further incubation at 37°C. is shown in row H (=hot).
Haemolysis after overnight refrigeration is shown in row C (=cold).

Series II. Similar to the above except that one drop of α toxin was added in place of one drop of saline.

Series III. Similar to Series II except that the drop of α toxin was added to each tube immediately prior to the addition of the drop of sheep cell suspension Readings were made after one hour at 37°C. and after overnight refrigeration.

		Final dilution of β toxin.									
		1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Series I	H	-	-	-	-	-	-	-	-	-	-
	C	++++	++++	++++	++++	++++	++++	+++	++	±	-
Series II	H	-	-	±	±	±	-	-	++	++++	++++
	C	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Series III	H	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	C	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

++++ indicates complete haemolysis
+++ " 75% "
++ " 50% "
± " trace of " (less than 5%).

(e) Inhibition of α Haemolysis by β Toxin.

A zone of β haemolysis was produced on sheep blood agar by inoculating with strain S32a and incubating for 24 hours. The non-lipolytic α toxin-producing strain, S210, was inoculated on and near this zone. After 24 hours' further incubation the colonies of S210 were surrounded by typical zones of α haemolysis only when they were beyond the zone of β haemolysis; when within the zone of β haemolysis there was no sign of clearing round them (Fig. 5). Even after refrigeration overnight no indications of lytic action due to α toxin occurred round the colonies of S210 within the partly lysed zone of β haemolysis.

A lipolytic strain, S61, which produced α toxin but no β toxin, was treated in the same way as strain S210. Its colonies were surrounded by large typical zones of α lysis when they occurred in medium free of β toxin; colonies situated within β areas had much smaller zones due to the action of the lipase produced by strain S61.

Inhibition of α haemolysis by β toxin was also demonstrated in test-tube experiments using cell-free filtrates. Eight drops of successive doubling dilutions of β toxin were added to a row of $3\frac{1}{2}$in. x $\frac{1}{2}$in. test-tubes. One drop of 10 p.c. washed sheep cell suspension was added to each tube and the rack placed in a water-bath at 37°C. After an hour one drop of saline was added to each tube. Readings of haemolysis were made after incubation for another hour and again after over-night refrigeration. A second row was prepared like the first, but with one drop of a diluted α toxin substituted for the drop of saline. (The amount of α toxin added was shown by preliminary titration to be double that required to lyse the cells completely.) A third series of tests was performed in which the α toxin was added to the β toxin preparations just prior to the addition of the cell suspension.

The results are shown in Table 2. Haemolysis occurring after one hour's incubation at 37°C. was due to the action of α toxin. Further haemolysis on cooling indicated the action of β toxin. The results in Series I showed the degree to which the β toxin could be diluted before it ceased to cause "hot-cold" lysis. The results in Series II showed that the α toxin could only lyse the cells previously treated with β toxin when the concentration of the latter was too low to affect them. Series III showed that the mere presence of β toxin did not inhibit the lytic action of α toxin.

(f) Effect of β Toxin on Cells Already Attacked by α Toxin.

The α toxin-producing strain, S210, was inoculated on sheep blood agar. After 24 hours' incubation at 37°C. a zone of clearing with the slight opacity and indefinite boundary described in Section (d) appeared around the colony. The β toxin-producing strain S32a was inoculated on the medium near this zone. After 24 hours' further incubation at 37°C. a zone of β haemolysis had formed round the growth from strain S32a. This zone overlapped the zone of α lysis. The overlapped area was distinct from the remainder of the zone round each growth in that it was glassy-clear. The arc of the circumference of the zone of β haemolysis in this area was sharply defined by the contrast between the opacity present in the zone of α lysis on the outside and the glassy clearness within (Fig. 6). On microscopical examination, the area which appeared glassy-clear to the naked eye was slightly opaque, but there were no unlysed cells present as in the normal zone of α lysis. That β toxin could lyse such cells showed that some change had been induced in them by α toxin since β toxin will not lyse normal sheep red cells at 37°C.

It was also noticed that the zones of α lysis occurring on sheep blood agar round colonies of αβ strains differed from those round colonies of α strains producing no β toxin; they were

glassy-clear, had a sharp boundary. Such zones therefore should not be regarded as zones of α lysis but of $(\alpha+\beta)$ lysis.

The action of β toxin in augmenting the action of α toxin on sheep cells was also shown using cell-free filtrates. A drop of α toxin produced a slightly opaque zone of clearing on a sheep blood-agar plate after one hour at 37°C. A drop of β toxin was allowed to fall on the edge of this zone. After one hour's further incubation the area of α lysis overlapped by the drop of β toxin was much clearer than the area of α lysis alone.

(g) The Action of the Toxins On the Colouring Matter of the Red Cells.

Several small experiments showed that the loss of colour in zones of α lysis round colonies on blood agar was partly or completely inhibited when diffusion of haemoglobin from these zones was prevented. No visible change occurred around colonies grown on lysed blood agar plates. A blood agar plate flooded with α toxin showed complete lysis but no loss of colour. Rigdon (1939) reported that staphylococcal toxin had no effect on lysed blood agar, although it caused areas of clearing on normal blood agar.

Spectroscopic examination of a sheep red cell suspension lysed by α toxin did not show absorption bands of altered haemoglobin. A red cell suspension treated at 37°C. for one hour with β toxin, and also a saline extract from sheep blood agar which had been discoloured by treatment for one hour with β toxin, showed no indication of the presence of altered haemoglobin. However, a red cell suspension left in contact with β toxin at 37°C. overnight and then lysed by cooling showed the absorption bands of methaemoglobin. It seems probable that this methaemoglobin resulted from some secondary reaction not necessarily involving the toxin. When a zone of β haemolysis on sheep blood agar is viewed obliquely by reflected light it appears lighter than the rest of the medium, suggesting that the darkening seen with transmitted light is due

to a change in the opacity of the cells rather than to an alteration in the colouring matter.

DISCUSSION

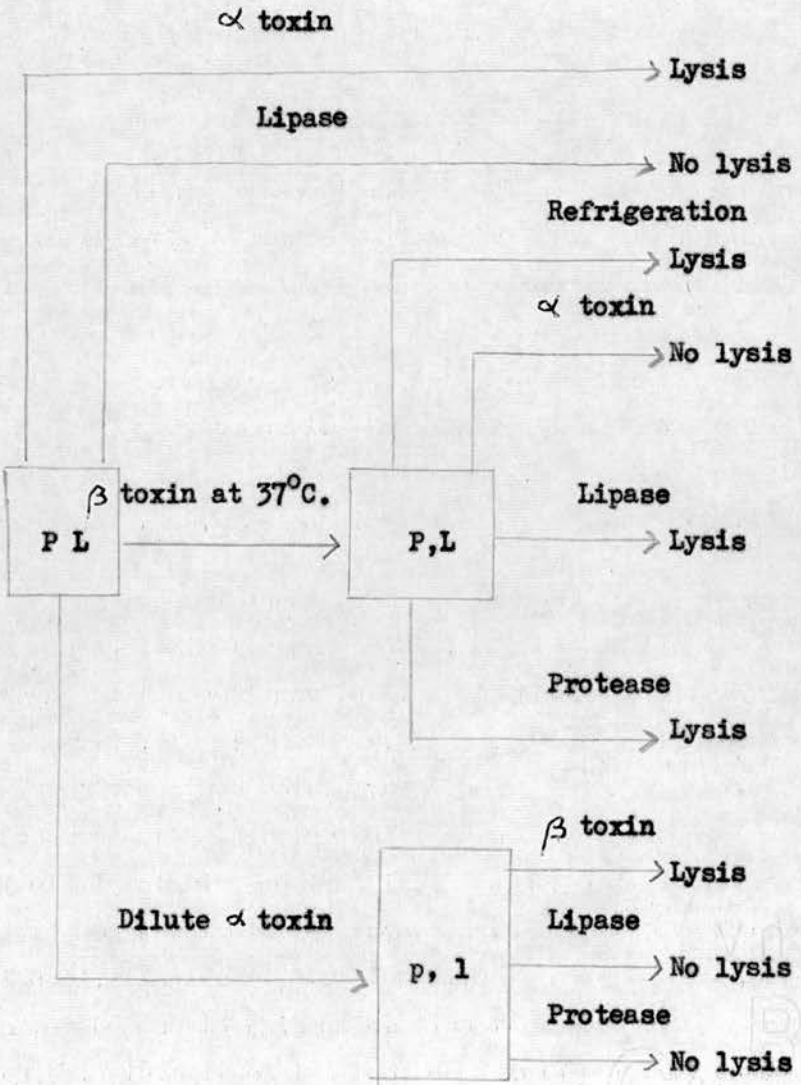
As Rigdon has reported, staphylococcal α toxin does not attack the haemoglobin of the red cell. The loss of colour which occurs round colonies of staphylococci on blood agar is due to diffusion of the liberated haemoglobin into the surrounding medium in a manner similar to that described by Brown (1919) to explain the clearing round colonies of streptococci. The destruction of haemoglobin found by Clauberg (1929) in test-tube experiments, by which he explained the clearing on blood agar, was possibly due to further bacterial action.

From general considerations it would appear that α and β toxins are somewhat similar substances, capable of linking up with some substrate in the cell envelope. This occurs primarily as an adsorption (Levine, 1938; Forssman, 1939), following which the mutual relationship between the protein and lipoid of the cell envelope is modified according to the nature of the toxin. The β toxin alters the protein-lipoidal material so that it is susceptible to staphylococcal lipase and to some proteolytic enzymes having no apparent effect on the normal cell. That cells treated with β toxin are no longer lysed by α toxin may be due to this alteration in the substrate normally attacked by α toxin. It could also be explained by assuming that the β toxin has occupied most of the available linkages in the substrate, leaving insufficient points of attack for the α toxin. Such an explanation has a parallel in the case of inhibited adsorption catalysis and in the "blockade immunity" hypothesis evolved to explain the action of weakly lytic bacteriophages in protecting against related and more potent bacteriophages (White, 1937). A further possibility is that, after attack by β toxin, the permeability of the envelope is so altered that α toxin is prevented from reaching the internal structure of the

envelope, which we must then presume to be its point of attack. The change in the opacity of the cell wall and the subsequent formation of methaemoglobin, though not inconsistent with either of the other theories, support the theory of altered permeability. From experiments with toxic filtrates on sheep blood agar, it appears that the action of β toxin on the envelopes of the red cells is completed rapidly. This conclusion is supported by the almost instantaneous lysis which follows rapid cooling of a suspension of sheep cells treated for one hour with β toxin. This lysis seems to be the result of a secondary change, possibly purely physical, and not caused by further action of the toxin.

The microscopic examination of red cell suspensions and of blood agar plates treated with α toxin shows that there is a variation in the resistance of individual cells to haemolysis. The resistant cells are altered, however, by the α toxin so that they are haemolysed by β toxin. This increase in the haemolytic power of β toxin suggests the presence of an activator, possibly the lecithin of the cell, which exerts a markedly accelerating effect on haemolysis of red cells, as demonstrated with snake venoms and certain other haemolytic agents (Herrmann and Rohner, 1925; Kellaway and Williams, 1933).

Our results showing the effect of these staphylococcal exudates on sheep red cells may be summarized diagrammatically as under:



P L	represents the normal sheep red cell.
P,L	represents the sheep red cell with protein-lipoid relationship modified by β toxin.
p, l	represents the sheep red cell with protein-lipoid modified by α toxin.

Our inability to confirm the correlation found by Birch-Hirschfeld between lipase and haemolysin production may be due to the larger number of strains in our series or to the differences in technique.

SUMMARY

A simple method of detecting lipase production by staphylococci is described.

In an examination of 48 strains of staphylococci, lipase was produced independently of haemolysin. There was no correlation between lipase production and pathogenicity.

Using blood agar plates it was shown that staphylococcal lipase will lyse sheep red cells treated with staphylococcal β toxin, although it will not lyse normal sheep red cells; it does not augment the lytic action of staphylococcal α toxin.

Using blood agar plates and also by test-tube experiments with toxic filtrates it was shown that sheep red cells, after treatment with staphylococcal β toxin, are resistant to the lytic action of staphylococcal α toxin.

On the other hand it was shown that under suitable conditions staphylococcal β toxin, which does not lyse normal sheep cells, will lyse sheep cells previously treated with α toxin.

The mode of action of staphylococcal toxins on sheep red cells is discussed.

ACKNOWLEDGEMENTS

We are grateful to Dr. F. G. Morgan, Director of these Laboratories, and to Dr. F. M. Burnet, of the Walter and Eliza Hall Institute, for helpful advice and criticism, and also to Mr. F. J. Dempster for preparing the photographs.

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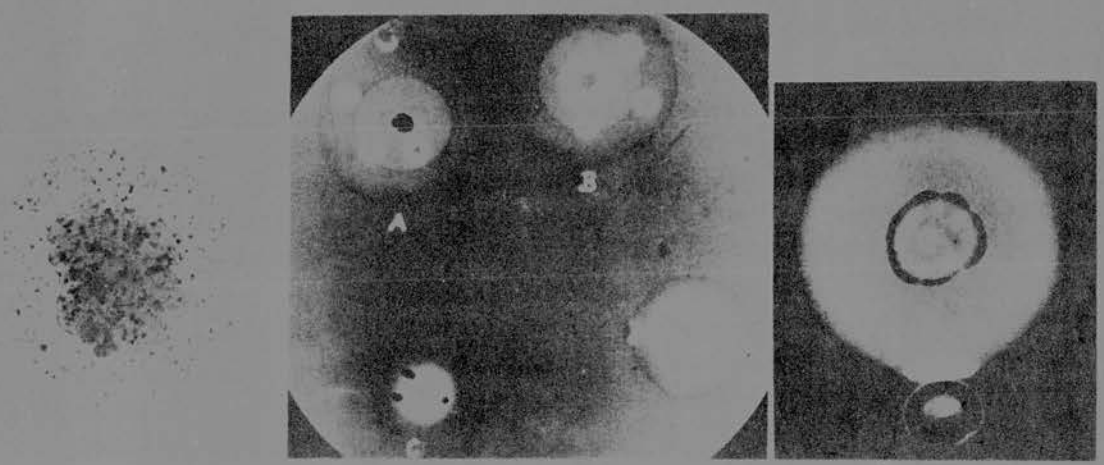

Fig. 1. Fig. 2. Fig. 3.

Fig. 1. Colony of a lipolytic staphylococcus (strain S36) on butter-fat neutral red agar after 48 hours' incubation at 37° C. The dark particles (actually red) consist of butter-fat partly hydrolysed by lipase.

Fig. 2. (a) A colony of staphylococci (strain S32a) on sheep blood agar surrounded by two concentric zones of β haemolysis. Four colonies of a non-haemolytic lipolytic staphylococcus (strain S36) are causing clearing in these zones. Three colonies of strain S36 producing no haemolysis appear in the toxin-free parts of the plate.

(b) Colony of staphylococci on sheep blood agar showing a clear zone of α haemolysis surrounded by two zones of β haemolysis. Three colonies of strain S36 appear in the zones of β haemolysis.

(c) A colony of α toxin-producing staphylococci (strain S210) on sheep blood agar showing three colonies of strain S36 within the area affected by α toxin.

(d) Colony of staphylococci on sheep blood agar showing a large zone of α haemolysis surrounded by a zone of β haemolysis. Three colonies of strain S36 appear within the zones of haemolysis.

Fig. 3. Colony of staphylococci (strain S32a) on sheep blood agar, surrounded by a large zone of β haemolysis showing the clearing caused within this zone by the lipase from the colony of lipolytic staphylococci (strain S36).

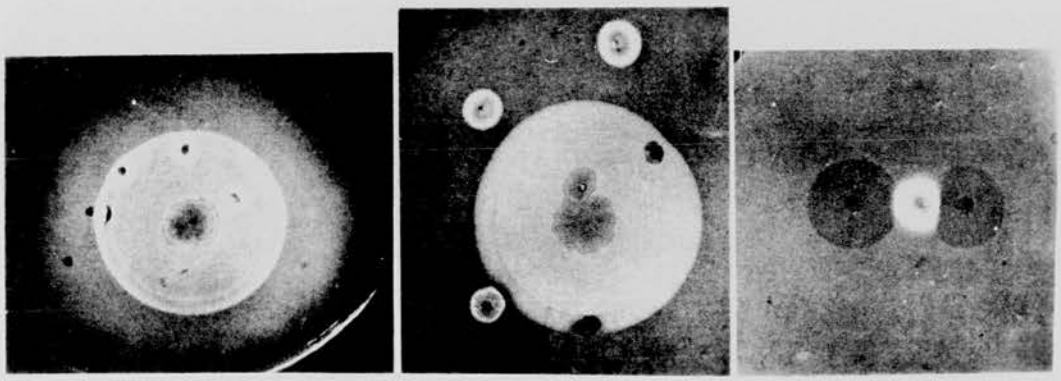

Fig. 4. Fig. 5. Fig. 6.

Fig. 4. Colony of staphylococci (strain S32a) on sheep blood agar surrounded by zones of β haemolysis, showing the clearing caused within these zones by a non-haemolytic air-borne cocco-bacillus. Five colonies of the latter appear.

Fig. 5. Colony of staphylococci (strain S32a) on sheep blood agar surrounded by two zones of β haemolysis. Five colonies of a strain (S210) producing α toxin show zones of clearing only when they are beyond the area of β haemolysis.

Fig. 6. Colony of α toxin-producing staphylococci (strain S210) on sheep blood agar with two colonies of β toxin-producing staphylococci (strain S32a) showing the extra clearing produced where β toxin acts on cells already affected by α toxin.

OBSERVATIONS ON STAPHYLOCOCCAL BETA TOXIN AND ITS PRODUCTION BY STRAINS ISOLATED FROM STAPHYLOCOCCAL LESIONS IN MAN

by R. CHRISTIE and E. A. NORTH

(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 12th September, 1941.)

Staphylococci may produce at least two exotoxins, α toxin and β toxin. α toxin is lethal for rabbits and mice, is formed by all or nearly all pathogenic strains of human origin, and is regarded as the agent chiefly responsible for the pathological disturbances in staphylococcal infections.

Morgan and Graydon (1936) have shown that this α toxin may consist of two separate components, α_1 and α_2 toxins, and that these are distinct from the β toxin of Glenny and Stevens (1935) which, though noted in their series, was relatively uncommon. They suggested, moreover, that conditions of cultivation influence the production of the α_2 component irrespective of the strain.

β toxin, which causes the "hot-cold" lysis of sheep red cells, is practically non-lethal for rabbits and mice, and is said to occur only rarely in strains of human origin. No strain producing β toxin only has yet been reported as responsible for disease in the human being. Cowan (1938) found that of 82 strains of human origin, only 3 produced β toxin. Christie and Keogh (1940) found 18 of 141 human haemolytic strains produced β as well as α toxin. β toxin occurs more frequently in animal strains (Minett, 1936; Cowan, 1938) and may

occur there without the α toxin. It is regarded of little significance in human infection.

Bryce and Rountree (1936) showed that sheep blood agar was suitable for determining the haemolytic nature of staphylococci. α strains produce on this medium colonies surrounded by zones of clearing. β strains produce colonies surrounded by zones of darkening while $\alpha\beta$ strains produce colonies surrounded by a zone of clearing within a zone of darkening. Rountree (1936) showed that β variants could be produced from some α strains by suitable methods of cultivation.

During an examination of a number of strains in this laboratory, using titration of toxins against rabbit and sheep red cells as well as appearance of colonies on sheep blood agar to identify the toxins, the findings of Bryce and Rountree and of Rountree were confirmed, whilst some additional information regarding β toxin was obtained.

EXPERIMENTS AND RESULTS

(a) Haemolysis on Sheep Blood Agar Plates.

Strains of staphylococci were spot-inoculated on sheep blood agar and incubated at 37°C. for 24 hours. The α strains showed zones of complete or nearly complete clearing around their colonies but it was noticed that there were three varieties of these zones:

1. A zone of clearing with a hazy boundary. (See Figure 1 (B).)
2. A zone of clearing with a fairly sharp edge, the medium in this zone appearing glassy-clear as compared with the faintly opaque zone obtained with the first type.
3. An inner zone of clearing surrounded by a zone of partial lysis, even in degree over its whole area, and having quite sharply defined inner and outer margins. (See Fig. 1(A).)

Cooling for some hours on the bench or at 4°C. followed by a further 24 hours' incubation caused no change in the first

variety of zone beyond some extension. The second variety usually developed an outer zone of partial lysis while the third variety showed extension of both the clear and the partly clear zone with in some cases, formation of a third zone a little darker than the second but still showing some lysis. Further cooling and incubation accentuated the difference between the first variety and the other two.

The possibility was examined that β toxin, in quantity insufficient to pass beyond the α zone and produce a typical zone darker than the rest of the medium, was responsible for the appearance in the second and third varieties. Considerable evidence was found in support of this view.

Anaerobic growth on sheep blood agar inhibits the appearance of the α zones but still allows the β zones to appear. A pure β strain, a strain of the first variety and a strain of the third variety were grown for three days anaerobically on sheep blood agar. Zones of darkening showed around the colonies of the pure β strain and of the strain of the third variety. No lysis appeared around the colonies of the first variety. That the two zones of darkening were caused by β toxin was confirmed by incubating the colonies for 24 hours further after inoculating a non-haemolytic, lipolytic strain (S36) on the dark zones. In both cases, zones of clearing appeared around the colonies of strain S36 within the zones of darkening (Christie and Graydon, 1941).

A two-months-old agar slope culture of this strain of the third variety was plated out on sheep blood agar to give single colonies. Several of these were definite $\alpha\beta$ variants while some of the others were of the second variety, thus suggesting that strains of the second variety were essentially the same as those of the third variety but produced less β toxin. That the appearance in strains of the second variety is due to α and β toxin is suggested by the findings of Christie and Graydon (1941) who showed that β toxin

may augment the action of α toxin on sheep red cells.

Support for the view that the outer partly-lysed zone shown by the third variety of strains was due to β toxin was obtained by plate experiments with cell-free toxins. A drop of pure β toxin caused a zone of darkening on a sheep blood agar plate while a drop of α toxin caused a zone of clearing with a hazy edge. Mixtures of α and β toxin were prepared, ranging from 5 parts of α toxin and 95 parts of β toxin at the one end to 95 parts of α toxin and 5 parts of β toxin at the other. Drops of these mixtures were allowed to fall on a blood agar plate and the plate left at 37°C. for an hour. Where the β toxin was in excess a dark zone formed. As the α toxin increased, a zone of partial clearing appeared in the centre of the dark zone until a point was reached where the outer zone instead of being darker than the rest of the medium appeared lighter. This was identical with the appearance of the third variety described above. With still lower proportions of β toxin appearances approximating to those of the second variety were observed.

Seventy-two old strains, originally recorded as showing a haemolysis from the appearance of their colonies on sheep blood agar were spot-inoculated on sheep blood agar, alternately incubated and cooled daily for a few hours and examined at intervals. Seven gave the definite appearance of $\alpha\beta$ strains, the outer zone being darker than the rest of the medium, 14 gave the appearance of the first variety and the other 51 were of the second and third varieties. The strains were plated out on sheep blood agar, incubated for 24 hours and examined. 22 of the strains showed definite $\alpha\beta$ variants.

Agar slope cultures of each strain were prepared from single colonies which showed no definite signs of darkening indicative of β toxin. Efforts were made to prepare $\alpha\beta$ variants from these by growing in broth under increased CO_2 tension, in peptone water

and anaerobically in broth for several days. We were able to obtain either $\alpha\beta$ variants or variants of the third variety from all except 15 of the strains. Many of the strains gave variants showing β and no α toxin. Non-haemolytic and visibly rough colonies also appeared.

These results suggest that β toxin is more commonly produced by staphylococci of human origin than was formerly thought, and that most strains generally regarded as solely α toxin producers are either actual or potential producers of β toxin as well. It will be convenient to refer to the strains of the second and third varieties as $\alpha(\beta)$ strains.

Strain "Mills" and strain "Denis", two of the strains used by Morgan and Graydon (1936) in detecting the two components of α toxin, were examined. Strain "Mills" ($\alpha 1$) was of the first variety, i.e. a pure α strain. Strain "Denis" ($\alpha 1 \alpha 2$) was an $\alpha(\beta)$ strain. It seemed not improbable that the toxin in strain "Denis" might have had an influence on the rabbit red cell lysis by α toxin similar to the α and β lysis of sheep red cells found by Christie and Graydon (1941) and that $\alpha 2$ toxin was actually β toxin. This view was supported by Roy's (1937) statement that β toxin affects the neutralization power of α antitoxin for α toxin. If this were so, however, the addition of β toxin to α toxin ought to increase the degree to which the latter will lyse rabbit cells. This was tested by adding varying quantities of β toxin to a fixed quantity of α toxin and titrating the mixtures so obtained against rabbit cells. The results indicated that β toxin had no effect on the lytic power of α toxin for rabbit red cells. Thus there was no evidence that $\alpha 2$ and β toxins were the same.

Since all the strains used in these tests had been maintained on artificial media for a considerable time, it was possible that the instability of the strains and tendency to form haemolytic

variants were peculiarities which would not be shown by freshly-isolated strains. To test this, strains were obtained from boils and examined without delay. Of the 43 strains examined, 39 were of the $\alpha(\beta)$ variety and 8 of these showed definite $\alpha\beta$ variants on the primary isolation plate. Only 4 were pure α strains (first variety).

(b) Haemolysis on Human Blood Agar Plates.

Strain "Mills" which produces α toxin only and strain S32a (received from Dr. L. Bryce) which produces β toxin only were spot-inoculated on 2.5 p.c. human blood agar and incubated at 37°C. After 4 days no haemolysis showed around either colony. On further incubation a small zone of partial lysis formed around strain "Mills" with an outer zone of slight browning, whilst a narrow zone of very weak haemolysis formed around strain S32a. On the seventh day a zone of reddish darkening of considerable extent formed around the narrow inner zone; this resembled the β zone which forms on sheep blood agar within 24 hours but was fainter and was not affected by subsequent refrigeration. It was evident that the haemolysis seen and reported on human blood agar had a different significance from that caused by α or β toxin on sheep blood agar. Strains whose haemolytic nature was known from the appearance of their colonies on sheep blood agar were incubated on human blood agar plates. The only strains which showed zones of complete haemolysis within 24 hours were the definite $\alpha\beta$ strains. Those showing zones of partial clearing within 24 hours were of the $\alpha(\beta)$ type, many of these zones becoming clear on longer incubation. One strain which produced much β toxin and little α toxin showed only a small zone of slight haemolysis on the third day with little change on further incubation until the fifth day when the wide zone of slight darkening, apparently indicative of β toxin, appeared.

(c) The Action of β Toxin on Human Serum.

Strains of staphylococci were spot-inoculated on human serum agar plates and incubated at 37°C. Zones of opacity appeared in the otherwise clear medium around some of the colonies. Only strains producing much β toxin caused these zones to form. (See Fig. 2.) Thirty-nine animal strains were similarly inoculated. The 35 αβ strains caused definite zones whilst the 4 α strains did not.

Strain "Mills" caused no zone but strain S32a, the pure β strain, gave a marked zone.

The assumption that the effect was caused by β toxin was confirmed by placing a drop of cell-free β toxin on the medium and incubating. Within 4 hours a zone of opacity marked the spot where the β toxin was placed. A drop of β toxin neutralized by β antitoxin caused no opacity; normal serum did not inhibit the phenomenon. A drop of α toxin had no effect on the medium.

A cell-free piece of agar cut from a turbid zone around a colony and placed on a second plate produced a second zone of opacity on the fresh medium. A piece of this turbid agar placed on a third plate caused a third zone of opacity.

The opacity could be produced in liquid serum in test-tubes by adding β toxin to it. On diluting the toxin, however, the power to cause the opacity in liquid serum was readily lost. The opacity did not appear on plates made from sheep, rabbit, guinea-pig or horse serum. In this sense as well as in the fact that various human samples differed in their sensitivity to the toxin, the reaction resembled the action of Cl. welchii toxin on serum (Nagler, 1939). To investigate the possibility as to whether the action of the two toxins was on the same constituent of the serum, three samples of human serum were tested against falling

dilutions of β toxin and of Cl. welchii toxin. Degree of sensitivity to β toxin did not parallel the degree of sensitivity to Cl. welchii toxin.

Serum agar plates made with 20 p.c. serum were found to be most suitable for the test. Seitz-filtration, inactivation and heating for one hour at 65°C. did not render the serum unsuitable for the test.

A piece of the opaque agar examined microscopically showed that the opacity was due to the presence of minute amorphous particles such as are formed when a weak albumin solution is heated. To find out which constituent of the serum was responsible for the reaction, the albumin and globulin were separated out by the usual fractionation methods using ammonium sulphate. The albumin and globulin were re-dissolved to give twice their former concentration. Albumin agar and globulin agar plates were prepared as well as albumin-globulin agar plates in which the albumin and globulin were present in the concentrations in which they were present in serum agar. The opacity appeared much denser on the albumin than on the globulin plates.

Agar plates to which egg albumin had been added did not show the opacity.

Small zones of opacity formed around many of the colonies other than those referred to above. These differed from the zones formed by β toxin in that they had a faint bluish tinge and not a faint brownish one; they were very narrow and did not tend to increase after 24 hours; they had a sharp edge, not a hazy one, and appeared to some extent on serum other than human serum.

A plate of serum agar containing a zone of opacity caused by a drop of cell-free β toxin was cooled at 4°C. for an hour and reincubated. A second zone appeared outside the first with a fine zone of clear medium between the two zones. This resembled the

action of β toxin on sheep blood agar; a circular line of apparently unaltered blood marks the division between the two zones of β haemolysis formed under similar circumstances.

A third appearance given only by strains of the third variety described above showed only after 3 or 4 days' incubation. Narrow concentric zones of marked opacity formed around the colonies of these strains on human serum agar and albumin agar.

DISCUSSION

The observations recorded above showing the frequency with which actual or potential β toxin formation can be demonstrated in strains of staphylococci isolated from lesions in human beings, are of some interest. They tend to confirm Rountree's (1936) work and extend it by showing that the bulk of strains isolated from boils form β as well as α toxin. Bryce and Rountree (1936) found that almost all of 65 random Wassermann samples of serum from human adults had a definite β anti-toxin content. It is possible that previous infection with β toxin-forming staphylococci was responsible for this anti-toxin.

The specific action of β toxin on human serum suggests the possibility of a reliable test for αβ toxin-producing strains in which the β toxin is insufficient to give the typical appearance round the colonies on sheep's blood agar plates.

Further, some speculations as to the possible role played by β toxin in human staphylococcal infections appear to be justified. Gengou (1933), as a result of very convincing experiments, concluded that the clotting of plasma and the subsequent digestion of the fibrin (fibrinolysis) were simply stages in the one process and caused by a single substance or enzyme secreted in the staphylococci. This is the substance known as coagulase. This process of clotting followed by digestion of the fibrin was found by Gengou to be influenced by the concentration of globulin and more particularly of the albumin in the

plasma. With high concentration of these proteins the process stopped short at the stage of coagulation. Lowering their concentration hastened the process, so that in some cases the clotting stage might be missed altogether.

It has been suggested that localization of staphylococcal infection is caused by the coagulase of the organism. Menkin and Walston (1934-35), however, have shown that by precipitation with glacial acetic acid, the coagulase may be separated out from a staphylococcal filtrate, free from the principle which causes lymphatic blockage.

As a big proportion of strains of staphylococci immediately after isolation from boils (39 out of 43) were found to produce at least some β toxin in addition to α, it is reasonable to suspect that the β toxin may play some role in the evolution of the lesion. It is suggested that the β toxin formed by the infecting cocci may assist in inhibiting the spread of the lesion by denaturation of the tissue proteins and/or serum proteins, especially the albumin. At the same time the staphylococci are secreting an enzyme, coagulase which, by clotting fibrinogen, may help in the early stage to wall off the lesion. As the concentration of the albumin and globulin in solution is lowered by the precipitating action of the β toxin, the fibrin is digested and liquefaction ensues. Further work would be necessary to substantiate or disprove this hypothesis.

The mechanism by which β toxin augments the lytic action of α toxin on human erythrocytes is not clear, and an explanation of the phenomenon will not be attempted at the present juncture.

SUMMARY

In an examination of 72 old strains of α toxin-producing staphylococci, it has been shown that 58 were either actual or potential producers of β toxin. Of 43 strains examined immediately after isolation

from boils, 39 were found to produce β toxin.

A plate method using sheep blood agar for detecting the difference between such strains and strains which produce only α toxin, is described.

β toxin has been shown to cause an opacity in human serum which is probably due to denaturation of the serum proteins.

β toxin has been shown to cause haemolysis of human red cells in human blood agar in the presence of sufficient α toxin within 24-48 hours, although neither toxin will do so alone.

One possible role of β toxin in the evolution of lesions such as boils is suggested.

ACKNOWLEDGMENT

We are indebted to Mr. F. J. Dempster for preparing the photographs.

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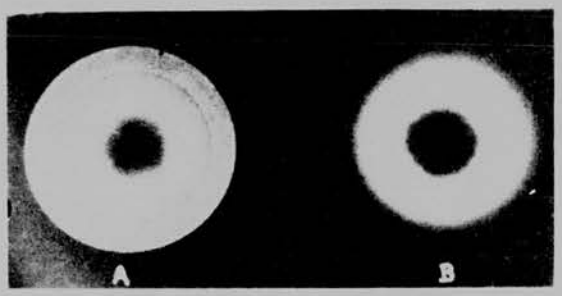

Fig. 1 (A). A colony of staphylococci on sheep blood agar after 24 hours at 37° C., showing an inner zone of clearing and an outer zone of partial lysis, both zones having entire borders (really $\alpha(\beta)$ haemolysis).

Fig. 1 (B). A colony of staphylococci on same plate as (A) showing the surrounding zone of clearing with a hazy boundary.

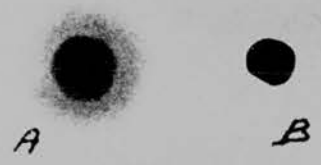

Fig. 2 (A). A colony of staphylococci (S62, an $\alpha\beta$ strain) on human serum agar surrounded by zone of opacity.

Fig. 2 (B). A colony of staphylococci (S210, a pure α strain) on same plate with no change in surrounding medium.

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A TEST OF STAPHYLOCOCCAL FIBRINOLYSIS

by R. CHRISTIE and HAROLD WILSON

(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 20th August, 1941).

The technique of Tillett and Garner (1933) for demonstrating streptococcal fibrinolysis appears to be unsuited to staphylococci on account of the length of time taken by some strains to liquefy the clot, the uncertainty of the results when the test is repeated and the occurrence of retraction or of liquefaction of the sterile uninoculated control clot during prolonged incubation. The reports of Madison (1935-6), Fisher (1936), Neter (1937) and Saski and Fejgin (1937) suggest that fibrinolysis goes with coagulase production and is characteristic of pathogenic staphylococci of human origin.

A test is here described in which lysis by staphylococci of heat-precipitated fibrinogen incorporated in nutrient agar may be observed. Among staphylococci of human origin such lysis was caused only by pathogenic strains.

MATERIALS AND METHODS

Nutrient agar was melted and cooled to about 50°C., 12 p.c. of sterile oxalated plasma was added, the mixture was heated at 56°C. for 10-15 minutes in order to precipitate the fibrinogen and plates were poured. This medium was turbid.

Spot-inoculation was done by touching the surface of the medium once with a platinum loop charged with staphylococci. By this means six strains were grown readily on a 9 cm. plate. Incubation was at 37°C.

One hundred and forty-one strains of human origin and 57 strains of animal origin were examined. Coagulase tests were carried out as previously described (Christie and Keogh, 1940). Fibrinolysis was tested for by the technique of Tillett and Garner. α and β toxins were recognised by cultivation on sheep blood-agar plates (Bryce and Rowntree, 1936) and, in many cases, by the action on sheep red cells of sterile filtrates of four days' "sloppy agar" cultures which had been incubated under increased CO_2 tension. Dermonecrosis was demonstrated by injecting similar culture filtrates into the skin of rabbits. Leucocidin was demonstrated for us by Dr. E. A. North using Valentine's (1936, 1939) technique.

TABLE 1.

Staphylococci of Human Origin

Relationship between clearing of fibrinogen-agar, coagulase and kind of toxin.

	Coagulase.		
Fibrinogen-agar	Positive	Negative	Total
Clearing	92 (α,90;neg.,2)	0	92
No clearing	7 (α,1;$\alpha\beta$,5;neg.,1)*	42 (all neg.)	49
	99	42	141

α, $\alpha\beta$, β or negative, in parenthesis, refer to the number of strains which produced the respective toxin or which showed negligible or no haemolysis.

*The α strain (S39, mentioned in the text) and the "negative" strain both cleared rabbit fibrinogen-agar.

RESULTS

After incubation overnight on fibrinogen-agar plates the colonies of some strains were surrounded by zones of clearing, which became wider after further incubation, and in which the precipitated fibrinogen was digested or dissolved, whereas the medium surrounding colonies of other strains retained its original turbidity (Fig. 1, A and B). A few strains which failed to cause clearing overnight did so after further incubation up to 48 hours.

Plates made with rabbit plasma were more sensitive than those containing human, sheep or horse plasma. The results here described were obtained with sheep plasma.

Many strains which caused clearing of fibrinogen-agar failed to liquefy the fibrin clot which resulted when calcium chloride was added to oxalated plasma or to liquefy the fibrin clot which they themselves had produced by means of coagulase, but all strains which liquefied fibrin clot caused clearing of fibrinogen-agar. The results of fibrinogen-agar and coagulase tests are shown in Tables 1 and 2.

TABLE 2.

Staphylococci of Animal Origin.

Relationship between clearing of fibrinogen-agar, coagulase and kind of toxin

	Coagulase		
Fibrinogen-agar	Positive	Negative	Total
Clearing	4 (all α)	1 (negative)	5
No clearing	39 (all $\alpha\beta$ or β)	13 (all neg.)	52
	43	14	57

cont'd..

Table 2 cont'd...

α , $\alpha\beta$, β or negative, in parenthesis, refer to the number of strains which produced the respective toxin or which showed negligible or no haemolysis.

The following considerations suggest that clearing of fibrinogen-agar is indicative of fibrinolysis:

(i) The same results were observed when a solution of fibrinogen in physiological saline was substituted for plasma.

(ii) As shown in Table 3 there is some agreement between the results of the two tests, using human plasma, as applied to β haemolytic streptococci of human origin. We are indebted to Mr. R. T. Simmons for applying the test of Tillett and Garner to the streptococci. In groups A and B there was complete agreement; in groups C and G, however, some strains which liquefied fibin clot failed to cause clearing of fibrinogen-agar. Cultivation on fibrinogen-agar is not recommended as a test of fibrinolysis by streptococci for which the technique of Tillett and Garner is more satisfactory. The zones of clearing surrounding colonies of streptococci were not as obvious as those surrounding colonies of staphylococci.

(iii) Other workers (mentioned above) have found indications that fibrinolysin is characteristic of pathogenic staphylococci of human origin. Our results with strains of human origin (Table 1) show a similar but not invariable connection between pathogenicity (as judged by coagulase production) and clearing of fibrinogen-agar. The few exceptions in this group and the many exceptions among strains of animal origin (Table 2), which will be discussed below, are associated with the presence of β toxin.

TABLE 3.

Beta Haemolytic Streptococci of Human Origin

Relationship between fibrinolysis (Tillett and Garner) and clearing of fibrinogen-agar

Group	Fibrinolysis and clearing	No fibrinolysis and no clearing	Fibinolysis but no clearing	Total
A	39	2	0	41
B	0	21	0	21
C	6	3	3	12
G	6	0	12	18
			TOTAL	92

RELATION TO α AND β TOXINS

Tables 1 and 2 show that of the 97 strains which caused clearing of fibrinogen-agar, 94 formed α toxin, 3 formed little or no toxin. Of the 101 strains which did not cause clearing of fibrinogen-agar, 44 formed β toxin, 56 were non-haemolytic, one formed α toxin. The width of the clearing around colonies on fibrinogen-agar was, however, not invariably proportionate to the width of the zones of α haemolysis around colonies on sheep blood-agar. Moreover, the one coagulase positive strain (S39) which formed α toxin but which did not cause clearing of fibrinogen-agar (Table 1) was a strong producer of α toxin[1]; α toxin, therefore, was apparently not identical with the factor causing clearing of fibrinogen-agar, although clearing of fibrinogen-agar was generally associated with the presence of α toxin.

Whether α toxin were present or not, no strain which formed

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1 But see footnote to Table 1.

β toxin in appreciable quantity caused clearing of fibrinogen-agar, suggesting that β toxin prevented clearing of fibrinogen-agar in the same way as it prevents lysis of sheep red cells by α toxin (Christie and Graydon, 1941). The following experiment, however, seemed to disprove this suggestion: A strain producing β toxin only was cultivated for 48 hours on the surface of fibrinogen-agar. No clearing occurred. A strain producing α toxin was then grown close to a colony of that strain. The clearing caused overnight by this latter strain (producing α toxin) was as great as that caused by colonies of this strain grown on another plate of fibrinogen-agar which was free from β toxin.

RELATION TO COAGULASE

Only one of the 97 strains which caused clearing of fibrinogen-agar was coagulase-negative. This strain (S159) was isolated from a cow's udder; it produced no α or β toxin, it was not agglutinated by any available serum (Christie and Keogh, 1940; Christie, 1940) and it was strongly caseinolytic. The zone of clearing around this singular strain differed from that around all other strains in that it had a diffuse, hazy outer margin in contrast to the sharp margin of other strains (Fig. 1) suggesting that the factor responsible for clearing by strain S159 was different from that responsible for clearing by the remaining 96 strains.

Forty-six of the 101 strains which failed to cause clearing of fibrinogen-agar were coagulase-positive but 44 of these produced β toxin; one of the remaining two strains produced neither α nor β toxin, one produced α toxin.

DISCUSSION

These observations appear to indicate that the factor "F", responsible for clearing of fibrinogen-agar is associated with coagulase and with α toxin and that it is inhibited by, or its effect is masked by, β toxin, a relationship which may be stated thus, αF - α(β)F - αβ - β

and which is illustrated by Fig. 2.

There are, however, among the 198 strains referred to in Tables 1 and 2, four exceptions to this hypothetical relationship between fibrinolysin, coagulase and α and β toxins: of the 97 strains which caused clearing of fibrinogen-agar all but three produced both coagulase and α toxin without demonstrable β toxin; of the 101 strains which failed to cause clearing of fibrinogen-agar one produced both coagulase and α toxin without demonstrable β toxin.

Clearing of fibrinogen-agar was apparently unrelated to leucocidin, dermonecrotoxin or to the enzyme which causes liquefaction of gelatine.

If strains of human origin only are considered, clearing of fibrinogen-agar is an indication of pathogenicity. Exceptions to this relationship are found with strains producing much β toxin, but such strains are rare in human lesions. The experience of Madison (1935-6), who found that many strains of human origin, but no strains of animal origin, were fibrinolytic, supports this contention, since most pathogenic animal strains produce much β toxin.

Cultivation on fibrinogen-agar of recently isolated staphylococci of human origin is suggested as a possible test of pathogenicity. It affords, in most instances, indirect evidence of the presence of α toxin, coagulase and fibrinolysin.

SUMMARY

Nutrient agar, rendered turbid by the presence of heat-precipitated fibrinogen, was used as a means of detecting fibrinolysis by staphylococci. Zones of clearing surrounding colonies on the surface of this medium are believed to indicate the presence of fibrinolysin.

Ninety-two out of 99 coagulase-positive strains of human

origin, but none of 42 coagulase-negative strains caused clearing of fibrinogen-agar. Of strains of animal origin, 4 out of 43 coagulase-positive strains and one out of 14 coagulase-negative strains caused clearing.

In both groups most of the coagulase-positive strains which failed to produce clearing were strong producers of β toxin; the discrepancy between the results with human and animal strains is related to the comparative rarity of β toxin in the former and to its frequency in the latter.

With human strains, clearing of fibrinogen-agar generally runs parallel with the presence of coagulase and with pathogenicity.

ACKNOWLEDGEMENT

We are indebted to Mr. F. J. Dempster for preparing the photographs.

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Fig. 1. Photograph of colonies, actual size, of staphylococci after 48 hours' cultivation on the surface of fibrinogen-agar: A, clearing; B, no clearing.

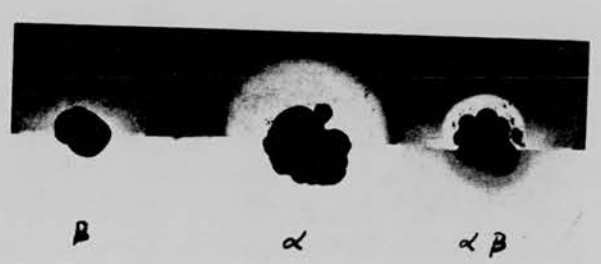

Fig. 2. Photograph of colonies, actual size, of coagulase-positive strains of staphylococci after 40 hours' cultivation on the junction between 5 p.c. sheep blood-agar in one half of a Petrie dish and fibrinogen-agar in the other half of the dish.

Colonies producing α, αβ or β toxin have caused the usual appearance in sheep blood-agar but only the colony which is free from demonstrable β toxin has caused clearing of the fibrinogen-agar.

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A NOTE ON A LYTIC PHENOMENON SHOWN BY GROUP B STREPTOCOCCI

by R. CHRISTIE, N. E. ATKINS and E. MUNCH-PETERSEN

(From the Commonwealth Serum Laboratories, University Bacteriology Department and C.S.I.R. Animal Health Research Laboratory, Melbourne.)

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In a recent outbreak of Scarlet Fever in a country district it was suspected that the milk was the vehicle of infection. Five samples of the milk supplied to the area were submitted to examination for haemolytic streptococci. After centrifugation, the mixed cream and sediment were stroked out on the surface of sheep blood agar plates which were incubated over-night at 37°C. From three of the samples streptococci were obtained which were apparently haemolytic on the primary plates but non-haemolytic on sub-culture.

Examination of the primary plates revealed the presence of many staphylococci of the $\alpha\beta$ type, that is, their colonies were in the centre of a clear zone, which in turn was surrounded by a large darkened zone, where the staphylococcal β toxin had altered, but not lysed, the sheep red cells. Wherever there were colonies of the streptococcus within these zones of darkening, the colonies were surrounded by an area of complete haemolysis, whilst elsewhere on the plates they produced no distinct haemolysis.

To confirm this observation, a colony of these staphylococci was grown by spot-inoculation on the centre of a sheep blood agar plate. After 24 hours' incubation the streptococci were inoculated on to the darkened zone. Within two hours haemolysis was visible around the streptococcal growth.

In the case of a staphylococcus producing α toxin only and giving therefore an area of partial haemolysis around a zone of clear haemolysis no variation of the haemolysis was caused by growing the streptococcus in or near the zone of partial haemolysis, but in the dark zone around staphylococci producing β toxin only, haemolysis was readily produced.

When the streptococcus was grown near the edge of one of these uniform dark zones, clearing of the medium could be seen at the point nearest to the streptococcus colony, suggesting that an invisible agent, or the products of such an agent, had reached the red cells already altered by the staphylococcal β toxin (Fig. 1).

Under anaerobic conditions the effect was more marked, and definite pitting of the medium could be seen in position coinciding with the clearing, indicating that some constituent or constituents of the medium had been altered to give diffusible substances. The pitting was too deep to be due entirely to migration of the haemoglobin liberated by red cell lysis. Furthermore, the clear area was glassy-clear and not faintly turbid, as is usually found when there is cell lysis only.

To demonstrate the lysis, sheep and ox blood were found suitable. No lysis was obtained when horse, human, rabbit or guinea-pig blood was used.

A number of strains of streptococci were then tested. Of 55 from various human sources (throat, sputum, urine, skin, tooth and nose), some haemolytic, others non-haemolytic, and 5 non-haemolytic

anaerobic strains of human origin (received through the courtesy of Miss H. Butler), none gave the reaction when grown with the staphylococcus, although some showed a narrow area of incomplete lysis. Forty-three strains from cases of suspected and nineteen from cases of definite bovine mastitis were tested. Of these, all but three gave the reaction. Serological examination then revealed that of all the strains tested only those which belonged to Group B Lancefield gave the reaction, and none belonging to Group B failed to give it. Five Group B strains of human origin (tonsils, vagina and skin lesion) also gave the positive reaction. Three representative strains of each of the Groups A, C and G. were tested. None was positive. Where normal haemolysis occurred, as with the Group A strains, the haemolytic area was no more extensive where it overlapped the staphylococcal zone than elsewhere in the medium. To determine whether lysis, similar to that caused by non-haemolytic Group B strains, was occurring but being masked by the ordinary streptococcal haemolysis, the boiled broth technique, described later, was used. The results were still negative. No Group B strain produced sufficient lysis by itself to interfere with the reading of the haemolysis induced within the staphylococcal zone.

The presence of the active agent in the solid medium could be detected fully half a centimetre from the streptococcal colonies. There was no detectable difference in the degree to which the separate strains produced the agent, although 13 serological sub-types were represented in the strains tested.

It is known that certain non-haemolytic staphylococci, when grown on blood agar near a staphylococcus with a zone of β toxin, produce lysis within this zone just as do the Group B streptococci (Christie and Graydon, 1941). Some evidence has been given to show that the active agent from the staphylococcus is staphylococcal lipase. Group B and other streptococci were therefore tested for lipolytic activity by growing on cream blood agar (Orcutt and Howe, 1922) and on a butter-fat neutral red agar, but none could be detected.

Many non-haemolytic air-borne organisms are known to cause lysis around β haemolytic staphylococci but whereas these contaminants also cause similar lysis in the medium close to colonies of Cl. welchii, the Group B streptococci do not do so. Furthermore, the contaminants near the staphylococcal colonies produce a haemolytic zone with a sharply defined edge, whereas the streptococci produce a zone with an indefinite edge. Thus, there must be at least three bacterial agents capable of causing lysis of red cells which have been treated with staphylococcal β toxin, namely those from Group B streptococci, and from non-haemolytic staphylococci (which are not identical), and that from some air-borne contaminants (which again acts in a different manner).

Group B streptococci are known to be non-fibrinolytic. Eight strains producing the above lysis (3 of animal and 5 of human origin) were tested against human fibrin and found to be non-fibrinolytic. A strongly fibrinolytic streptococcus, not of Group B, was unable to induce the above-mentioned lysis. Fibrinolysin was therefore ruled out as the responsible lytic agent.

Since Group B streptococci commonly occur in milk, take part in a reaction of an apparently digestive nature, and are not lipolytic, their ability to digest casein was examined in an unsuccessful effort to find a test for the lytic agent other than the above test with sheep cells. They were grown on agar rendered slightly turbid with fat-free milk (4 p.c.), and they showed only faint traces of clearing, much less than was shown by many streptococci of other serological groups; e.g. Str. faecalis; which does not produce the haemolytic reaction, gave large clear zones, its digestive power for the casein being visible after two hours.

A strain of Group B streptococci was grown in meat infusion broth for 18 hours and the broth filtered through a Seitz EK pad. Tests proved the filtrate to be sterile. The initial pH of the broth was 7.6 and the final pH 7.0. A drop of the filtrate on the dark

zone around a staphylococcus colony caused lysis, just visible after 10 minutes and quite definite after one hour. The clearing was most complete at the edge of the zone. Readjustment of the pH of the filtrate to 7.6 had no effect on the amount of clearing it could produce.

Heating of the filtrate at 56°C. for 30 minutes, or at 60°C. for 10 minutes, altered it so that it produced only a trace of lysis and further heating at 56°C. and 60°C. for 30 minutes had no more effect. On the other hand, a sample heated at 100°C. for 5 minutes gave almost as strong a reaction as the unheated filtrate. Heated and unheated fresh broth gave no reaction. The unexpected positive result with filtrate heated at 100°C. suggested that the samples, partly inactivated by heat at 56°C. or 60°C. would be re-activated by heating to 100°C. On testing, this was found to be the case. The test was repeated with two other Group B strains, with similar results.

Landsteiner and von Rauchenbichler (1909), using crude staphylococcal α toxin, found that this was inactivated when heated to 65°C. for 30 minutes, with reactivation to one-quarter of the original titre after five minutes' further heating at 100°C. The inactivation at 65° C. was due, not to destruction of the toxin, but to the combination of the toxin with some constituent of the broth, a combination that was disrupted by heating at 100°C. This phenomenon seems to be related to that found with the streptococcal filtrate mentioned above.

It was found possible to reproduce these findings in test-tube titrations, using Seitz-filtered staphylococcal β toxin and the streptococcal broth filtrate. Diluted samples of the filtrate were added to constant volumes of the β toxin, and then washed sheep red cells were added. After incubation for one hour at 37°C., the cells were found to be lysed in the tube containing the undiluted filtrate, but the power of the filtrate to induce lysis was quickly lost on dilution. An undiluted sample which had been heated at 56°C. for

30 minutes caused only a trace of lysis, but a sample heated at 56°C. for 30 minutes and then maintained at 100°C. for five minutes, had a lytic potency similar to that of the unheated filtrate. A sample heated for five minutes at 100°C. was as potent as the raw filtrates (see Table 1). When an undiluted sample of the filtrate was allowed to act on the red cells for 30 minutes at 37°C., lysis occurred immediately on addition of the β toxin.

TABLE 1.

Results of haemolysis titrations, using streptococcal filtrate, staphylococcal β toxin and sheep red cells.

SERIES I. Ten drops of each diluted preparation of streptococcal filtrate were added to the corresponding tube. Ten drops of β toxin (raw, filtered toxin diluted 1:10 with normal saline) were added to each tube. Two drops of a 10 p.c. suspension in saline of washed sheep red cells were then added and the tubes incubated at 37°C. for 1 hour.

SERIES II. As above, except that the filtrate was first heated to 56°C. for 30 minutes.

SERIES III. As in series I, except that the filtrate was first heated to 56°C. for 30 minutes, and then to 100°C. for five minutes.

SERIES IV. As in series I, except that the filtrate was first heated to 100°C. for five minutes.

SERIES V. As in series I, but with saline substituted for the β toxin.

SERIES VI. As in series I, but with saline substituted for the streptococcal filtrate.

cont'd...

Table 1 cont'd...

	Initial Dilution of Filtrate				
	10/10	8/10	6/10	4/10	2/10
Series I	++++	++++	++++	++++	+
Series II	+	+	+	-	-
Series III	++++	++++	++++	+	-
Series IV	++++	++++	++++	++++	+
Series V	-	-	-	-	-
Series VI	-	-	-	-	-

++++ Indicates complete lysis.
+ Indicates 25 p.c. lysis.
- Indicates no lysis.

The quantity of the agent present in the filtrate was not found to be increased by incubation for longer than 18 hours.

The staphylococci most commonly found in animals are predominantly β toxin-producers (Minett, 1936; Cowan, 1938). It is only with these staphylococci that Group B streptococci produce the lysis described above. Not infrequently staphylococci and Group B streptococci are found together in the bovine udder; there may be some significant connection between this occurrence and the fact that together they have a lytic power which neither has independently.

The results obtained with the Group B streptococci of human origin give further evidence of the close relationship of the animal and human strains of this group, in spite of the differences in serological type and in some other characteristics established by Simmons and Keogh (1940).

The negative results obtained when horse blood was substituted for sheep blood suggest that in routine tests for haemolytic streptococci, horse blood only should be used, particularly where β toxin-producing staphylococci are to be expected, as in milk samples.

The number of members of the various groups tested was not large, but the results obtained so far indicate that the phenomenon may provide a relatively simple confirmatory or substitute test for Group B streptococci.

SUMMARY

Strains of Group B streptococci, of animal and human origin, produce an agent which will lyse sheep and ox, but not human, horse, rabbit or guinea-pig red cells, when these cells have been altered by staphylococcal β toxin.

The agent is extracellular, filtrable, and thermostable.

Streptococci of human and animal origin, belonging to Groups other than Group B, have not been found to produce this agent.

ACKNOWLEDGEMENTS

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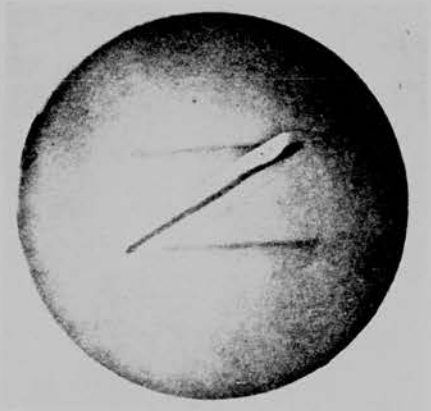

Fig. 1. The central streak is due to growth of staphylococci on sheep blood agar. The zone around it is due to β toxin. The lower streak shows a growth of Group B streptococci with haemolysis where it adjoins the staphylococcal zone. The upper streak shows growth of non-Group B streptococci.

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STAPHYLOCOCCAL FIBRINOLYSIN

by R. CHRISTIE, J. J. GRAYDON and E. F. WOODS

(From the Commonwealth Serum Laboratories, Melbourne).

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The ability of staphylococci to lyse fibrin clot is shown by some of the pathogenic strains. The tube test to detect fibrinolysin production by streptococci (Tillett and Garner, 1933) cannot be used satisfactorily with staphylococci because of the length of time taken for lysis of the fibrin, the difficulty of reproducing results and the tendency of sterile clots to lyse. Christie and Wilson (1941) showed that agar plates made turbid with heat-precipitated fibrinogen could be used to detect lysis of fibrinogen by staphylococci and gave reasons for assuming that the agent which lysed heat-precipitated fibrinogen was fibrinolysin.

Aoi (1932) and Fisher (1936) obtained fibrinolysin in culture filtrates; Neter (1937) found that commercial staphylococcal antitoxin would inhibit fibrinolysis. Tests carried out here have confirmed and extended these observations as well as thrown some light on the nature of the Muller phenomenon (see below).

EXPERIMENTAL

Production of Fibrinolysin.

Strains of fibrinolytic staphylococci were grown in a

modified Dolman's medium (Morgan and Graydon, 1936) for two days at 37°C. in an atmosphere of 80 p.c. oxygen and 20 p.c. carbon dioxide. The broth was then clarified by centrifugalization and 0.02 p.c. merthiolate added to prevent further growth of organisms. This procedure is similar to that used in the production of staphylococcal toxin.

Titration of Fibrinolysin.

Oxalated human plasma was heated for 5 minutes at 53-56°C. The precipitated fibrinogen was removed by centrifuging and re-suspended in a volume of merthiolated saline equal to that of the discarded supernatant fluid. Serial doubling dilutions of the fibrinolytic solution were made with merthiolated saline. Sixteen drops of each dilution were transferred to small test tubes, 2¼" x ⅜", four drops of the fibrinogen suspension were added and the tubes were placed in a water bath at 37°C. Lysis of the fibrinogen was apparent in the more concentrated solutions after four hours and final readings were made after 18 to 24 hours. In a typical titration complete lysis occurred at 80- to 160-fold dilution, with partial lysis at a 320- to 640-fold dilution. Sheep and guinea-pig fibrinogen gave similar end-points but the lysis in the more concentrated solutions occurred earlier than with human fibrinogen.

It seemed possible that fibrinogen in solution would be more susceptible to attack than in the precipitated form. A fibrinogen solution was therefore prepared from plasma by salting out the fibrinogen with 50 p.c. saturated sodium chloride solution and re-dissolving it, after washing, in distilled water. This solution was treated for 18 hours with serial dilutions of a culture filtrate prepared as above and the tubes then placed in a water bath at 53°C. to precipitate unaltered fibrinogen. Precipitates were obtained only in the tubes with higher dilutions of the filtrate, but the titre of the filtrate was similar to that obtained in a direct titration with heat-precipitated

fibrinogen.

This result, coupled with the fact that heat-precipitated fibrinogen which has been lysed by a filtrate is no longer precipitable by heat, suggests that when heat-precipitated fibrinogen is lysed the change is not merely a form of disaggregation as has been suggested to occur when fibrin is lysed by staphylococcal filtrates (Birch-Hirschfeld, 1940).

Effect of Filtration and Heating.

A broth culture was filtered through a Seitz E.K. filter, shown to be sterile and titrated against fibrinogen. The same end-point was reached as with unfiltered broth culture. Samples of the filtrate were heated for 5, 10, 15 and 30 minutes at 60°C., and for 5 minutes at 100°C. Slight precipitates caused by the heating were removed by centrifuging. No loss in fibrinolysin was detected on titrating the samples thus obtained. Fibrinolysin is evidently much more heat-stable than α toxin. Its ability to pass through an asbestos filter-pad differentiates it from coagulase.

Connection with the Staphylococcal Haemotoxins.

Most pathogenic animal strains are non-fibrinolytic (Madison, 1935-6; Christie and Wilson, 1941). These strains usually produce much β toxin and since the few strains tested here which were fibrinolytic (4 out of 43) were those which produced little or no β toxin, it seemed possible that β toxin was inhibiting the action of the fibrinolysin produced by the other strains. It has been shown that β toxin inhibits the haemolysis of sheep red cells by α toxin (Christie and Graydon, 1941).

However, when a drop of strong β toxin was added to each tube in a fibrinolysin titration row, no change in titre was obtained although the rate of lysis was reduced.

A suspension of heat-precipitated fibrinogen was then treated overnight with strong β toxin and used in the titration of a culture filtrate. The rate of lysis was reduced but the final end-point was the same as that obtained in a parallel titration using fibrinogen not previously treated with β toxin.

A strain producing much β toxin but showing no fibrinolysin on fibrinogen agar plates was grown in broth for two days. The filtrate showed no lytic activity towards fibrinogen either before or after neutralizing the β toxin with β antitoxin.

In the early part of this work it was suspected that toxin and fibrinolysin were identical but this was discounted by their different susceptibilities to heat, the results of the neutralization tests (see below) and finally by the fact that it was possible to produce a filtrate from one strain (S39) with a high α toxin content but no detectable fibrinolysin.

Neutralization Tests

Constant volumes of a culture filtrate were treated for 30 minutes with various dilutions of staphylococcal α antitoxin to determine whether fibrinolysin was neutralized by α antitoxin serum. Serum dilutions in 10 p.c. stages were used. An antitoxin, P31, containing 90 international units per ml. and a concentrate of this, R33, containing 240 units per ml. were used. The antitoxic content was determined by reference to standard serum containing 20 units per ml. R33 showed approximately $2\frac{1}{2}$ times as much neutralizing power for fibrinolysin as that possessed by P31, the anti-fibrinolytic power being parallel to the α antitoxin content. The anti-fibrinolytic factor and the antitoxic factor were either identical or had been precipitated during concentration in the same pseudo-globulin fraction.

A sample of standard serum showed less than a tenth of the neutralizing power for fibrinolysin possessed by P31 serum although

the antitoxic titres of the two were in the ratio of 1:4½. The standard serum is a purified product. Since this might have contained glycerine or borate buffer, the titration was repeated with preparations of the other sera to which glycerine and borate buffer had been added but no change in anti-fibrinolytic titre was obtained. Gengou (1933) showed that albumin and globulin influenced fibrinolysis. Normal horse serum which showed no anti-fibrinolytic activity was therefore added to standard serum but still with no effect on its neutralizing power for fibrinolysin.

It was therefore evident that α antitoxin was not the agent responsible for neutralizing the fibrinolysin but that fibrinolysin was antigenic and that immunization of horses with staphylococcal filtrates could produce an anti-fibrinolysin.

Anti-fibrinolysin in Human Serum.

Four samples of human serum (obtained through the courtesy of Miss H. Butler) were titrated for their anti-fibrinolytic ability as follows: Tubes containing one drop of a filtrate capable of causing lysis in a 40-fold dilution and 16 drops of dilutions of human serum were left at 37°C. for 30 minutes; 3 drops of fibrinogen suspension were then added to each tube and the whole left overnight at 37°C. The highest dilution of serum capable of inhibiting lysis was then noted. Two samples which were from people convalescent from severe infections with fibrinolytic staphylococci gave titres of 1/10 and 1/20. Two others from normal people gave titres of 1/5 and 1/10. There was thus no reason to suspect that recent infection gave a significantly high anti-fibrinolytic content in serum.

The Muller Phenomenon.

Muller (1927) reported that when certain staphylococci were grown on human blood agar plates in a continuous film for several days, discrete zones of clearing could be seen from the under side of

the plates. Burnet (1928) showed that the same phenomenon could be seen around colonies of staphylococci on human blood agar where it manifested itself as small discrete zones of apparent lysis beyond the main circular zone of haemolysis. Packalen (1941) showed that the reaction was one of haemodigestion and not of haemolysis since it showed better on lysed blood agar than on blood agar. All agreed that a thermolabile substance in serum was essential for the production of the discrete zones. Another manifestation of the same reaction shown by Packalen was the appearance of zones of digestion of serum, shown as small discrete pittings in the medium around colonies of staphylococci on inspissated serum plates to which unheated serum had been added.

We observed another form of the phenomenon with fibrinolytic staphylococci grown on fibrinogen agar plates. The periphery of the zones of clearing around the colonies were frequently irregular (see photographs), and small discrete zones of lysis were occasionally found outside the main zones. A similar observation has been made by Fisk and Mordvin (1943). With some samples of medium, zones of lysis with outlines in the shape of irregular polygons, some even with re-entrant angles, were obtained. A strain which produced very little fibrinolysin occasionally gave only small discrete zones, one, two or three, adjoining the colony, without the complete zone shown by more strongly fibrinolytic strains.

An examination of 79 strains of staphylococci (21 non-pathogenic and non-fibrinolytic, 37 pathogenic and fibrinolytic, 21 pathogenic and non-fibrinolytic) showed that all of the 37 fibrinolytic strains could produce the Muller effect on human lysed blood agar while none of the nonfibrinolytic strains were able to do so.

These figures, together with the appearances of the discrete zones on fibrinogen agar plates, led us to the assumption that fibrin-

olysin was the agent responsible for the Muller phenomenon and that the irregularities on the plates were another manifestation of the phenomenon.

Support for this assumption was got from the fact that a thermolabile substance in serum is necessary for the production of the irregularities on the plates, just as it is for the discrete zones on human blood agar plates. If fibrinogen is heat-precipitated, centrifuged off, re-suspended in saline and used to prepare plates, the zones of lysis around staphylococcal colonies have perfectly circular peripheries. The irregularities on fibrinogen agar are not obtained if the fibrinogen is precipitated by prolonged heating of the plasma (30 minutes) at 56°C.; a medium thus prepared gives extensive zones with circular peripheries. Heating at 53°C. for three minutes gives a medium which shows the irregularities and less extensive zones. Two plates of fibrinogen agar were prepared in which the medium had been heated for 30 minutes at 56°C. Before pouring, 2 ml. of unheated serum were added to one of them. Fibrinolytic staphylococci produced zones of lysis around their colonies with marked irregularities only on the medium containing the unheated serum.

The appearances suggested that the part played by the thermolabile substance was an inhibitory one, its antagonistic action being such as to prevent lysis by the staphylococcal agent. The well-defined edges of the zones of lysis, which were less marked when the inhibitory substance in the serum had been destroyed by heat, indicate that there was a fairly critical concentration below which the lysin could not act in the presence of unheated serum. Where lysis occurs in discrete areas beyond the general zone of lysis, local conditions must be favourable and the most probable assumption is that adsorbent particles act there either by concentrating the lytic agent or by removing the inhibitory one. Burnet found that in the centre of the Muller zones on blood agar a particle could sometimes be seen. We

occasionally found such particles in lysed zones and significance was imparted by the fact that, when present, they were always centrally placed. The absence of visible particles in many zones where lysis had occurred did not necessarily prove that they were not present originally.

The solution of Muller's problem has not yet been achieved but it seems probable that the explanation of the discreteness will be found along physical rather than chemical lines.

SUMMARY

A method is described for detecting and titrating staphylococcal fibrinolysin in broth cultures.

Fibrinolysin will pass through a bacterial filter. It is thermostable and anti-genic.

Antifibrinolysin can be detected in human serum and in the sera of immunized horses.

Fibrinolysin is the agent responsible for the Muller phenomenon. A new manifestation of this phenomenon is described.

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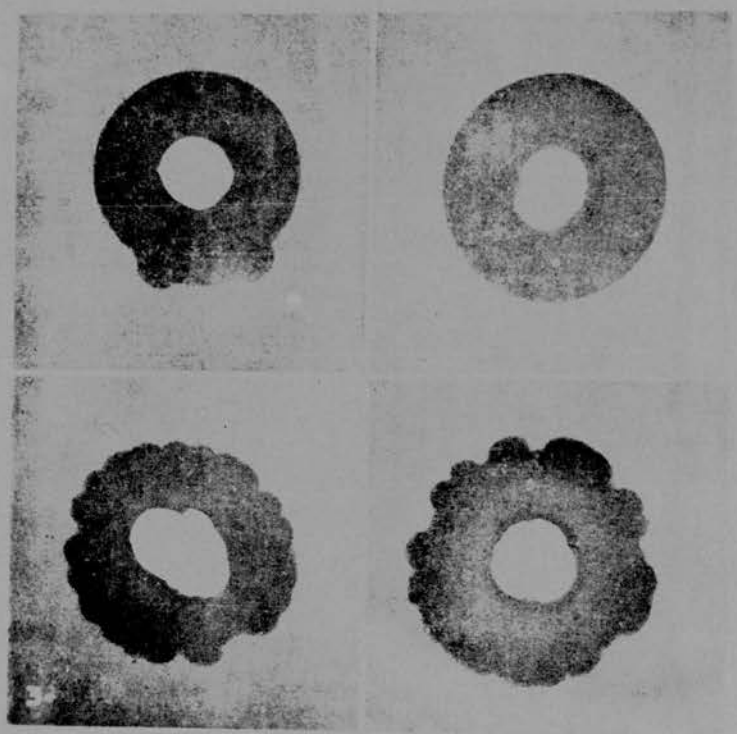

Figs. 1–4. Photographs of four colonies of staphylococci on fibrinogen agar, one showing a regular outline to the zone of lysis and the other three showing irregular outlines.

Photographs by Mr. F. J. Dempster.

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FURTHER NOTES ON A LYTIC PHENOMENON SHOWN BY GROUP B STREPTOCOCCI

by E. MUNCH-PETERSEN, R. CHRISTIE and R. T. SIMMONS with the technical assistance of H. A. BEDDOME

(From the C.S.I.R., Division of Animal Health and Production, Animal Health Research Laboratory, and the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 28th May, 1945).

In a previous paper (Christie, Atkins and Munch-Petersen, 1944) it was shown that group B streptococci, haemolytic and non-haemolytic, of human or animal origin produced an extracellular, filtrable, thermostable agent which lysed sheep or ox red cells in the presence of staphylococcus β toxin.

The agent was most easily detected when colonies of group B streptococci on sheep or ox blood-agar plates were within the neighbourhood of colonies of β toxin-producing staphylococci. Each of the latter was surrounded by a zone of discolouration caused by the β toxin (Bryce and Rountree, 1936). Where colonies of a streptococcus and a staphylococcus were sufficiently close, an area of lysis appeared between the two colonies and within the discoloured zone.

Sixty-four strains of group B streptococci and 72 of other groups were examined.

The purpose of this paper is to report observations made in connection with the medium used in the tests and also the results obtained with a much larger number of streptococci than was used in the original work.

MATERIAL AND METHODS

Preparation of Staphylococcus β Toxin.

The strain Staphylococcus, S32a, used originally by Bryce and Rountree, was grown in nutrient broth containing 0.1 p.c. agar for 3 days at 37°C. in an atmosphere of 80 p.c. carbon dioxide and 20 p.c. oxygen. The broth was then clarified by centrifugation and further growth was inhibited by the addition of merthiolate in final concentration of 0.02 p.c.

To estimate the strength of the toxin, serial doubling dilutions were prepared; 8 drops of each dilution were placed in small test-tubes and a drop of a 10 p.c. suspension in saline of washed sheep red cells added to each. After one hour's incubation at 37°C. followed by refrigeration overnight at 4°C., the highest dilution with complete lysis was noted. This dilution was found to be 1:1,600.

Titration of Staphylococcus β Anti-toxin of Serum.

Serial doubling dilutions of the serum to be tested were prepared; 8 drops of each dilution were placed in small test-tubes and eight drops of a 1:200 dilution of the β toxin added. The tubes were kept at 37°C. for one hour, after which one drop of a 10 p.c. suspension of washed sheep red cells was added to each. After a further hour at 37°C., the tubes were left at 4°C. overnight. The titre of the serum was taken as the highest dilution at which no lysis occurred.

Test for the Lytic Phenomenon.

Plates of nutrient agar containing 5 p.c. by volume of

defibrinated sheep blood were prepared. A streak was made across the centre of each plate with a loopful of a culture of the staphylococcus (S32a). At an angle to this were streaked the strains of streptococci to be tested, care being taken to avoid contact with the staphylococci.

Eight strains were tested on each plate. Readings were made after overnight incubation at 37°C.

Strains of Streptococci Tested.

Group A. 213 strains representing the 26 Griffith's types, 13 local types (Keogh, Simmons and Wilson, 1941), and 27 untyped strains.

Group B. 200 strains, viz., 80 of human origin (Simmons and Keogh, 1940) and 120 of bovine origin.

Group C. 93 strains representing those described by Simmons and Keogh, (1940) and also Griffith's types 7, 20 and 21.

Group D. 2 strains, obtained from Dr. R. Lancefield.

Group E. 2 strains, obtained from Dr. R. Lancefield.

Group G. 64 strains (Simmons and Keogh, 1940).

Group H. 1 strain, obtained from Dr. R. Lancefield.

Ungrouped 20 strains, which might be classed as Str. dysgalactiae and Str. uberis on biochemical grounds, but not as group B.

RESULTS

Preliminary efforts to prepare sheep blood-agar which would show zones of discolouration around colonies of staphylococci known to produce β toxin were not always successful. Alterations in the constituents of the basic medium did not correct the irregularity, which was finally found to lie in the nature of the sheep blood used.

Some samples of sheep blood gave a medium which showed large zones of discolouration, others showed none at all and intermediate results between these two extremes were found. All blood samples gave satisfactory results if red cells only were used. The addition of serum from a "suitable" sheep to the red cells from an "unsuitable" sheep did not render the red cells unsuitable.

The obvious assumption that "unsuitable" blood samples contained much β anti-toxin was confirmed when the sera of a number of sheep were tested for the presence of this anti-toxin. Defibrinated blood samples were collected from 41 sheep. A blood agar plate was prepared from each sample; a β toxin-producing staphylococcus was sown on the surface and the size of the characteristic darkened zone, if present, was noted. Serum from each sample was then tested by titration for β anti-toxin as described above. The blood samples giving a suitable medium contained little or no anti-toxin and the unsatisfactory blood samples were those with a high anti-toxin content. The highest titre obtained was 1:64; 9 sera when tested undiluted showed no anti-toxin. Only 11 of the 41 samples were considered satisfactory for preparation of media for this test. Four samples of blood taken at weekly intervals from a sheep with a high anti-toxin content had the same titre of β anti-toxin. All the 41 sheep tested appeared healthy and had no known history of staphylococcal infection.

Samples of blood from 55 female cattle were similarly examined. Blood from only 19 of them was suitable for preparation of blood agar plates to detect β toxin production, and these corresponded with a low β anti-toxin content in the sera. Four of the sera, used undiluted, showed no anti-toxin detectable by the above technique. The highest titre obtained in the 55 sera was 1:8,200. This figure was given by serum from a cow in the 2nd lactation period having a staphylococcal infection in each of the four quarters of the udder.

The series of tests with the strains of streptococci was

therefore carried out on blood-agar to which had been added blood from sheep known to be suitable for the test. All of the 200 strains belonging to group B gave a definitely positive reaction in the test for the lytic agent referred to above. The 80 strains of human origin were also tested on agar plates with suitable ox blood and gave a typical positive reaction. None of the 395 strains not of group B gave the reaction in a manner comparable with that given by group B strains. They were either frankly negative or showed a narrow zone of lysis which in every case was partial only; in no instance was there difficulty in distinguishing it from the group B reaction.

There was no difference in degree of ability to produce the lytic agent shown by group B streptococci. All group B streptococci did so and non-group B streptococci either did not produce the agent or did so to a negligible degree. This complete parallelism, coupled with the fact that a strain of streptococcus either produced the agent strongly or not at all, suggested to us that the agent must be something as intimately connected with group B streptococci as the group B specific antigen but we were unable to obtain confirmation of this.

Of the streptococcal groups A, B, C and G. only members of group B produce intense browning on horse blood agar containing 1 p.c. maltose (Simmons and Wilson, 1941). The characteristics of this reaction, however, do not suggest that it is caused by the same agent as that which produces the above-mentioned lysis.

SUMMARY

A total of 200 strains of streptococci belonging to group B all produced an agent which lysed sheep red cells in the presence of staphylococcus β toxin.

A total of 395 strains of streptococci belonging to other groups did not produce this agent. Group A was represented by 213

strains, group C by 93, group D by 2, group E by 2, group G by 64 strains and group H by one strain. The remaining 20 strains were of animal origin but not of group B.

Sheep or ox blood for the preparation of blood agar to demonstrate production of β toxin by staphylococci must not contain too much β anti-toxin. This also applies to the sheep or ox blood to be used in the lytic test for group B streptococci.

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OBSERVATIONS ON THE SENSITIVITY OF STAPHYLOCOCCI TO PENICILLIN

by E. A. NORTH and R. CHRISTIE

From the Commonwealth Serum Laboratories, Melbourne.

The continued pathogenicity of staphylococci which have become resistant to penicillin is of interest epidemiologically and also from the point of view of treatment. As a result of the work of Abraham et alii (1941) it has been generally assumed that organisms with this acquired penicillin resistance retain their pathogenicity and may therefore be dangerous in situations where cross-infection can occur.

Spink, Ferris and Vivino (1944) compared the virulence of staphylococci with that of resistant variants developed from them by cultivation in penicillin broth. To test the organisms for virulence, they examined their resistance to the bactericidal action of defibrinated normal human blood. Their results indicated that penicillin-resistant strains were less virulent than the parent sensitive strains. They agreed, however, with the findings of Abraham et alii that a strain which had acquired resistance showed no fundamental change in biological or metabolic activities as compared with the parent, except that the rates at which these activities were carried out were slower for the resistant strain.

The present work was undertaken with a view to ascertaining whether the possession or absence of any of the commoner properties of

staphylococci would show correlation with the degree of their sensitivity to penicillin.

STRAINS EXAMINED

One hundred and twenty-eight strains of staphylococci of known biological and metabolic characteristics, collected from various sources before the advent of penicillin treatment, were tested for penicillin resistance. They included strains from various lesions - osteomyelitis, carbuncles, furuncles et cetera - as well as others from healthy organs and tissues. Seventy of the strains had been tested for bacteriophage sensitivity with the four staphylococcal bacteriophages Au1, Au2, Au3 and Au4 used by Burnet and Lush (1935). Definite differences in sensitivity to bacteriophage action were found.

Thirty-one strains from hospital wards in which penicillin has been used extensively, particularly in local application to wounds, were also tested for resistance to penicillin. In addition, their biological and metabolic characteristics were compared with those of the 128 strains mentioned above.

A standard reference penicillin-sensitive strain (F.D.A. 209) and a known resistant strain (Crotty) were included as controls. Both of these strains came originally from America.

TECHNIQUE

Tests for haemolysins (α, $\alpha\beta$ and β)were made on 5% sheep blood-agar medium (Christie and North, 1941).

Tests for coagulase were made with human plasma diluted ten times with normal saline solution (Cruickshank, 1937), readings being made after four and after eighteen hours.

Fibrinolysin was detected on fibrinogen agar (Christie and Wilson, 1941).

Lipase production was detected on butter fat neutral red agar medium (Christie and Graydon, 1940).

Mouse pathogenicity was estimated as follows:

> The organisms from an overnight agar slope culture were scraped off and added to normal saline solution. The suspension was standardized to 4,000,000,000 organisms per millilitre by means of Burroughs Wellcome standard opacity tubes. One millilitre of this suspension was injected intra-peritoneally into each of five mice weighing 18 to 22 grammes.

Previous work with this inoculum and further trial tests with 18 pathogenic strains and 12 non-pathogenic strains had shown that if the strains were coagulase-producing and from a pathological source, the five mice would die within twenty-four hours, whereas if the strain did not produce coagulase and were from a non-pathological source, no mice would die.

Mannite fermentation was detected by the inoculation of mannite broth tubes, daily examination being made during incubation at 37°C.

Serological typing was carried out by means of Cowan's (1939) slide agglutination method.

Pigment formation was detected by cultivation of the organisms on milk-agar medium (Fujita and Yoshikka, 1938).

Penicillin sensitivity was estimated by the inoculation into ten-millilitre samples of nutrient broth containing different quantities of penicillin of one drop (from a fine-tipped Pasteur pipette delivering approximately 0.033 millilitre) of an overnight

broth culture. The inoculated tubes were examined after twenty-four hours' incubation at 37°C. and the tube containing the minimum quantity of penicillin with no visible growth was noted. The stock penicillin solutions used were obtained direct from the penicillin assay department, and all dilutions were made with phosphate buffer solution. To ensure uniformity of penicillin concentration, bulk quantities of each penicillin broth were prepared and dispensed in quantities of ten millilitres in tubes.

RESULTS

Of the 128 strains from "non-penicillin" sources, 123 were inhibited by one-eighth of a unit or less of penicillin per millilitre, two were inhibited by one-quarter of a unit, and three were inhibited by half a unit (see Table I). The five strains showing slightly increased resistance were non-pathogenic. The series included strains known to vary in such properties as haemolytic (α, β and $\alpha\beta$) power, ability to produce pigment, fibrinogen, lipase and coagulase, fermentation of carbohydrates (mannite, trehalose and mannose) and sensitivity to bacteriophage action, while nine serological types (Christie and Keogh, 1940) were represented. In spite of this variety in properties, sensitivity to penicillin did not vary appreciably from strain to strain.

Of the 31 strains from patients in penicillin treatment wards, 18 showed considerable resistance to the drug (see Table I). All were pathogenic, in that they produced coagulase, α toxin, fibrinogen and golden yellow pigment, and also they were found to be lethal for mice. All strains fermented mannite within two days, except two which required three days, these two being penicillin-sensitive.

In a further search for some property which might be influenced by penicillin treatment, the ability of these 31 strains to produce the enzyme phosphatase was examined. Bray and King's (1943)

phenolphthalein phosphate medium was used. All 31 strains were found to give an equally strongly positive indication of phosphatase production.

It seemed possible that the resistant strains might have had a mixed population, part of which was responsible for growth in penicillin broth, while the remainder were pathogenic to mice. To test this, cultures from four resistant strains in penicillin broth were plated out on blood-agar medium. No haemolytic variants were seen. Sub-cultures were made from single colonies from all four strains and tested for pathogenicity to mice by the above method. All gave positive results. Plate cultures from resistant strains made on blood-agar containing one-quarter of a unit of penicillin per millilitre showed as many colonies as similar cultures from the same strains on control plates without penicillin.

The possibility that all 18 resistant strains were from a common parent and that cross-infection from a single source had occurred was discounted when slide agglutination of the strains showed that at least three serological types were represented. The evidence, however, did not exclude the possibility that some cross-infection had occurred.

The results shown in Table I with the 119 pathogenic strains are represented graphically in Figure I, the number of strains being shown along the ordinate and the inhibiting unitage of penicillin along the abscissa. The resulting graph, with its two maxima, suggests that staphylococci under the action of penicillin in vivo rapidly acquire resistance of a fairly constant degree (five to ten units), further resistance being much more slowly acquired. Efforts to confirm this in vitro were unsuccessful, the resistance having been found to increase steadily to a much higher degree.

TABLE I.

Comparison of Sensitivity of Staphylococci Collected Prior to the Use of Penicillin, with Staphylococci Isolated from Patients in a Hospital where Penicillin Treatment is Given.

Strains	Minimum Inhibiting Concentration of Penicillin in Broth. (Units per Millilitre).											Total
	80	40	20	10	5	2½	1	½	¼	1/8	1/16	1/32	1/64	
Strains from non-penicillin sources:														
Coagulose-producing	0	0	0	0	0	0	0	0	0	53	30	5	0	88
Coagulose non-producing	0	0	0	0	0	0	0	3	2	3	26	6	0	40
Strains from hospital where penicillin was used (all coagulose-producing)	1	0	1	13	2	0	1	0	0	11	2	0	0	31
TOTAL	..	..	..	..	..	..	..	..	..	..	..	..	..	159

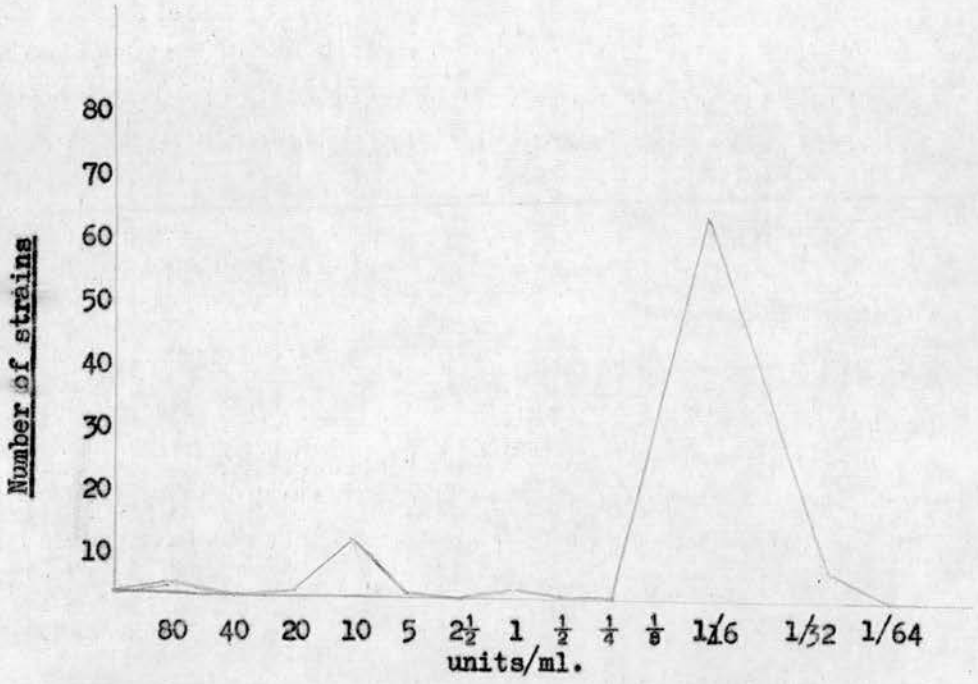

FIGURE I.

Graph connecting number of strains of staphylococci with minimum unitage of penicillin that will inhibit growth in broth. (Staphylococci not producing coagulase are not included).

DISCUSSION

The finding of such a large proportion of penicillin-resistant strains (17 of 31 strains being resistant to 2.5 or more units of penicillin per millilitre) in cultures received from penicillin-treated lesions was unexpected. The contrast between this group and the group of 88 coagulase-producing strains from the original collection (no strain was resistant to more than one-sixteenth of a unit per millilitre) is striking.

It is clear that naturally occurring penicillin-resistant pathogenic staphylococci are uncommon; it would seem equally clear that a large proportion of resistant strains - pathogenic, by the usual

criteria of pathogenicity - may appear in wounds in wards where penicillin is used intensively and over long periods of time.

No difference in the rate of biological or metabolic activities of the penicillin-resistant as compared with penicillin-sensitive strains was found. However, we have not yet compared a resistant strain developed in the test tube with the parent culture, as has been done by Abraham et alii and by Spink, Ferris and Vivino. It does not necessarily follow that changes induced in vitro will develop in vivo.

To reiterate, our only definite findings are that a highly significant proportion of penicillin-resistant staphylococci have been obtained from lesions of patients in wards in which penicillin treatment has been given. It is not known whether these resistant strains develop mainly from sensitive parent organisms in the same wound or are the result of cross-infection, either from carriers or air-borne. Further, from this preliminary study it has not been possible to determine to what extent these resistant strains have delayed the sterilization and healing of wounds.

Further work is necessary and is being done to determine the importance of these preliminary findings from the aspects of epidemiology and prognosis.

SUMMARY

In an examination of 159 strains of staphylococci no correlation was found between resistance to penicillin and other biochemical properties.

The only strains showing resistance to penicillin came from patients in wards in which penicillin treatment had been given. These strains showed no differences in common properties from normal strains.

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CRITERIA OF PATHOGENICITY IN STAPHYLOCOCCI

by R. CHRISTIE, E. A. NORTH and BARBARA J. PARKIN

(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 16th November, 1945).

The coagulase reaction is generally accepted as the best single test of pathogenicity in a staphylococcus. Other tests used include pigment-production, haemolysis, toxin-production, agglutinability with anti-sera prepared from pathogenic strains and mannitol fermentation. Chapman et al. (1937) have described three tests using special media. Fibrinolysin-production is another indication of pathogenicity, although all pathogenic strains are not fibrinolytic (Madison, 1933; Christie and Wilson, 1941). Recently North and Christie (1945) have shown that the ability of a strain to kill mice could be adapted to give another convenient test.

Cowan (1938) subdivided staphylococci into two groups, a haemolytic group with three sub-divisions depending upon the type of toxin produced, and a non-haemolytic group sub-divided on the basis of mannitol fermentation. Field and Smith (1945) suggested that staphylococci be divided into two groups, Staphylococcus pyogenes and Staphylococcus saprophyticus.

A number of strains of staphylococci have been tested in these laboratories for ability to produce coagulase, haemolysin, toxin, pigment and fibrinolysin and to ferment mannitol, the original purpose being to investigate how far pathogenicity for mice would correlate

with other indications of pathogenicity. The results indicate that on the basis of the coagulase-test and of haemolysis on sheep blood agar, staphylococci may be divided into three broad groups, viz., coagulase-positive strains of definite pathogenicity, coagulase-positive strains of doubtful pathogenicity and non-pathogenic strains. They agree with the usual view that staphylococci form one large group of organisms with varying biochemical and pathogenic properties. The tendency shown by many strains to throw variants with fewer of the properties characteristic of pathogens than those possessed by the parent strains suggest that the fully pathogenic staphylococcus showing all these properties is the original organism, and that as one property after another is lost, the organism degenerates to the typical non-pathogen with none of the special properties possessed by the typical pathogen.

Chapman et al (1937) found that the commonest form of degeneration was loss of haemolytic power. Singer (1945) also reports this loss.

MATERIALS AND METHODS

Strains Used.

1,027 strains of staphylococci were examined. Those of human origin came from cases of osteomyelitis, soft tissue wound infections, boils, furuncles, throat swabs, urines, etc. Of those of animal origin, 3 were from nasal swabs of dogs with distemper, one was from an abscess on a horse, 3 were from rabbits with ear abscesses, 2 were from a calf with a ruptured, infected cyst, 5 were from milk of sheep with mastitis, and 114 from milk of cattle with mastitis. The non-pathogenic strains in the collection were mainly encountered in the examination of specimens for pathogens.

Coagulase Test.

Fresh, oxalated human plasma, diluted ten times with

normal saline, was dispensed in 2 ml. quantities in small tubes. The inoculum used was a scraping from a 24 hours agar slope culture. A positive and a negative control and an uninoculated tube were included in each set of tests. Readings were made after 4 and after 24 hours' incubation at 37°C.

Haemolysis.

Plates were poured from nutrient agar to which 5 p.c. defibrinated sheep blood had been added. These were "spot-inoculated" from agar slope cultures, 8 strains being tested on each plate. After 24 hours' incubation at 37°C., a zone of lysis with an indeterminate edge was taken as a sign of α toxin-production, a zone with a sharp edge as evidence of production of α toxin plus some β toxin, while a zone of lysis surrounded by a darkened zone meant production of α toxin with a considerable amount of β toxin (Bryce and Rountree, 1936) (Christie and North, 1941).

Toxin Production.

Nutrient broth containing 0.1 p.c. glucose and 0.1 pc. agar was inoculated with the strain to be tested, and incubated for 3 days at 37°C. in an atmosphere of 20 p.c. oxygen and 80 p.c. carbon dioxide. The liquid, was then clarified by centrifugation and 0.02 p.c. merthiolate added. A series of doubling dilutions was then prepared and tested against rabbit and sheep cells as described by Bryce and Rountree (1936).

Pigment.

One part of sterile whole milk was added to 4 parts of liquid nutrient agar and plates poured. After inoculation, the plates were kept at 37°C. for 2 days and at room temperature near a window for 3 further days.

Fibrinolysin.

One part of oxalated human plasma was added to 7 parts of liquid nutrient agar at 50°C. The mixture was heated to 56°C. for 5 minutes and plates poured. Strains were inoculated on to these plates, 8 per plate, and incubated overnight. Zones of clearing around colonies were taken to indicate fibrinolysis.

Fermentation of Mannitol.

Peptone water containing 1 p.c. mannitol, with brom-cresol-purple as indicator, was used. Readings were made daily up to 14 days.

Mouse pathogenicity.

Suspensions in saline containing 4,000 million organisms per ml. as measured against Burroughs Wellcome standard opacity tubes were prepared from 24 hours agar slope cultures. One ml. of this was given intraperitoneally to each of five mice weighing 18-22 gm. The mice were examined 24 hours after inoculation.

RESULTS

The strains were divided on the basis of their biochemical reactions and the results are shown in Table 1.

TABLE 1

Results of Tests for Production of Coagulase, Haemolysin, Fibrinolysin and Pigment and for Fermentation of Mannitol, Showing Number of Strains With Each Set of Properties

	Coagulase	α Toxin*	β Toxin	Fibrinolysin	Mannitol	Pigment	Number of Strains: Human	Number of Strains: Animal
Pathogens	+	+	+	+	+	+	36	-
	+	+	-	+	+	+	588	6
	+	+	+	-	+	+	30	55
	+	+	-	-	+	+	3	23
	+	+	-	+	+	-	11	-
	+	+	+	-	+	-	1	4
	+	+	+	-	-	-	-	4
	+	+	-	+	-	+	2	-
Doubtful pathogens	+	±	±	+	+	+	3	1
	+	±	±	±	+	+	-	1
	+	±	+	-	+	+	-	11
	+	±	-	±	+	+	2	-
	+	-	+	-	+	-	-	1
	+	±	-	+	+	+	21	-
	+	±	±	+	+	-	1	-
	+	-	-	+	+	+	2	1
	+	-	+	-	+	+	-	5
	+	±	-	-	+	+	-	4
	+	-	-	-	+	+	-	1
Non-pathogens	-	-	-	+	+	+	1	-
	-	-	-	-	+	+	25	5
	-	-	-	-	+	-	21	3
	-	-	-	+	-	+	-	1
	-	-	-	-	-	+	5	-
	-	-	-	±	-	-	1	-
	-	-	-	-	-	-	146	2
						TOTAL	899	128

* Strains showing only a trace of haemolysis on sheep blood agar are entered as in the α toxin column.

Haemolysin and Toxin.

With nearly all haemolytic strains, there was no difficulty in deciding whether they produced α or β toxin from the appearances around their colonies on sheep blood agar. Toxic filtrates from 100 of these, titrated against rabbit and sheep cells, gave results which agreed with the plate haemolysis.

The detection of β toxin in the presence of much α toxin, however, was uncertain with many strains, as there is no sensitive test for this toxin in filtrates or on blood agar. Christie and North found that at least 85 p.c. of strains produced β toxin as well. The percentage may prove to be much higher when a sensitive test is devised. In view of this and also because of the lack of apparent significance of β toxin, strains producing α toxin and little β toxin have been grouped with the α strains, and only those producing sufficient β toxin to form a zone of darkening around the clear zone of α lysis have been classed as $\alpha\beta$ strains.

Four strains labelled as pure β strains from plate tests were found to produce α toxin as well when their toxic filtrates were examined. When grown on blood agar under conditions more favourable to production of α toxin, i.e. in an atmosphere with increased CO_2 tension, α lysis was evident. The failure of this to show normally was probably due to the β toxin being produced in such quantity as to inhibit lysis of the red cells by α toxin (Christie and Graydon, 1941). One of these strains, when first isolated several years ago, gave a toxin which lyzed rabbit but not sheep cells at 37°C. and caused considerable "hot-cold" lysis of sheep cells. The latter was due to β toxin but the rabbit cell lysis, in the absence of sheep cell lysis at 37°C. suggested that this may have been due to a toxin distinct from α toxin and resembling that mentioned by Flaum and Forssman (1936). Toxins prepared with one and two days' incubation behaved as $\alpha\beta$ toxins, while one prepared with three days' incubation again caused lysis of

rabbit cells but no lysis of sheep cells at 37°C. It seemed more probable that an interfering agent caused the abnormality in the three-day toxin than that the α toxin had changed to a new one. After toxoiding with formalin until no detectable haemolysin was present, the liquid was still toxic for rabbits, 0.5 ml. given intravenously killing four rabbits overnight, while 0.1 ml. killed one out of two rabbits, the survivor being very sick for several days before it recovered. Recent attempts to produce this abnormal haemolysin with the organism recovered from the dried condition have failed, nor has any toxicity been demonstable in toxoided filtrates.

With strains producing only a trace of haemolysis on blood agar, it was difficult to decide whether the trace was due to α toxin or to some other agent. Many coagulase-negative strains showed a similar trace, especially if incubation were extended to 48 hours. Twelve of these strains were inoculated into toxin-broth and the filtrates, after 3 days' incubation, titrated against rabbit red cells. Eight caused lysis, but only in low dilution. Of these eight, the lysis was only inhibited in three by treatment with α anti-toxin. As this anti-toxin was not definitely specific for α toxin, no conclusion was drawn. Two other coagulase-negative strains, both isolated from acne pustules, gave more than a trace of haemolysis on blood agar although less than that given by the usual toxigenic strains. Yet neither produced more than a trace of haemotoxin in broth.

No strain showing considerable haemolysis on blood agar failed to coagulate plasma. Only one strain was found which produced no haemolysis at all and no soluble toxin, but which coagulated plasma.

Fermentation of Mannitol.

This was found to agree fairly well with coagulase-production as has been frequently reported. 811 of 817 coagulase-positive strains and 53 of 210 coagulase-negative strains fermented mannitol. From Table

1 it will be seen that the agreement was less marked with strains of animal origin than with those of human origin.

Of 400 recently-isolated coagulase-positive strains, only 3 failed to ferment mannitol within 24 hours. Two of these did so within 48 hours, while the third failed to do so within 14 days. Of 106 coagulase-negative strains isolated at the same time as this, one fermented mannitol within 24 hours, 8 within 2 days, 8 within 3 days, 1 in 4 days, 2 in 5 days and 2 in 6 days. Fermentation of mannitol has therefore considerable value as a presumptive test for pathogenicity if readings are made within 24 hours.

Pigment-Production.

This also agreed with the coagulase test fairly well, but many of the strains required the special medium and several days' incubation before the presence of pigment was definite. All degrees of pigmentation were found, from the usual deep orange to a shade difficult to distinguish from that of albus strains. The production of lemon-yellow or citreus pigment was shown by some strains which were typically pathogenic by other tests, as well as by some non-pathogenic strains. Such strains were included with the aureus strains in Table 1, since there was no obvious reason for classifying them separately.

The results agree with the conclusion of Field and Smith (1945) that pigment has little value as an indicator of pathogenicity, but with the qualification that strains which quickly produce much pigment are most probably pathogenic.

Fibrinolysin Production.

With strains of human origin, 651 of 685 coagulase-positive strains were fibrinolytic. Of the 34 non-fibrinolytic strains, 31 were strong producers of toxin, some being old strains and others freshly isolated.

With strains of animal origin, 9 of 117 coagulase-positive strains were fibrinolytic. Of these 9, 3 were isolated from abscesses in the ears of rabbits which had been subjected to much bleeding from ear veins and therefore may have come from human sources. Of the 108 non-fibrinolytic strains, 80 were strong producers of β toxin while 6 of these 80 produced no appreciable quantity of α toxin. Seventeen of the 108 strains were tested for fibrinolysin using ox, horse and sheep plasma as well as the human plasma used in the routine tests. Negative results were again obtained except with one strain which was positive with sheep plasma only. This strain also showed the peculiarity that it coagulated sheep plasma but not that of ox, horse or man.

The difference in fibrinolytic activity shown by strains of human origin as compared with those of animal origin was so definite as to suggest that some strains were specific for humans and others for animals, and that a serological difference, as shown by the streptococci, might be detectable. Many of the animal strains were from sources which did not necessarily imply any more pathogenicity than that required by a secondary invader (e.g. nasal swabs from dogs with distemper, milk from cattle with sub-clinical mastitis, or milk from cattle with clinical mastitis when Str. agalactiae was also present), but such as were obtained from sources which indicated pathogenicity were also non-fibrinolytic, with the exception of the three rabbit strains mentioned above. An anti-serum was prepared from a non-fibrinolytic $\alpha\beta$ strain isolated post-mortem from an abscess in the udder of a cow which died from acute staphylococcal mastitis. The serum agglutinated several human fibrinolytic α strains to the same degree as the original animal strain. It gave a very strong precipitin reaction with the supernatant fluid from a three days' broth culture of the parent strain but an equally strong one with 6 of 9 human strains selected on the grounds of differing biochemical properties.

Coagulase Production.

No strain which was definitely haemolytic and of human origin failed to coagulate human plasma. Several strains, however, whose toxigenicity, as tested on blood agar or against mice, was slight, were coagulase-positive.

Three animal strains, toxigenic on sheep blood agar and against mice, failed to coagulate human plasma. When tested against horse, ox and sheep plasma, none of them failed to coagulate at least one. A small series was tested against all four kinds of plasma with the results shown in Table 2. Smith and Hale (1944) found that an activator present in plasma took part in the clotting of plasma by staphylococci and thus explained why some samples of plasma were more easily coagulated than others, the amount of activator varying with the sample. This alone does not explain the lack of uniformity shown in Table 2.

TABLE 2.

Results of Coagulase Test With Different Plasmas.

Origin of Strain	Type of Plasma				Number of Strains
	Human	Ox	Sheep	Horse	
Human	+	-	+	+	2
Ox	+	+	+	+	4
Ox	+	-	-	-	1
Dog	-	+	+	+	2
Dog	+	+	+	+	3
Dog	+	-	+	+	1
Horse	-	-	+	+	1

A canine strain failed to coagulate each of 6 samples of human plasma, but coagulated each of 6 samples of horse plasma, while three control human strains coagulated all 12 samples.

Mouse Tests.

When mice were inoculated with strains that were pathogenic as indicated by the usual tests, death occurred within 24 hours. With strains having the properties of typical non-pathogens, no deaths occurred nor, with few exceptions, were signs of sickness evident. Clear-cut results of this nature were obtained with 120 coagulase-positive strains and 130 coagulase-negative strains, five mice being used to test each strain.

Preliminary tests had shown that an inoculum of 4,000 million organisms would kill mice. Smaller doses of some strains will also kill mice, but the dose chosen for the routine tests was found to be the one most suitable to kill all five mice when a number of typical pathogens were investigated.

Fifty-three coagulase-positive strains failed to kill all five mice, the number of deaths being four or less, in 25 cases nil. All of these strains produced little or no α toxin as shown on sheep blood agar. The ability to produce coagulase, pigment, β toxin or fibrinolysin or to ferment mannitol did not mean that a strain was pathogenic, while inability to produce β toxin, pigment or fibrinolysin or to ferment mannitol did not prevent a strain from being pathogenic to mice, provided it produced sufficient α toxin. These properties, while possibly playing a role in invasiveness or survival of the organism, are minor ones compared with α toxin-production.

Two coagulase-negative strains caused death to mice, but neither strain killed all five. One strain was weakly and the other definitely fibrinolytic. A third fibrinolytic strain, however, which was coagulase-negative showed no pathogenicity to mice while many of

the non-fibrinolytic, coagulase-positive strains were fully pathogenic.

Four coagulase-positive strains, pathogenic by all these properties but unable to kill all five mice, were further examined. All were old strains which, when plated out, showed the presence of variants of the kind usually found in old strains; the colonies were mixtures of α, $\alpha\beta$, β and non-haemolytic forms. When sub-cultures were made from β haemolytic colonies, they were found to be fully pathogenic to mice.

Two further coagulase-positive strains, sufficiently haemolytic to suggest that they should be fully pathogenic, were unable to kill mice, nor did they show mixed colonies. One of these strains, when grown in toxin-broth, produced only one-quarter of the α toxin produced by the usual pathogen. This toxin was neutralizable by α anti-toxin. The strain required 5 days to ferment mannitol. The work of Warmer and Amluxen (1945) suggests that ability of the staphylococcus to grow within mice is necessary if death is to follow, and it seems probable that the reduced metabolic rate of the above strain enables the defences of the animal to cope with it before it can produce sufficient toxin to affect the animal, although it is able to produce sufficient toxin in vitro. No such obvious explanation could be found for the second strain, however. This strain, which was of animal origin and from a source which did not necessarily indicate pathogenicity, was non-fibrinolytic but fully α haemolytic and fermented mannitol within 24 hours. It produced as much α toxin in broth as the usual pathogen; this toxin was neutralizable with α anti-toxin, and when given intraperitoneally to mice in graded doses was as lethal as the toxins from two typical pathogens which gave the same titres when titrated against rabbit red cells. The live organisms killed none of 20 mice. 0.5 ml. of a 24 hours broth culture, given I.V., did not affect a rabbit. The only unusual feature of the strain, apart from lack of fibrinolysin, was that its suspension in saline showed at times,

though not invariably, auto-agglutinability, a property more common with non-pathogens than with pathogens. If the conclusion arrived at, from the results with all the other strains, that α toxin is the lethal agent, is not to be rejected, it must be assumed that this strain has some peculiarity which renders it susceptible to attack by the animal before it can generate its toxin.

A strain producing so little α toxin that it should have shown no pathogenicity to mice was found to kill some but not all of the five animals. When plated out, it yielded yellow and white colonies, both types showing the same slight degree of haemolysis and giving cultures which coagulated plasma. A broth culture from the white colonies after 14 days incubation yielded white, non-haemolytic colonies of coagulase-negative organisms. Cultures from all three forms, the parent and the two variants, were equally though not fully pathogenic to mice. The nature of this lethal power is uncertain since even the parent strain did not produce sufficient toxin on blood agar or in broth, to kill any of the five mice, as judged by the behaviour of other strains. The strain, in degenerating, lost its pigment first, but that this is not the invariable order of degeneration is seen from the fact that coagulase-negative strains with pigment are not uncommon.

The tentative assumption that pathogenicity to mice was an indication of general pathogenicity agreed with pathogenicity as indicated by source. The coagulase-positive strains which failed to kill all five mice came chiefly from nose and throat swabs, milk samples and war wounds, while such of these strains as came from boils, furuncles and osteomyelitis were old strains which had been maintained for years on artificial media and had also been stored in the dried condition.

The strains in Table 1 have been divided into three groups on the basis of α toxin-production as indicated by haemolysin on sheep blood agar. It was not practicable to test all strains against mice, but fully a quarter have been so tested. Had the results of the mouse

test been used to group strains, at least two in the first group and two in the third group would have fallen into the second group, while 25 coagulase-positive strains in the second group would have been classed with the non-pathogens.

DISCUSSION

The significance of the presence or absence of the different properties of the strains examined has been indicated above. It has been shown that, of the various tests employed, the production of α haemolysin on sheep blood agar plates agrees most closely with the results of the mouse pathogenicity tests. Definite discrepancies were found between the tests for coagulase and haemolysin, several strains that coagulated plasma failing to produce toxin, although the reverse was not found.

While the results of animal experiments cannot be regarded as a certain indication of what would happen in human infections, it is perhaps significant that the strains that kill mice are those that produce α toxin - a product that certainly kills both humans and animals.

Schwabacher et al. (1945), who compared the pathogenicity of strains as judged by source with certain other properties, concluded, as we have, that α toxin-production appeared to be the property most closely associated with pathogenicity in man.

In addition to its greater reliability as a criterion of pathogenicity, the test for haemolysis on sheep blood agar has certain technical advantages over the coagulase test. If used for primary isolation instead of nutrient agar, strains showing definite haemolysis need not be tested for coagulase, the occasional pathogenic non-pigmented strain is less likely to be ignored, the presence of more than one kind differentiated by type of haemolysis can be detected in a manner not possible on nutrient agar plates, while mixed infection with an organism

which grows poorly or not at all on nutrient agar plates will be noted. If a series of strains, already isolated, is to be tested, as many as sixteen can be incubated on one plate, while the medium can be stored for at least seven days at 4°C. Sheep blood is readily obtained in standard laboratories. An occasional error in the coagulase test is possible where fibrinolysis of the clot occurs rapidly after formation, while with animal strains, an error is possible if an unsuitable plasma is used.

SUMMARY

1,027 strains of staphylococci have been tested for ability to produce coagulase, haemolysin, fibrinolysin and pigment and to ferment mannitol. 312 of these strains have been tested for pathogenicity to mice.

Production of α toxin is necessary for full pathogenicity. A strain may produce coagulase and still be non-pathogenic. It may fail to produce β toxin, fibrinolysin or pigment or to ferment mannitol and still be pathogenic.

If sheep blood agar is used in primary isolation of staphylococci from human beings, presence or absence of definite haemolysis will decide pathogenicity. Further tests are only necessary where the degree of haemolysis is less than that given by the usual pathogenic strains.

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ACQUIRED RESISTANCE OF STAPHYLOCOCCI TO THE ACTION OF PENICILLIN

by E. A. NORTH and R. CHRISTIE

(From the Commonwealth Serum Laboratories, Melbourne).

Spink, Hall and Ferris (1945) failed to find a strain of staphylococcus not previously exposed to penicillin that was not inhibited by one unit of penicillin per millilitre, although more than 100 strains were tested. This is in accord with our own findings (North and Christie, 1945), that whilst naturally occurring penicillin-resistant staphylococci are at the most rare, a large proportion of the strains isolated from wounds in patients undergoing prolonged penicillin treatment showed considerable resistance as judged by standard sensitivity tests. There was, however, no definite evidence to indicate whether the resistant strains were the direct descendants of sensitive organisms already in the wounds or were the result of cross-infection, either with naturally resistant strains or with odd strains that had acquired resistance and had become disseminated through the wards.

In the course of further work (to be published) some penicillin-resistant staphylococci were encountered which with little reasonable doubt could be regarded as variants of sensitive parent organisms, which had developed their resistance to penicillin in vivo.

The finding of such variants enabled us to compare the reactions, particularly in laboratory animals, of the variants which had apparently developed resistance to penicillin in vivo with variants which had acquired resistance in vitro.

The observations recorded in this communication confirm the general conclusions reached by Spink, Hall and Ferris (1945) with regard to the difference between variants whose resistance has been developed in vivo and variants whose resistance has been developed in vitro. They also show that the former retain full pathogenicity as judged by animal experiments, and that the penicillin resistance demonstrated by tests in vitro can also be shown in tests in laboratory animals - that is, in vivo.

MATERIALS AND METHODS

Penicillin-Resistant Variants of Normally Sensitive Strain Developed in Vivo.

Three cultures, S948, S962 and S988, were isolated on the dates shown in Table I. This table also shows their various biological and metabolic characteristics. Serological typing was done by slide agglutination (Christie and Keogh, 1940), whilst tests for penicillin resistance were carried out as previously described (North and Christie, 1945). S962 and S988 are regarded as true penicillin-resistant variants of S948.

In the animal experiments to be described, the reactions of S988 were compared with those of S948.

Penicillin-Resistant Variants of Standard Sensitive Strain F.D.A. 209, Developed in Vitro.

Penicillin-resistant variants of standard sensitive strain F.D.A. 209 were developed in vitro by daily subculturing of strain F.D.A. 209 in increasing concentrations of penicillin in nutrient

broth. As the strain developed increasing resistance, agar slopes were inoculated from the tube containing the highest concentration of penicillin in which growth occurred. Such cultures were preserved under paraffin for future reference and testing, and were designated F.D.A. 209/1, F.D.A. 209/10, and so on, up to F.D.A. 209/160, the denominator indicating the highest unitage of penicillin per millilitre of broth in which the variant would grow.

Mouse Pathogenicity Tests.

Mouse pathogenicity tests were carried out as previously described (North and Christie, 1945), except that a range of dosages was employed to find the actual killing effect of the strains compared. The dose was always made up to a volume of one millilitre with normal saline solution and its strength was estimated by the use of Burroughs Wellcome standard opacity tubes.

Comparison of Results of Penicillin Therapy in Staphylococcal Infections In Mice with Sensitive (S948) and Resistant (S988) Strains.

The infecting dose was that used in our standard mouse pathogenicity test (4,000,000,000 organisms), and the penicillin dosage was based on the report of Warmer and Amluxen (1945), in which they found that it was necessary to give 200 units per gramme of body weight every one and a half hours to maintain a concentration of one unit per millilitre. Further details will be given when the actual experiment is being described.

Standard Biochemical Tests.

Tests for coagulase production, fibrinolysis, haemolysis, pigment formation and penicillin sensitivity were carried out as described previously (North and Christie, 1945; Christie, North and Parkin, 1945).

TABLE I

Characteristics of Staphylococci Isolated from Leg Wound of Patient W33

Date Isolated	Serial Number of Strain	Coagulase Formation	Fibrinolysis	Haemolysis[1]	Pigment Formation[2]	Mannitol Fermentation[3]	Serological Typing[4]	Penicillin Resistance[5]
7.6.45	S948	+	+	α	Aureus++++	+1	III-VI	0.6 unit
15.6.45	S962	+	+	α	Aureus++++	+1	III-VI	2.5 units
21.6.45	S938	+	+	α	Aureus++++	+1	III-VI	10 units

1 Haemolysis is recorded as observed on 5% sheep blood agar.

2 Pigment is graded with regard to intensity ("Aureus++++", indicates intense pigment formation).

3 The "+" sign indicates fermentation, and the numeral the day on which fermentation is complete.

4 The Roman numerals indicate the type sera which cause agglutination.

5 Resistance is recorded as the highest concentration in units per millilitre of penicillin in broth in which growth is apparent (even if slight) after twenty-four hours.

RESULTS

Comparison of the pathogenicity for mice of strains S948 (penicillin-sensitive) and S988 (penicillin-resistant) shows (Table II) that their killing power is identical within the limits of experimental error. Further reference to Table I shows that this similarity is combined with only one observable difference - resistance to penicillin as tested in vitro.

TABLE II

Effect on Mice of Living Cultures of Penicillin-Sensitive Staphylococcus S948, and of Penicillin-Resistant Variant, S988.

Culture	Number of Organisms. (Millions)	Number of Mice	Result After Twenty-four Hours[1]
S948	4,000	5	+++++
S948	2,000	5	++++S
S948	1,000	5	++++S
S948	500	5	SSSSS
S988	4,000	5	+++++
S988	2,000	5	+++++
S988	1,000	5	++SSS
S988	500	5	SSSSS

1 The symbol "+" indicates death; "S" indicates survival of mouse.

Once the fact was established that S948 and S988 were equally lethal for mice, the ability or otherwise of penicillin to prevent death in mice was investigated. Four groups of mice, A, B, C and D, each consisting of ten animals of equal weight (20 grammes), were used. Groups A and C were left untreated, whilst the mice of groups B and D were given 4,000 units of penicillin intraperitoneally at 8.45 a.m. and again at 10.15 a.m. Mice of groups A and B were then infected with S948 organisms intraperitoneally, and mice of groups C and D with S988 organisms (penicillin-resistant variants). The order of infecting the mice was such that the penicillin-treated animals received the living staphylococci fifteen to twenty minutes after the second dose of penicillin. The mice in groups B and D received a further 17,000 units spread over three doses at intervals of three hours, the first dose being given one hour after the infection with staphylococci. The penicillin solution used contained 25,000 units per millilitre, and each mouse treated received one millilitre in divided doses.

The result of this experiment (Table III) is similar to that of one recorded by Warmer and Amluxen (1945), in that penicillin saved the mice infected with a penicillin-sensitive strain, whilst similar treatment failed to protect mice against infection with a penicillin-resistant staphylococcus. Our experiment is of added interest, since the resistant staphylococcus is apparently a direct descendant of the sensitive strain S948.

The penicillin-resistant variant F.D.A. 209/160, developed in vitro, was found to differ considerably from the parent strain F.D.A. 209 in its biological and metabolic characteristics. It grew poorly on nutrient agar and the colonies were smaller than the usual staphylococcal colonies. Little pigment was formed, and some β haemolysis was evident on sheep's blood agar. Mannitol fermentation was only slightly slower, but the variant still produced coagulase, although not so rapidly as F.D.A. 209. On the other hand, in poorness

of growth, reduced size of colonies and almost complete lack of opacity in colonies, it could not be said to resemble closely any strain - pathogen or non-pathogen - that we have isolated from living tissues.

On repreated subculture in nutrient broth of strain F.D.A. 209/160, its characteristics reverted to those of the parent strain, including its sensitivity to penicillin. The same alteration in characteristics was shown by the less resistant variants F.D.A. 209/1 and F.D.A. 209/10. These changes did not run parallel with increased resistance to penicillin; they appeared to precede it.

The killing doses in mice of living cultures of F.D.A. 209 and its resistant variants, particularly F.D.A. 209/160, were investigated with the result shown in Table IV. It will be noticed that strain F.D.A. 209 kills within exactly the same range as S948 and S988 (see Tables II and IV), whilst F.D.A. 209/160 is almost completely avirulent; (Suspensions of killed organisms from non-pathogenic strains are lethal for mice in doses of 100,000,000,000).

The killing power of strain F.D.A. 209/10 was not tested in a higher dosage than 6,000,000,000, as many non-pathogenic strains cause mice to appear ill with this dosage. After injection of organisms F.D.A. 209/10 the mice remained perfectly well.

TABLE III

Demonstration of Penicillin Resistance in Staphylococci by Mouse Inoculation

Group of Mice	Strain of Infecting Staphylococci	Penicillin Resistance in Vitro	Number of Organisms. (Millions)	Total Units of Penicillin Administered	Number of Mice	Result[1]
A	S948	0.06 unit[2]	4,000	Nil	10	++++++++++
B	S948	0.06 unit	4,000	25,000	10	SSSSSSSSSS
C	S988	10 units	4,000	Nil	10	++++++++++
D	S988	10 units	4,000	25,000	10	++++++++++

1 The result was read twenty-four hours after the mice were infected intraperitoneally, and seventeen hours after groups B and D had their final doses of penicillin. The symbol "+" indicates death; "S" indicates survival of mouse.

2 The standard penicillin-sensitive strain F.D.A. 209 used as a control when resistance was being estimated in vitro gave the same result as S948.

TABLE IV

Fatal Dose for Mice of Living Culture of Standard Penicillin-Sensitive Strain F.D.A. 209 and of Three Variants with Resistance to Penicillin Developed in Vitro.

Culture	Infecting Dose (Millions of Organisms).	Number of Mice	Result After Twenty-four Hours.[2]
F.D.A. 209[1]	4,000	5	+++++
F.D.A. 209	2,000	5	++++S
F.D.A. 209	1,000	5	++++S
F.D.A. 209	500	5	SSSSS
F.D.A. 209/160	32,000	2	++
F.D.A. 209/160	16,000	6	++SSSS
F.D.A. 209/160	8,000	7	SSSSSSS
F.D.A. 209/160	4,000	5	SSSSS
F.D.A. 209/10	6,000	1	S
F.D.A. 209/10	4,000	5	SSSSS
F.D.A. 209/1	4,000	5	SSSSS

1 For particulars of F.D.A. 209 and its variants, see text.

2 Symbol "+" indicates death; "S" indicates survival of mouse.

A rabbit was injected intravenously with one millilitre of a twenty-four hour broth culture of F.D.A. 209.160. On the day following the injection the rabbit looked perfectly well. An attempt to recover the organism from the blood was successful, and the staphylococcus was found to be still resistant to 160 units of penicillin per millilitre. Further attempts at blood culture failed, and the rabbit suffered no apparent ill effects.

As a further check on the validity of the results obtained in mice, 0.5 millilitre of a twenty-four hour broth culture of F.D.A. 209 was injected intravenously into a rabbit, which died within twenty hours. Another rabbit similarly dosed with a culture of F.D.A. 209/10 remained perfectly well.

It has been shown that a penicillin inhibitor can be extracted from staphylococci that have acquired resistance in vivo (Spink, Hall and Ferris, 1945) but not from resistant variants developed in vitro. By two methods, both differing from that referred to by Spink et alii, we have confirmed this.

Selected strains were grown overnight in broth, and penicillin was added in graded amounts to quantities of 10 millilitres of the sterile filtrates. The tubes were then inoculated with the standard sensitive strain, F.D.A. 209, and reincubated. Growth occurred in the two filtrates from resistant strains developed in vivo, when the penicillin concentration was as high as 2.5 units per millilitre. With the filtrates from the standard sensitive strain itself and from a resistant variant (F.D.A. 209/160) developed in vitro, the highest concentration of penicillin in which growth occurred was 1/32 unit per millilitre.

Two resistant strains developed in vivo and one developed in vitro were "spot inoculated" on the centre of nutrient agar plates containing 2.5 units of penicillin per millilitre and incubated over-

night. A streak inoculum of the standard sensitive strain (F.D.A. 209) was then made across the medium, passing close to each central colony. On further incubation growth from the streak inoculum of the sensitive strain occurred near the colonies of the resistant strains developed in vivo. No growth occurred near the colony of the resistant variant (F.D.A. 209/160) developed in vitro.

DISCUSSION

Spink, Ferris and Vivino (1944) stated that "probably of considerable clinical importance is that an increased resistance to penicillin is accompanied by the development of strains which are more susceptible to the bactericidal action of whole blood, and possibly to the other defence mechanisms of the host". This conclusion appears to have been based mainly on experiments conducted on resistant variants developed in vitro, and Spink and his associates (1945) have recently modified this view. They now consider that the resistance to penicillin which has been developed by in vitro methods is only a temporary characteristic of the organisms, whilst resistance acquired in vivo as a result of therapy with penicillin appears to be a more permanently acquired property. They state that the organisms which have become resistant to penicillin in the human body are as resistant to the bactericidal action of human blood as the penicillin-sensitive parent strains - in other words, they retain their full pathogenicity.

Our results tend to confirm the later views of Spink and his associates, especially with regard to the invasiveness of the strains which have developed resistance in the human body. In our experience it has not been easy to trace the direct development of resistance in a strain in infected war wounds. However, there is little room for doubt in the instance recorded by us, as strains serologically resembling S948, S962 and S988 were uncommon.

So far there is little evidence that these resistant strains

developed in vivo have been a major cause of failures with penicillin. However, Anderson, Howard and Rammelkamp (1944), reporting on the penicillin treatment of a series of patients suffering from chronic osteomyelitis, tested the sensitivity of the organisms before therapy; at intervals the organisms which persisted in the lesions were tested again. In two cases cultural examinations made a year after completion of penicillin therapy showed the organisms to be still resistant to penicillin.

Six resistant strains isolated from infected wounds treated with penicillin have been subcultured in this laboratory every day in nutrient broth for a period of thirty days. At the end of that time they showed the same resistance to penicillin as at the beginning, and they also showed all their former properties indicating full pathogenicity and invasiveness. One of these strains was three times subjected to animal passage, being given intravenously to a rabbit and recovered post mortem; after the third passage its resistance to penicillin as tested in vitro had not altered.

Warmer and Amluxen (1945), in experiments (already referred to) using mice, found that a penicillin-resistant haemolytic staphylococcus proved to be resistant to concentrations of penicillin in vivo comparable with those in vitro. The strain which was isolated from an abscess following osteomyelitis treated with penicillin was found by laboratory tests to be resistant to concentrations of penicillin below 10 units per millilitre. The experiment (see Table III) carried out by us, and based on Warmer and Amluxen's report, completely confirmed their findings.

Evidence such as that contained in the reports of Spink et alii (1945), Anderson et alii (1944), Warmer and Amluxen (1945, together with our own experience, suggests that penicillin-resistant staphylococci may become a clinical problem of the future.

CONCLUSIONS

1. Penicillin-resistant staphylococci developed in vivo are as highly pathogenic for laboratory animals as the sensitive parent organisms.

2. Resistant variants developed in vitro are not pathogenic for laboratory animals.

3. Staphylococci in which resistance has been developed in the human body following penicillin treatment kill mice in spite of the administration of large amounts of penicillin.

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PENICILLIN-RESISTANT STAPHYLOCOCCI IN WOUNDS: A REPORT BASED ON A STUDY OF 59 CASES OF INFECTED WAR WOUNDS

by A. E. NORTH and R. CHRISTIE

(From the Commonwealth Serum Laboratories, Melbourne).

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B. K. RANK,

Lieutenant-Colonel, Australian Army Medical Corps,
Surgeon in Charge, Plastic Unit, Heidelberg Military Hospital.

This paper records a survey of bacteriological changes in a series of war wounds, in their later stages and under penicillin treatment. The survey was undertaken because of a previous finding of a high proportion of penicillin-resistant strains of staphylococci among cultures from such wounds - 18 amongst 31 tested (North and Christie, 1945). The incidence of such penicillin-resistant staphylococci in penicillin-treated wounds has already been confirmed. Selbie, Simon and McIntosh (1945) found that of a collection of 157 strains of coagulase-positive staphylococci, 9% were relatively resistant. In only an occasional instance, however, was the resistance as high as in our series. Selbie and his co-workers also detected an increase in the resistance of the infecting organism during systemic treatment with penicillin in four out of 25 cases. Gallardo (1945), in a study

of 108 strains from traumatic wounds, found that 24 (22.2%) were either naturally resistant to penicillin or became penicillin-fast during the course of treatment.

In our series nearly all these resistant strains were noted to come from cases in one hospital ward. It was in this ward that the present survey was undertaken. It was anticipated that investigation of the biological and serological characteristics of the strains isolated might indicate the manner in which penicillin-resistant staphylococci replaced penicillin-sensitive strains. It was also hoped to determine what effect the presence of penicillin-resistant staphylococci in the wounds had on sterilization and healing.

Clinical Material and Source of Bacteriological Specimens.

The wounds investigated were all of the open, infected type in patients admitted, during a period, to one ward of a plastic surgery unit where routine treatment of these wounds included the use of antiseptics. These were used either to aid rapid spontaneous healing or to cover active surgical procedures, many of which would not have been undertaken so soon or in such radical fashion prior to the advent of penicillin. The majority of the patients had already had penicillin therapy, both local and general, for intermittent or continuous periods prior to their admission to the plastic surgery ward. Many of the patients had received vast quantities of penicillin. They came from many different hospital sources on the lines of communications.

The wounds themselves were of two main types. Some were superficial or uncomplicated soft tissue wounds highly suited to the local use of penicillin, the others were soft tissue wounds complicated by bone sepsis or by the sequestration process in its various stages. These latter were not suited to the local use of penicillin, and for them penicillin was used parenterally, chiefly in relation to surgical operation ranging from simple sequestrectomies to complex bone grafting and soft tissue plastic operations. In this latter group penicillin

per se was not regarded as having any specific effect in reducing healing time, but it rendered certain operations feasible and safe at an earlier phase in the management of such wounds.

Routine swabs were taken from all wounds at their first inspection on the patients' admission to the ward, and further swabs were taken at least once each week. All wounds from which staphylococci were isolated were treated with penicillin locally and/or parenterally. Penicillin was used parenterally chiefly for active, spreading sepsis or in relation to surgical operations in the presence of sepsis. The technique of ward management and dressing routine was conducted essentially in accordance with procedures as prescribed by the Medical Research Council. The methods used and the staff were unchanged during the period of observation.

ISOLATION OF STAPHYLOCOCCI

On arrival at the laboratory, the swabs were moistened with sterile physiological saline solution and rubbed over the surface of freshly prepared 5% sheep's blood agar plates, which were then incubated overnight. Single colonies of haemolytic staphylococci were picked off and transferred to two nutrient agar slopes. The growth from one slope was used for the immediate routine tests; the other was covered with sterile paraffin (Simmons, 1942) and used later as the source of material for serological typing and, when necessary, for checking previous findings. Occasionally colonies with obvious differences of form, pigmentation, or zone of haemolysis were found on the same plate, and a colony of each of these was picked off. In the event of a completely confluent growth further plating was undertaken until single colonies were obtained. When a plate containing haemolytic staphylococci was overgrown with Bacillus proteus, a suspension of a sample was spread on nutrient agar containing phenol (0.1%) to obtain uncontaminated single colonies of staphylococci.

INVESTIGATION OF STRAINS ISOLATED

Tests for haemolysins (α and β) were made on 5% sheep's blood agar plates (Christie and North, 1941).

Tests for coagulase were made with human plasma diluted ten times with normal saline solution, readings being made after four and eighteen hours. Coagulase-negative strains were rejected.

Fibrinolysin was detected on fibrinogen agar (Christie and Wilson, 1941).

These for mouse pathogenicity were made by injecting each of five mice weighing 18 to 22 grammes with one millilitre of a saline suspension containing 4,000,000,000 organisms from an overnight agar slope culture (North and Christie, 1945).

Mannitol fermentation was detected in mannitol broth, which was examined each day for fourteen days during incubation at 37°C.

Pigment formation was detected by cultivation of the organisms on milk agar (Fujita and Yoshioka, 1938), and strains of staphylococci were graded as Albus, Aureus I, Aureus II, Aureus III, Aureus IV, or Citreus, according to the depth and colour of pigment.

Penicillin sensitivity was estimated by inoculating 10 millilitre samples of penicillin broth with one drop of an overnight broth culture from a fine-tipped Pasteur pipette, and examining the tubes for turbidity after twenty-four hours' incubation at 37°C. (North and Christie, 1945).

Serological typing was carried out by slide agglutination (Cowan, 1939; Christie and Keogh, 1940).

The above mentioned examinations, with the exception of

the serological typing, were carried out within a few days of isolation. No strain was considered to be pathogenic unless it produced an appreciable amount of ∝ haemolysis (Christie, North and Parkin, 1946). All tests except the mouse pathogenicity test were carried out on each strain. The mouse test was used only on strains of doubtful pathogenicity as indicated by their degree of haemolysis or on strains which were either non-agglutinable or auto-agglutinable.

Serological typing of the strains from each patient was carried out after swabs ceased to arrive. The whole series was then typed at the one time.

The total number of patients included in the survey was 59 and from these 328 cultures of haemolytic staphylococci were isolated and tested as indicated. Cases were numbered in chronological order and were observed bacteriologically for periods ranging from one to twenty-two weeks, the average time being six weeks.

RESULTS

Analysis of the results shows that, with regard to penicillin-fastness of staphylococci, the cases fall into four groups:

Group A: Those associated only with penicillin-sensitive staphylococci in the wounds: 10 cases (Table I).

Group B: Those in which penicillin-resistant staphylococci replaced penicillin-sensitive organisms during the period observed: 10 cases (Table II).

Group C: Those associated only with penicillin-resistant staphylococci in the wounds: 35 cases (Table III).

Group D: Those in which penicillin-resistant and penicillin-sensitive strains alternated: 4 cases (Table IV).

Total - 59 cases.

The cases of group A (Table I) were for the most part those in which superficial and uncomplicated wounds were present - wounds in which rapid healing would naturally be expected with correct treatment. Their relative sterilization to render the common grafting procedures effective was usually easy. This explains the short period of infection.

The cases of groups B, C and D (Tables II, III and IV) included those in which the wounds were complicated by factors which in themselves would retard healing and render any permanent sterilization impracticable. They could not be expected to heal even if they were free of staphylococci. It was in these cases that staphylococci persisted, and that practically all penicillin-resistant strains occurred.

It would seem possible, both from the clinical nature of the wounds harbouring penicillin-resistant strains and from the fact that the subjects had previously received large amounts of penicillin, that these strains represented the residuum of organisms in wounds by their very nature incapable of complete sterilization, in which the sensitive strains were previously eliminated by penicillin treatment. This possibility will be discussed later.

There was nothing to suggest that the presence of these penicillin-resistant strains in any way retarded the clinical progress of the wounds. Their healing was not a primary function of antiseptic treatment or absolute bacteriological sterilization. They were healed only after natural processes or time-accepted surgical methods ultimately brought them into the category of simple, uncomplicated wounds, when they were rapidly healed, as were the wounds in group A, whether they harboured penicillin-resistant staphylococci or not.

In the attempt to decide whether or not all the strains in one series originated from the strain first isolated, the biochemical tests were found to be of limited value only as most of the 328 strains

were strongly haemolytic, deeply pigmented and fibrinolytic. When successively isolated strains from a patient were found to be indistinguishable biochemically and serologically, it was assumed that they were descendants of the strain first isolated. When some unusual biochemical characteristic was found to be associated with strains of one serological type, this assumption was strengthened. In successive strains isolated from two patients, one series of which consisted of strongly haemolytic albus strains and the other of non-fibrinolytic strains, the biochemical characteristics might have been of value in deciding the origin of penicillin-resistant strains. However, both series belonged to group C. The laboratory findings in the series from the latter case (Case 19, Table III) are shown in Table V.

There is a statistically significant difference between the distribution of serological types encountered in the wounds of group A patients and those in groups B, C and D. The data are summarized in Table VI. In cases of group A, type VI-VIII-III organisms were not found in any of the wounds, whilst in cases of the other three groups they occurred frequently.

For the purpose of presentation of results, all strains that were definitely agglutinated by type VI and by type VIII serum, together with a smaller number that were in addition agglutinated by type III serum - usually a minor agglutination - are regarded as belonging to type VI-VIII-III. A protocol giving the results of serological typing of strains from a characteristic case (Case 52, group C, Table III) is shown in Table VII.

A few cases were encountered early in which strains were isolated that were agglutinated only with type III and type VI serum. When these strains were retested later with freshly absorbed sera, they could not be separated from type VI-VIII-III and are included in it. With regard to other characteristics, most type VI-VIII-III strains

closely resembled one another. They all, with but two exceptions (Case 2, Table IV, and Case 43, Table III), produced a deep golden pigment. In the two exceptions weakly pigmented strains were isolated from the wounds and naso-pharyngeal swabs; but the usual deeply pigmented type VI-VIII-III organisms were also isolated from the wounds of both patients.

In addition to the type VI-VIII-III strains shown in Table VI, there were three cases (Tables III and IV) in which strains giving major agglutination with type VI or type VIII serum were also agglutinated by type V serum (two cases) or by type IV serum (one case). In other characteristics they resembled the type VI-VIII-III strains.

Type I strains were isolated from the wounds of thirteen patients and were distributed among all groups of cases (Tables I, II, III and IV). They differed considerably from one another in degree of agglutinability and in other characteristics, particularly pigmentation. Type II strains, some with minor agglutination with type IV serum, were the only others found sufficiently frequently to warrant separate classification. They were found in four wounds (one in each group) and comprised both penicillin-sensitive and penicillin-resistant strains.

Routine nose and throat swabbing was not carried out during the whole period of the investigation. Table VIII contains a summary of the results obtained amongst those patients recorded as having had nose and throat swabs examined. Of the thirty patients from the noses or throats of whom pathogenic staphylococci were isolated, in seventeen the organisms resembled those in the wounds. Thirteen of the strains isolated from the nose or throat belonged to type VI-VIII-III strains, but in two cases corresponding organisms were not isolated from the wound. None of the type VI-VIII-III strains came from group A patients (TableI). The strains from the naso-pharynx of the patients that differed from those in the wounds were made up as shown in Table IX.

TABLE I.

Cases in Which Only Penicillin-Sensitive Staphylococci were Isolated from Wounds (Group A).

Case Number	Period During which Swabs were Received	Duration of Wound Before First Swab	Diagnosis: Clinical Condition	Progress of Patient	Serological Type and Colour of Staphylococci Isolated	Result of Naso-pharyngeal Swabbing	Additional Information re Penicillin Treatment
6	3 weeks	Nil	Multiple burns	Normal healing	Type V, Aureus IV	No swab taken	Local penicillin treatment only.
12	9 weeks	Years	Infected eye socket surface infection only.	Plastic operation, uncomplicated.	Type I, Aureus III	No swab taken	No pathogen isolated after six weeks; had local penicillin treatment only.
14	2 weeks	2 days	Hand wound	Rapidly healed by graft	Type I, Aureus III	No swab taken	Local penicillin treatment only.
17	1 week	3 weeks	Multiple burns	Normal healing	Not agglutinated by type sera I to IX, Aureus IV.	No swab taken	Local penicillin treatment only.
20	1 week	3 weeks	Multiple burns	Normal healing	Type VII, Aureus IV	Type VI, Aureus IV, penicillin-resistant.	Penicillin given intramuscularly before first swabs.
34	2 weeks	3 weeks	Gun-shot wound of the hand.	Normal healing	Not agglutinated by type sera I to IX, Aureus IV.	No pathogen	Penicillin given intramuscularly and locally before admission to hospital.
39	4 weeks	1 day	Gun-shot wound of the hand.	A primary graft was satisfactory finally healed soon after dead bone and tendon separated.	Type II - IV, Aureus II.	Type II - IV, Aureus II, penicillin-sensitive.	-
40	1 week	Days.	Infection about skeletal pin.	Subsided rapidly after pin was removed.	Not agglutinated by type sera I to IX, Aureus III.	As in wound, Aureus III, penicillin-sensitive.	No penicillin before first swab taken.

Table I cont'd...

Table I cont'd...

48	1 week	Nil	Acute abscess in old bone wound of leg.	Did well after drainage and penicillin treatment	Type I - V, Aureus IV.	No swab taken.	Has had penicillin intramuscularly.
53	1 week	4 weeks	Shoulder wound	Normal healing	Type V, Aureus IV	No pathogen	Has had penicillin intramuscularly.

1 Average period of observation for 10 cases, 2.4 weeks.

TABLE II

Cases in Which Penicillin-Sensitive followed by Penicillin-Resistant Staphylococci were Isolated From Wounds (Group B). [1]

Case Number	Period During Which Swabs Received	Duration of Wound Before First Swab	Diagnosis: Clinical Condition	Progress of Patient	Serological Type and Colour		Results of Naso-pharyngeal Swabbing	Additional Information re Similarity or otherwise of Penicillin-Sensitive and Penicillin-Resistant Strains
					Penicillin-Sensitive Staphylococci	Penicillin-Resistant Staphylococci		
3	1 week	3 weeks	Compound hand injury - multiple small wounds.	Rapidly healed by graft.	Type VI-VIII-III, Aureus III.	Type VI-VIII-III, Aureus III.	No swab taken	From comparison of characteristics may be change from penicillin-sensitive to penicillin-resistant.
4	22 weeks	1 year	Chronic leg ulcer	Remained indolent even with penicillin treatment. Recurred after healing. Pinch grafts 100%.	Not agglutinated by type sera I to IX, Aureus IV.	Type VI-VII-III, Aureus IV	Penicillin-sensitivity, similar to penicillin-sensitive strain from wound.	Serologically penicillin-sensitive and penicillin-resistant strains differ, but otherwise resemble one another.
8	13 weeks	Years	Ulceration of nose.	Radical operation and skin graft, uncomplicated.	Type VI-VIII-III, Aureus IV	Type VI-VIII-III, Aureus IV	Lesion involved nose, penicillin resistant type VI-VIII-III.	Although serologically the same, other characteristics differ - for example, degree of haemolysis.
11	21 weeks	7 weeks	Acute finger infection; direct flap to original wound.	Normal progress	Type I, Aureus III.	Type VI-VIII-III, Aureus III	No pathogens	Gradual change serologically and in other characteristics.
15	4 weeks	Nil	Electric burns of hand.	Residual wound after primary excision, rapid healing by graft.	Type VI-VIII-III, Aureus IV.	Type VI-VIII-III, Aureus III	No pathogens	Serologically identical, but other characteristics differ slightly - for example, pigment.

Table II cont'd...

Table II cont'd...

25	4 weeks	Nil	Acid burns of thigh.	Progress not influenced by infection.	Type VI-VIII-III, Aureus IV	Type VI-VIII-III, Aureus IV	No pathogens	Serologically and otherwise identical, but characters differed after subculture from under paraffin.
27	3 weeks	3 to 4 days	Burns on hand.	Normal healing	Type V-IX, Aureus IV.	Type V-VI-VIII-IX, Albus.	No pathogens	Differed more in other characteristics - for example, pigment - than serologically.
30	6 weeks	3 weeks	Multiple caustic burns	Normal healing	Type VI-VIII-V, Aureus III	Two strains: (a) type VI-VIII-V, Aureus III; (b) type II-IV, Aureus I.	Penicillin-sensitive, type I.	Type VI-VIII-V strains similar.
32	5 weeks	2 weeks	Gun-shot wound of face involving bone.	Wound closure, bone graft et cetera uncomplicated.	Not agglutinated by type sera I to IX	Mixed strains serologically including type I.	Penicillin-resistant differing from wound organism type VI-VIII-III, Citreus.	None of the three penicillin-resistant strains from wounds resembled the penicillin-sensitive strains.
37	1 week	Years	Chronic ulcer of leg.	Plastic operations all uncomplicated	Type VI-VIII-III, Aureus III.	Type VI-VIII-III, Aureus III.	No swab taken	Similar serologically and otherwise.

1 Average period of observation for 10 cases, eight weeks.

TABLE III.

Cases in Which Only Penicillin-Resistant Staphylococci Were Isolated From Wounds (Group C)[1]

Case Number	Period During Which Swabs Were Received	Duration of Wound Before First Swab	Diagnosis: Clinical Condition	Progress of Patient	Serological Type of Penicillin-Resistant Staphylococci Isolated	Colour or Other Characteristics of Strain	Result of Naso-pharyngeal Swabbing
1	18 weeks	5 weeks	Compound hand injury	Operation wound and plastic wound broke down. Subsequent healing with pinch grafts. Result poor.	Type VI-VIII-III	Aureus IV	Similar penicillin-resistant type VI-VIII-III repeatedly.
5	2 weeks	Years	Chronic leg wound	Direct flap, uncomplicated	Type VI-VIII-III	Aureus IV	No swab taken.
7	18 weeks	3 weeks	Gunshot wound of face, multiple plastic procedures; bone grafts.	Did well despite infection flare-up controlled by penicillin.	Type VI-VIII-III	Aureus IV	Similar penicillin-resistant type VI-VIII-III repeatedly.
9	13 weeks	2 weeks	Gun-shot wound of face involving bone.	Secondary suture and bone graft, uncomplicated.	(a) Type V-IX (b) Type VI-VIII-III	Aureus IV (a) Aureus IV (b)	Similar to (b), penicillin-resistant type VI-VIII-III.
10	4 weeks	1 week	Infected operation wound; pedicle from chest.	Subsequent operations uncomplicated.	Type VI-VIII-III	Aureus IV	No pathogens.
13	13 weeks	4 weeks	Compound jaw injury; abscess	Rapidly subsided after drainage and penicillin treatment; subsequent plastic operation satisfactory	Type I	Aureus IV	No swab taken

Table III cont'd...

Table III cont'd...

16	10 weeks	4 weeks	Gun-shot wound of hand	Rapidly healed by graft	No agglutination types I to IX	Albus	Similar penicillin-resistant
18	4 weeks	6 weeks	Unhealed amputation stump - arm.	Normal healing after sequestrum removed.	Type VI-VIII-III	Aureus IV	No swab taken.
19	11 weeks	14 weeks	Gun-shot wound of leg - plastic flaps	Plastic operation, uncomplicated.	Type II-IV	Aureus II, non-fibrinolytic	No swab taken.
22	1 week	5 weeks	Multiple burns	Rapid healing by graft; subsequent plastic operation satisfactory.	Type VI-VIII-III	Aureus IV	Different penicillin-resistant type V, Albus.
24	2 weeks	3 days	Recent burns - arm.	Normal healing	Type VI-VIII-III	Aureus IV	No swab taken.
26	1 week	5 weeks	Gun-shot wound of face and jaw soft tissue plastic operation and bone graft.	Plastic operation and bone graft uncomplicated.	Type VI-VIII-III	Aureus IV	Similar penicillin-resistant type VI-VIII-III.
28	8 weeks	4 weeks	Multiple shrapnel wounds	Operation on unhealed wound did well.	Type VI-VIII-III	Aureus IV	No swab taken.
29	5 weeks	7 weeks	Infected wound - finger.	Normal healing after tendon slough	(a) Type I. (b) Type VI-VIII-III.	(a) Aureus IV (b) Aureus IV	Different penicillin-sensitive type II.
31	12 weeks	6 weeks	Compound facture, gun-shot wound of forearm.	Normal healing when bone sequestration complete.	Type VI-VIII-III	Aureus IV	No pathogens.

Table III cont'd...

Table III cont'd...

33	4 weeks	8 weeks	Gun-shot wound of hand, involving bone.	Normal healing after sequestration; subsequent result good.	Type VI-VIII-III	Aureus IV	Penicillin-resistant type I repeatedly.
35	6 weeks	4 weeks	Compound hand wound.	Rapid healing by graft.	Type VI-VIII-V	Aureus IV	Similar penicillin-resistant type VI-VIII-V.
36	9 weeks	2 weeks	Infected operation wound, back of hand.	Subsequent operation, including tendon grafts, uncomplicated.	Type VI-VIII-III	Aureus IV	Similar penicillin-resistant type VI-VIII-III.
38	2 weeks	6 weeks	Infected eye socket, surface infection only.	Infection cleared rapidly	Type VI-VIII-III	Aureus IV	Similar penicillin-resistant type VI-VIII-III.
41	10 weeks	8 weeks	Gun-shot wound of leg.	Direct flap after sequestration, uncomplicated.	Type VI-VIII-III	Aureus IV	No pathogens.
42	4 weeks	5 weeks	Infected wound - elbow.	Cellulitis controlled by penicillin.	Type I	Aureus II	Similar penicillin-resistant type I.
43	3 weeks	9 weeks	Osteomyelitis of jaw.	Radical operation; did well; subsequent bone graft uncomplicated; a hip sinus healed well.	(a) Type VI-VIII-III. (b) Type VI-VIII-III.	Aureus IV (a) Aureus I (b)	Penicillin-resistant similar to (b) type VI-VIII-III.
44	2 weeks	2 weeks	Gun-shot wound of face.	Normal healing; operation uncomplicated.	Type V-VIII.	Aureus IV	Different penicillin-resistant Aureus IV auto-agglutinable.

Table III cont'd...

Table III cont'd...

45	2 weeks	3 weeks	Gun-shot wound of jaw.	Normal healing; uncomplicated bone graft.	Type I	Aureus II	Different penicillin-resistant type V.
46	3 weeks	Nil	Multiple burns	Normal healing	Type VI-VIII-III	Aureus IV	Different penicillin-resistant Aureus IV auto-agglutinable.
47	10 weeks	2 weeks	Colossal wounds (burns) of legs	Sepsis controlled; multiple grafts all did well.	(a) Type I (b) Type VI-VIII-III.	(a) Aureus IV (b) Aureus IV	No pathogens.
49	1 week	8 weeks	Multiple gun-shot wound.	Operation uncomplicated.	Type I	Aureus II	Different penicillin-sensitive Aureus II, not agglutinable.
51	1 week	3 weeks	Gun-shot wound of leg.	Direct flap after sequestrectomy, did well.	Type VI-VIII-III	Aureus IV	Different penicillin-resistant type I, Aureus II.
52	7 weeks	4 weeks	Burns, infected ear.	Slow healing after drainage; chondritis.	Type VI-VIII-III	Aureus IV	(a) Penicillin-resistant similar type VI-VIII-III. (b) Different penicillin-resistant type I, Aureus II.
54	1 week	9 weeks	Multiple wounds.	Thigh rapidly healed by pinch grafts.	Type VI-VIII-III	Aureus IV	Penicillin-resistant similar type VI-VIII-III, Aureus IV.
55	2 weeks	8 weeks	Unhealed amputation stump	Wound rapidly healed, but has chronic skin disease.	Type VI-VIII-III	Aureus IV	Different penicillin-resistant not agglutinable Aureus IV.
56	1 week	10 weeks	Chronic ulcer - leg.	Direct flap, uncomplicated	Type V-VIII	Aureus IV	No pathogens.
57	2 weeks	5 weeks	Burns to hand	Rapid healing with pinch grafts, subsequent plastic operation uncomplicated.	(a) Type I (b) Type VI-VIII-III.	(a) Aureus II (b) Aureus IV	No swab taken.

Table III cont'd...

Table III cont'd...

58	6 weeks	9 weeks	Gun-shot wound of foot.	Rapid healing but subsequent breakdown; chronic bone sepsis.	Type VI-VIII-III	Aureus IV	No swab taken.
59	2 weeks	4 weeks	Infected operation wound; bone graft; did well after sequestration.	Penicillin controlled several exacerbations of acute infection	Type VI-VIII-III	Aureus IV	No swab taken.

1 Average period of observation for 35 cases, six weeks.

TABLE IV.

Cases in Which Penicillin-Resistant and Penicillin-Sensitive Staphylococci were Alternately Isolated From Wounds (Group D).[1]

Case Number	Period During Which Swabs Received	Duration of Wounds Before First Swab	Diagnosis: Clinical Condition	Progress of Patient	Characteristics of Staphylococci Isolated from Wounds		Result of Naso-pharyngeal Swabbing	Further Information re Sequence of Penicillin-Resistant and Penicillin-Sensitive Strains Isolated
					Penicillin-Sensitive	Penicillin-Resistant		
2	20 weeks	4 weeks	Gun-shot wound of face involving bone.	Local plastic operations including bone grafts, all uncomplicated.	Type II, Aureus IV	(a) Type VI-VIII-III, Aureus II. (b) Type VI-VIII-III, Aureus IV.	Penicillin-resistant similar to (a) type VI-VIII-III.	Swabs received from 4.4.45 to 20.8.45; penicillin-sensitive type II last isolated 19.7.45; penicillin-resistant type VI-VIII-III first swab, and again twice after 19.7.45. A penicillin-resistant type VI-VIII-III was constantly isolated from nose
21	20 weeks	6 weeks	Unhealed amputation stump, leg.	Wound breakdown and sequestration - bad result	Type I, Aureus IV	Type I, Aureus IV	Penicillin-resistant, various, including types II and VI-VIII-III	Swabs received from 17.5.45 to 4.10.45; penicillin-resistant type I at first swab, then on 21.6.45 and 28.6.45 penicillin-sensitive, followed by penicillin-resistant on eight subsequent swabbings.

Table IV cont'd...

Table IV cont'd...

23	3 weeks	4 weeks	Gun-shot wound of hand, also eye.	Normal healing	Type VI-VIII-IV, Aureus IV from eye socket.	(a) Type I, Aureus II. (b) Type VI-VIII-III, Aureus IV.	No pathogens	Penicillin-sensitive type VI-VIII-IV following penicillin-resistant type I in eye socket. Penicillin-resistant type VI-VIII-III only in hand.
50	3 weeks	8 weeks	Wound in thigh	Slow healing subsequent operation wound broke down with sepsis.	Type VI-VIII-III, Aureus IV	Type VI-VIII-III, Aureus IV	No swab taken	A penicillin-sensitive strain was isolated from the last swab received.

1 Average period of observation for four cases, 11 - 5 weeks.

TABLE V.

Characteristics of Staphylococci Isolated from Gun-shot Wound in Case 19, Group C.

Identification Number of Strain	Date of Isolation	Type of Haemolysis	Coagulase	Fibrinolysis	Time for Mannitol Fermentation	Pigment	Resistance to Penicillin	Serological Type
872	13.5.45	α (β)	Positive	None	1 day	Aureus II	10 units	II - IV
873	17.5.45	α (β)	Positive	None	1 day	Aureus II	10 units	II - IV
1034	28.6.45	α (β)	Positive	None	1 day	Aureus II	10 units	II - IV
1062	5.7.45	α (β)	Positive	None	1 day	Aureus II	10 units	II - IV
1114	19.7.45	α (β)	Positive	None	1 day	Aureus II	10 units	II - IV
1214	2.8.45	α (β)	Positive	None	1 day	Aureus II	10 units	II - IV

TABLE VI.

Distribution of Staphylococci According to Serological Type Amongst the Different Groups of Cases

Group of Cases	Number of Cases	Number of Strains in Serological Types								Total Number of Strains in Group			Number of Cases in Each Group in Which Type VI-VIII-III Found in Wound.	Statistical Significance re Type VI-VIII-III Strains Compared with Group A.
		I		II		VI-VIII-III		Others						
		P.S.[1]	P.R.[1]	P.S.	P.R.	P.S.	P.R.	P.S.	P.R.	P.S.	P.R.	Total		
Group A: Cases in which only penicillin-sensitive strains were isolated from wounds - Table I.	10	2	0	1	0	0	0	7	0	10	0	10	0/10	
Group B: Cases in which penicillin-sensitive strains followed by penicillin-resistant strains were isolated from wounds - Table II	10	1	1	0	1	5	7	4	3	10	12	22	5/10 (P.S. strains) 7/10 (P.R. strains) 7/10 (total cases)	Significant Highly Significant
Group C: Cases in which only penicillin-resistant strains were isolated from wounds - Table III.	35	0	7	0	1	0	26	0	5	0	39	39	26/35	Highly Significant
Group D: Cases in which a mixture of penicillin-resistant and penicillin-sensitive strains were isolated from wounds - Table IV	4	1	2	1	0	1	3	1	0	4	5	9	1/4 (P.S. strains 3/4 (P.R. strains 3/4 (total cases	Not sufficient data.

1 P.S. = penicillin-sensitive; P.R. = penicillin-resistant

TABLE VII.

Protocol of the Results of Serological Typing by Slide Agglutination of Strains Isolated from a Patient (Case 52) with Burns and Infected Ear.

Identification Number of Strain	Source	Serum Types									Other Characteristics of Interest
		I	II	III	IV	V	VI	VII	VIII	IX	
1244	Throat	+++[1]	-[1]	-	-	-	-	-	-	-	Aureus II, penicillin resistant.
1245	Nose	-	-	-	-	-	++[1]	-	+[1]	-	All Aureus IV strains are similar with regard to haemolysis. All are penicillin-resistant.
1247	Shoulder	-	-	-	-	-	+	-	+	-	
1248	Right hand	-	-	-	-	-	±[1]	-	±	-	
1250	Right arm	-	-	±-[1]	-	-	+	-	+	-	
1251	Ear	-	-	-	-	-	+	-	+	-	
1270	Right arm	-	-	-	-	-	±	-	±	-	
1271	Ear	-	-	-	-	-	±	-	±	-	
1283	Ear	-	-	-	-	-	±	-	±	-	
1284	Arm	-	-	-	-	-	±	-	± -	-	
1328	Arm	-	-	± -	-	-	+	-	+	-	
1337	Arm	-	-	-	-	-	±	-	± -	-	
982	(Control.)	+ +	±	-	-	-	-	-	± -	-	

1 "+++" = complete agglutination; "++" = heavy agglutination; "+" = definite agglutination seen with naked eye; "±" = agglutination confirmed with magnifying lens; "± -" = slight agglutination.

TABLE VIII.

Relation between Staphylococci Found in Nose and Throat Swabs and Staphylococci Found in Wounds

Group of Cases	Number of Cases in Which Nose and Throat Swabs were Examined	Number in Which Pathogenic Staphylococci were Found in Nose or Throat	Number in Which Staphylococci from Nose or Throat Resembled Those in Wound.	Number in Which Pathogens in Nose or Throat were of Type VI-VIII-III	Remarks
Group A: Cases in which only penicillin-sensitive strains were isolated from wounds - Table I.	5	3	2	Nil	One of the pathogens from nose and throat agglutinated with type VI serum only. It was penicillin-resistant.
Group B: Cases in which penicillin-sensitive strains followed by penicillin-resistant strains were isolated from wounds - Table II.	8	4	2	2	Both type VI-VIII-III strains were penicillin-resistant.
Group C: Cases in which only penicillin-resistant strains were isolated from wounds - Table III.	27	21	12	9	All nine type VI-VIII-III strains from nose or throat were penicillin-resistant.
Group D: Cases in which a mixture of penicillin-resistant and penicillin-sensitive strains were isolated from wounds - Table IV.	3	2	1	2	Both type VI-VIII-III strains were penicillin-resistant. In one case only (21) type I strains were isolated from wound and type VI-VIII-III from nose and throat.
TOTALS	43	30	17	13	

Only on one occasion were swabs taken from the noses and throats of the medical and nursing staffs of the wards. Routine examination of the staphylococci isolated revealed no penicillin-resistant pathogenic strains. Serological typing was not carried out.

All the sensitive strains closely resembled the standard strain "F.D.A. 209" with regard to their penicillin sensitivity, whilst the penicillin-resistant strains were mostly inhibited by from five to twenty units of penicillin per millilitre. This confirms our previous experience (North and Christie, 1945). Even in those cases (Group B, Table II) in which penicillin-resistant replaced penicillin-sensitive staphylococci during the period of observation, the change was sudden, and strains of intermediate sensitivity were not found. In one case (Case 25), (Table II), already reported by North and Christie (1946), there was a change within eight days from resistance to 0.06 unit of penicillin per millilitre to resistance to 2.5 units per millilitre.

TABLE IX

	Number of Strains
Type I	3
Type II	1
Types VI-VIII-III	2
Type V	2
Type VI	1
Auto-agglutinable	2
Non-agglutinable	2
TOTAL	13

Again, in cases associated with the presence of penicillin-fast staphylococci for a period of five months or more (Tables III and IV), staphylococci isolated from final swabs were not more highly resistant than those isolated at the beginning, despite the fact that the patients had been treated in some instances with large amounts of penicillin in the intervening period.

DISCUSSION

As has already been emphasized, the healing of wounds infected by staphylococci has been found to depend rather on the nature of the wound than on the reaction of the infecting strains to penicillin as determined by in-vitro tests. It might seem from this experience that the term penicillin-resistant staphylococci" was a misnomer when applied to the particular kind of staphylococci under consideration.

It has previously been reported (Kirby, 1944; Spink et alii, 1945; Gots, 1945; North and Christie, 1946) that penicillin-resistant staphylococci produced in vivo or occurring naturally develop a penicillin inactivator or penicillinase. By increasing the size of the inoculum we have been able to obtain growth with such a strain in broth containing as much as 400 units of penicillin per millilitre without changing the characteristics of the organism in any way. When tested later by the usual technique, a subculture from the penicillin broth was found to behave as the parent culture did, both showing inhibition of growth by ten units of penicillin per millilitre.

Anderson (1946) has further shown, by serially diminishing the inoculum, that these "in-vivo resistants" are really little more tolerant to penicillin than the typically penicillin-sensitive strains. They are not in themselves penicillin-fast, but elaborate a penicillinase which destroys penicillin. It has already been pointed out (North and Christie, 1946) that resistant staphylococci developed in vitro are actually tolerant to penicillin without producing a penicillinase

to destroy it.

Laboratory findings, together with the clinical experience of the present series of cases, suggest that if conditions are such that penicillinase-producing staphylococci cannot accumulate and produce an abundance of penicillinase, penicillin, if freely administered, should prove efficacious in treatment. This implies that all the infected area is open to access by penicillin either locally or by the vascular system. There must be free surgical drainage and no necrotic tissue mass which would harbour the accumulation of penicillinase-producing staphylococci. Our findings help to explain the common experience of surgeons, that in the case of staphylococcal infection penicillin therapy can never replace and must be combined with orthodox surgical methods if effective results are to be obtained.

It has been shown, however, that mice injected intraperitoneally with a certainly fatal dose of penicillinase-producing staphylococci were not protected against death by large doses of penicillin, whilst other animals similarly injected with a penicillin-sensitive strain were saved (North and Christie, 1946). In view of this and similar observations it would be unwise to conceive that in no circumstances would the presence of penicillin-fast strains in lesions infected by staphylococci adversely affect the response to penicillin therapy.

At the commencement of observation, in only 20 out of 59 cases were penicillin-sensitive strains isolated from the wounds. In nearly all the remainder penicillin had been administered both parenterally and locally in forward areas and along the lines of communication. This would account for the high proportion of cases in which penicillin-resistant organisms were present at the inception of our observations.

Amongst the cases in group B in which a change from penicillin-sensitive to penicillin-resistant staphylococci was found, cultures

consisted of either definitely penicillin-sensitive or penicillin-resistant organisms. Indefinite sensitivity or resistance to penicillin suggesting an intermediate stage of transition from one state to the other, was never observed. On the laboratory data we have accumulated from the cases shown in Table II, there is little apart from the results of serological typing to suggest that penicillin-sensitive strains produce penicillinase-forming variants (that is, "in-vivo resistants") during penicillin therapy.

Alternative hypotheses to the penicillinase-producing variant proposition include the following:

(i) elimination by intensive penicillin therapy of all but pre-existing penicillin-resistant staphylococci from the wounds;

(ii) contamination of the wounds from self-infection, cross-infection or infection from the surroundings by penicillinase-producing organisms.

There are serious objections to the acceptance of the first of these alternatives. If such penicillinase-producing strains were present from the beginning in any lesion infected by staphylococci, it is reasonable to assume that they would sometimes be isolated from lesions not treated by penicillin. Three hundred and ninety-six strains, of which 281 were coagulase-positive, collected prior to the institution of penicillin therapy, were examined for the production of penicillinase (Anderson, 1946). The only penicillinase producers found amongst them were a few non-pathogens. Further, our experience during the present investigation leads us to believe that penicillin-sensitive and penicillin-resistant staphylococci do not occur together in lesions. For a time we used penicillin blood agar as well as blood agar without penicillin for the isolation of strains. We obtained either the same amount of staphylococcal growth on the penicillin and non-penicillin plates, or growth on the latter with none in the presence of penicillin.

The objections just considered also apply to the possibility that added infection from sources outside the wound may account for the presence of penicillinase-producing staphylococci. There is, however, other evidence against such causes.

Miles et alii (1944) report that 40% to 50% of over 1,000 normal adults were carriers of Staphylococcus aureus in the anterior nares, and 10% to 20% of them carried this organism on the skin of the wrist. They regard both nasal and skin staphylococci as important in wound infection, and express the opinion that opportunities for self-infection by a wounded nasal carrier and cross-infection from carriers among hospital personnel occur frequently, but that it is likely that such a transfer can be prevented by relatively simple means.

The preponderance of type VI-VIII-III strains found by us in both wounds and naso-pharynx of patients of groups B, C and D, is strong evidence of cross-infection. However, there are factors that made this supposition less probable than it would at first sight appear to be.

Strict precautions against cross-infection and self-infection were observed in the wards from which the specimens came. The absence of any penicillin-resistant pathogenic staphylococci in naso-pharyngeal swabs from the staff (although the staffs were tested on only one occasion) suggests that cross-infection from patients to potential carriers was at least rare. It may be assumed that carriage of infection in the opposite direction (staff to patient) was also unusual. In most cases infection with these strains was already present when the patients arrived in hospital. Patients came from different forward areas and passed through different medical establishments en route to the base, and it seems that cross-infection from medical personnel would result in a mixture of serological types in the wounds. The same remarks would apply to possible contaminants from the air and also to self-infection. However, patients harbouring more than one

serological type of staphylococcus in wounds were rare.

Our small series of ten cases (Table I), in which only penicillin-sensitive organisms were isolated from the wounds, includes patients with a fairly representative collection of serological types. The only resistant strain found in the naso-pharyngeal swabs belonged to type VI (Case 20), and the patient had previously had penicillin by the intramuscular route.

Christie and Keogh (1940) found that of 57 strains of pathogenic staphylococci isolated in cases of osteomyelitis, blood infection and furuncles in Melbourne, only six gave major agglutination with any of the type sera III, VI or VIII, whilst of thirty strains from the nose, throat or sputum, six belonged to type III.

Whilst some type I and type II strains were obtained from patients in group B, C and D, the majority of the remainder belonged to type VI-VIII-III. It may be that a miscellaneous group of staphylococci, including many that are difficult to classify serologically, have their antigenic components altered under the influence of penicillin in the living body in such a way that they tend to be agglutinated by type VI and VIII serum.

This hypothesis finds some support in a laboratory animal experiment. An attempt was being made to produce variants, resistant in vivo, in mice which, after subcutaneous infection, received three daily intraperitoneal injections of penicillin. Penicillin-resistant variants did not develop, but there was a serological change from a type II organism to type VI-VIII-III in the later cultures made on succeeding days from aspirated material. A check on the connexion between strains that resemble one another serologically and in other characteristics by the more recent phage-typing technique (Fisk, 1942; Wilson and Atkinson, 1945) should help to elucidate the matter.

As has already been pointed out, there is little to confirm the supposition that the presence of penicillin-fast staphylococci in war wounds is simply due to their being present from the commencement of infection and subsequently being unmasked by the elimination of the penicillin-sensitive organisms by penicillin therapy. The same difficulties and others also discussed make the acceptance of self-infection and cross-infection appear unlikely as a frequent cause. Any satisfactory explanation must take into account the statistically significant different distribution of serological types among the penicillin-sensitive strains from group B patients as compared with the serological types found in strains from those in group A. It is possible that the influence of penicillin therapy in the human body may account for this change in antigenicity.

From what is known of bacterial variation, it seems possible that a few individual bacteria may have the tendency to produce a penicillin-destroying enzyme, and that under the stimulation of penicillin this tendency may become accentuated.[1] Such a phenomenon is well known amongst the Salmonellas and dysentery bacilli, strains of which may be accustomed to ferment some particular carbohydrate, though at first they show no tendency to do so.

One difficulty in accepting any of the possible explanations offered is our inability to isolate intermediate stages between penicillin-sensitive and penicillin-fast strains.

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1 Since this paper was submitted for publication, an article by E. S. Luria has appeared in The Proceedings of the Society for Experimental Biology and Medicine, January, 1946, in which he claims that sensitivity tests are influenced by the size of the inoculum on account of (a) the presence in sensitive strains of a minority of resistant individuals originating by mutation, and (b) the occurrence of penicillinase-producing strains whose cells are individually sensitive to penicillin.

It would seem certain, however, that, whilst penicillinase-producing strains, particularly non-pathogenic strains, sometimes occur independently of penicillin therapy, the bulk of pathogenic penicillinase-forming strains are found only in patients during or after intensive penicillin therapy.

Whilst we are awaiting further evidence, confirmatory or otherwise, it is suggested that the stimulation by penicillin to change potential penicillinase-producing staphylococci into actual penicillinase-producing variants offers the most satisfactory explanation.

SUMMARY AND CONCLUSIONS

1. The bacteriological changes in war wounds infected with Staphylococcus pyogenes and treated with penicillin have been observed in 59 cases for periods up to five months.

2. Individual wounds were usually found to harbour staphylococci biochemically and serologically identical during the period of observation.

3. After prolonged penicillin therapy, the staphylococci recovered from the wound as judged by tests in vitro were almost invariably found to be penicillin-resistant.

4. Such penicillin-resistance was not found to affect the clinical response to penicillin. This depended on the nature of the wound and not on the penicillin-resistance of the infecting staphylococci as judged by tests in vitro.

5. Possible explanations of the phenomena observed are discussed.

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576 . 8 . 077 . 35 : 576 . 851 . 21 (Streptococcus agalactiae)

ON THE EFFECT OF THE INTERACTION OF STAPHYLOCOCCAL β TOXIN AND GROUP-B STREPTOCOCCAL SUBSTANCE ON RED BLOOD CORPUSCLES AND ITS USE AS A TEST FOR THE IDENTIFICATION OF STREPTOCOCCUS AGALACTIAE.

by E. MUNCH-PETERSEN and R. CHRISTIE

From the C.S.I.R., Division of Animal Health and Production, Animal Health Research Laboratory, and the Commonwealth Serum Laboratories, Melbourne.

(PLATES XLIX AND L)

It is now well established that bovine mastitis is most frequently caused by Streptococcus agalactiae, group B Lancefield. Control measures which aim at eradicating this micro-organism from the cow's udder call for its ready detection in milk samples. Short-cut diagnostic methods have been suggested for this purpose: the Hotis test and direct microscopical examination, for example; but these are presumptive tests and not specific for group-B streptococci.

The more accurate method of plating milk samples in blood agar with or without selective inhibiting substances, followed by serological or biochemical testing of the streptococci, is time-consuming. Any test which will detect Str. agalactiae quickly and accurately on blood-agar plates is therefore useful. The test here described was developed from the observation that all strains of Str. agalactiae, whether haemolytic or non-haemolytic, produce an agent which can lyse sheep or ox red cells in the presence of staphylococci

producing β toxin (Christie, Atkihs and Munch-Petersen, 1944). The toxin itself gives rise to a darkened zone around the colonies on the surface of blood-agar plates, and colonies of Str. agalactiae growing close enough will cause lysis within the darkened zone (fig. 1).

In preliminary tests this phenomenon appeared only with 64 strains of Str. agalactiae and not at all with 72 strains of other streptococci. Agreement with these findings has since been recorded in tests with 200 strains of group B and 395 strains of other streptococci (Munch-Petersen, Christie and Simmons, 1945).

It was also observed that when blood-agar plates with colonies of Str. agalactiae were flooded with cell-free staphylococcal β toxin extensive zones of haemolysis developed within two hours at 37°C. This observation was the basis of the tests to be described.

MATERIALS AND METHODS

The Source of Micro-organisms.

A total of 650 milk samples was obtained aseptically from individual quarters of cows, most of which were suffering from acute or subacute mastitis. The bacteriological examination included plating of whole milk and of suitable dilutions in blood agar; usually 0.5 ml. of milk was added direct to a tube with 12 ml. of melted agar containing 5 per cent. ox or sheep blood; milk diluted 1 : 10, 1 : 100 or more in saline was also used to ensure a convenient number of isolated colonies and because undiluted milk often causes a confusing darkening of the medium. The plates were incubated at 37°C. and representative colonies were subcultured into serum broth when they had reached a suitable size - usually at 24 or 48 hours; the streptococci thus isolated were tested serologically.

The Medium.

The basic medium was nutrient agar enriched with sheep or

ox blood, but care was taken to use only blood which contained little or no staphylococcal β antitoxin. Tests for suitability were carried out by preparing plates, sowing on the surface a strain of staphylococcus known to produce β toxin, and incubating at 37°C. overnight. The development of a darkened zone around the staphylococcal colonies indicated that the blood was suitable. Such blood could be used equally well whether defibrinated, oxalated or citrated. If much staphylococcal antitoxin was present the red cells were spun down and washed; they were then used in the plates instead of blood.

The Staphylococcal β Toxin.

Staphylococcus S32a (Bryce and Rountree, 1936), which produces β toxin only, was grown in nutrient broth containing 0.1 per cent. agar and 0.1 per cent. glucose and was incubated at 37°C. for three days in an atmosphere of 20 per cent. oxygen and 80 per cent. carbon dioxide. The medium was then clarified by centrifugation, preserved by the addition of 0.02 per cent. merthiolate and stored at 4°C. Toxin stored for five years was as suitable as that freshly prepared; both the toxins used in the tests caused complete lysis of sheep red cells in a dilution of 1 : 1600. The toxin was used in the plate test either undiluted or diluted with saline; a dilution of 1 : 10 gave weak but distinct results, but only very weak reactions were obtained with a dilution of 1 : 15.

The Plate Test.

The plates were marked to show the position of colonies selected for isolation and a record was made of any haemolysis. One drop of β toxin, usually diluted 1 : 5, was dropped on or near such colonies. The plates were then left at 37°C. and readings were made of induced haemolysis at intervals of 30 minutes for two hours. Plates were not allowed to cool appreciably during these examinations; otherwise "hot-cold" reactions could lead to error. Lysis during this period

within the area covered by the β toxin was taken as proof of the presence of Str. agalactiae. The results were then compared with the microscopical appearance of the organisms on subculture and with their serological reactions.

RESULTS

According to the results of the group precipitin reaction on the streptococci isolated, 365, of the 650 milk samples contained Str. agalactiae. Staphylococcal β toxin applied to the plates which yielded these streptococci produced faint but distinct haemolysis around the colonies after 30 minutes at 37°C. The lysis was quite definite after one hour. Figs. 2, 3 and 4 illustrate some of these results.

All strains of Str. agalactiae produced this lysis, whether they were themselves weakly haemolytic (fig. 2), strongly haemolytic with double zones (fig. 3) or non-haemolytic (fig. 4); the phenomenon was observed even when the streptococci were growing outside the area covered by the staphylococcal β toxin, if the colonies were within 10 mm. of the edge (figs. 3 and 4).

The addition of crystal violet with either sodium azide or thallium nitrate or acetate in concentrations recommended for rendering blood agar more selective for streptococci did not interfere with the test. The presence of other micro-organisms, none of which gave the reaction, did not prevent or interfere with the lysis produced by Str. agalactiae in the area covered by the staphylococcal β toxin.

Only two samples of milk gave plates with colonies of β toxin-producing staphylococci and Str. agalactiae. Fig. 1 shows one of these. Neither micro-organism produced haemolysis alone, but where the streptococci were sufficiently close to the darkened zone surrounding the staphylococci definite zones of lysis were produced.

If the streptococcus colony was inside the darkened zone

caused by the staphylococcal toxin it was surrounded by a zone of clear haemolysis; if it was sufficiently far from the outer edge - more than 10 mm. - no interaction resulted. At intermediate distances clear lysis developed in the intersecting areas of influence, whose shape varied from that of a concavo-convex lens to a segment according to proximity of the colonies. Possible explanations of this are discussed later.

Plates prepared from 285 samples did not contain Str. agalactiae, although there was growth of other streptococci, micrococci, staphylococci and diphtheroid bacilli, either in pure culture or in mixtures. None of these bacteria gave the reaction with staphylococcal β toxin characteristic of group-B streptococci. All these plates were left in the 37°C. incubator overnight after the toxin was applied, and it was then found that two gave a weak reaction. Both contained non-haemolytic viridans streptococci which were not of group B by serological tests. Reactions of this kind (false positives) can be avoided if the final reading is taken not more than two hours after the application of the β toxin. Fig. 5 shows the reaction produced on one of these plates after overnight incubation compared with that on a plate containing a strain of non-haemolytic Str. agalactiae, one hour after the addition of the toxin.

In a small series of tests run in parallel with those just described, the staphylococcal toxin was incorporated in the medium at the time of plating (one or two drops to 12 ml. of agar). In one such test the plate containing toxin clearly showed the position of the group B streptococci after overnight incubation at 37°C., although the colonies themselves were hardly visible to the naked eye; the control plate without the toxin, prepared with the same dilution of milk, gave barely visible colonies even after 48 hours at 37°C. Fig. 6 shows these results. However, this method was discontinued, as it

was necessary to have control plates without toxin in order to determine the presence of micro-organisms, other than Str. agalactiae, which produce wide zones of normal haemolysis. Furthermore, some air-borne organisms will give a lytic reaction similar to that produced by Str. agalactiae (Christie and Graydon, 1941), and it might not always be possible to exclude such contaminants from the samples.

DISCUSSION

A study of figs. 1 and 2 would give the impression that the agent produced by Str. agalactiae does not always lyse in the same manner the darkened area caused by the β toxin on blood agar. This apparent variation calls for some comment.

Consider a colony of β toxin-producing staphylococci which is developing on a blood-agar plate within 10 mm. of a colony of Str. agalactiae. The β toxin is revealed by a darkened zone spreading regularly outwards from the colony; the streptococcal agent also spreads regularly but with no obvious alteration to the medium. Where the two zones overlap the lysed area appears. Now, the nearer the two colonies are the wider the lysed area of interception becomes, which might then be expected to take the shape of a true plane lune; in point of fact the area is a concavo-convex lenticule (fig. 2 - widest lysed area).

When the streptococcal colony is (say) 9 - 10 mm. outside the darkened zone the lysed area is almost a segment.

When two streptococcal colonies grow (say) 5 mm. from the β -producing staphylococcus colony and (say) 5 - 6 mm. from one another, the lysed area may become half-moon-shaped (fig. 1).

Possible explanations of this phenomenon are:

(1) That the streptococcal agent is being used up as it advances

through the darkened zone, perhaps by being adsorbed;

(2) that the red cells (or medium) in the darkened zone resist diffusion, or

(3) a combination of both.

When broth containing a 24-hour Str. agalactiæ culture is boiled to kill the cells and a drop placed on the darkened zone surrounding a staphylococcal colony so that the centre of the drop is on the edge of this zone, lysis will first appear on the edge, and slightly later extend towards the colony. As incubation proceeds, the darkened zone advances through the remainder of the drop and becomes lysed, but not to the same extent as the edge. Possibly the red cells just undergoing modification by the β toxin are lysed more quickly than those already modified for some time. Why the β toxin diffusing through medium with red cells already acted on by the streptococcal agent should not also lyse them completely is not clear. It is paralleled, however, by test-tube experiments with washed red cells, β toxin and streptococcal agent.

In a preliminary series of experiments it was found that the addition to blood agar of cholesterol or washed lysed red cells produced by freezing and thawing did not interfere with or modify the reactions described.

SUMMARY

A test is described for identifying colonies of Streptococcus agalactiæ on agar plates containing sheep or ox blood by the addition of staphylococcal β toxin. In less than two hours at 37°C. such colonies will lyse the red cells to a distance of 10 mm. within the area covered by the toxin.

ACKNOWLEDGEMENTS

Thanks are due to Drs. L. B. Bull and A. W. Turner for helpful criticism and advice, to Miss M. Monsbourgh for the serological typing of the streptococci, to Mr. H. A. Beddome for technical assistance, and to Mr. N. E. Southern for the photographs.

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J PATH. BACT.—VOL. LIX PLATE XLIX

STR. AGALACTIÆ

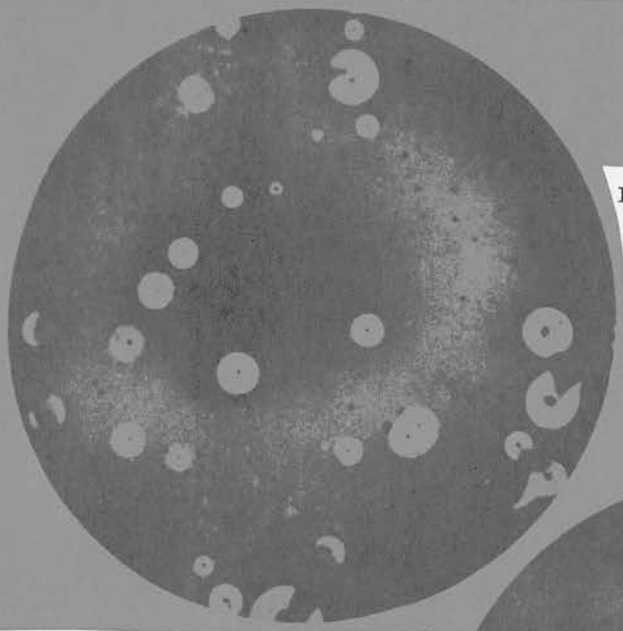

FIG. 1.—Blood-agar plate containing colonies of β toxin-producing staphylococci and non-hæmolytic *Str. agalactiæ* after incubation for 48 hours at 37° C. The darkened areas surrounding the staphylococci have been wholly or partly lysed where *Str. agalactiæ* grew sufficiently close. A probable explanation of the crescentic areas of lysis is given in the text. ×1.

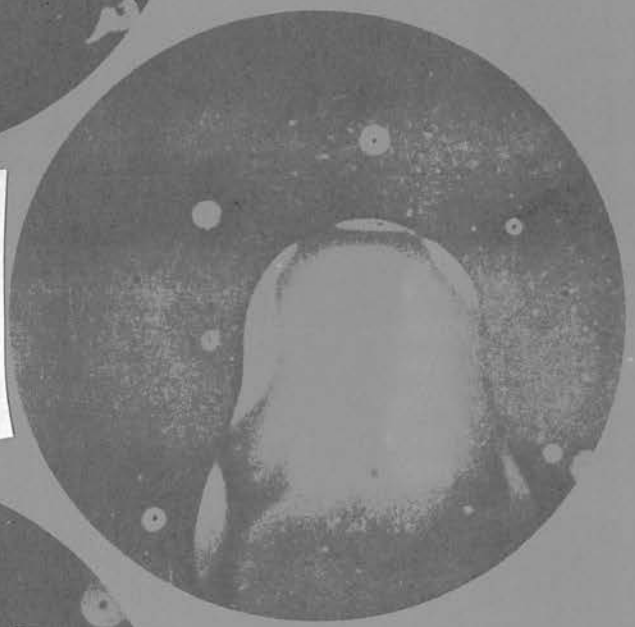

FIG. 2.—Crystal-violet blood-agar plate containing slightly hæmolytic colonies of *Str. agalactiæ*, some within and five clearly visible beyond the area covered by the staphylococcal β toxin. The margin of this area is clearly defined; lysis within it has occurred up to 10 mm. from each streptococcal colony. The plate was incubated at 37° C. for 48 hours and the photograph taken one hour after the application of the toxin. ×1.

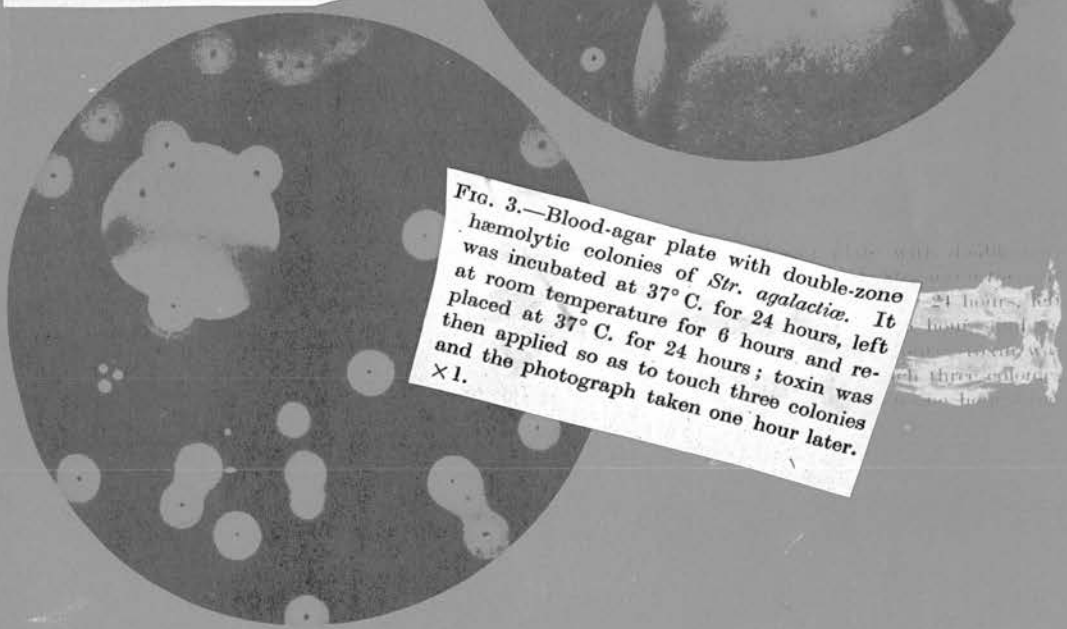

FIG. 3.—Blood-agar plate with double-zone hæmolytic colonies of *Str. agalactiæ*. It was incubated at 37° C. for 24 hours, left at room temperature for 6 hours and replaced at 37° C. for 24 hours; toxin was then applied so as to touch three colonies and the photograph taken one hour later. ×1.

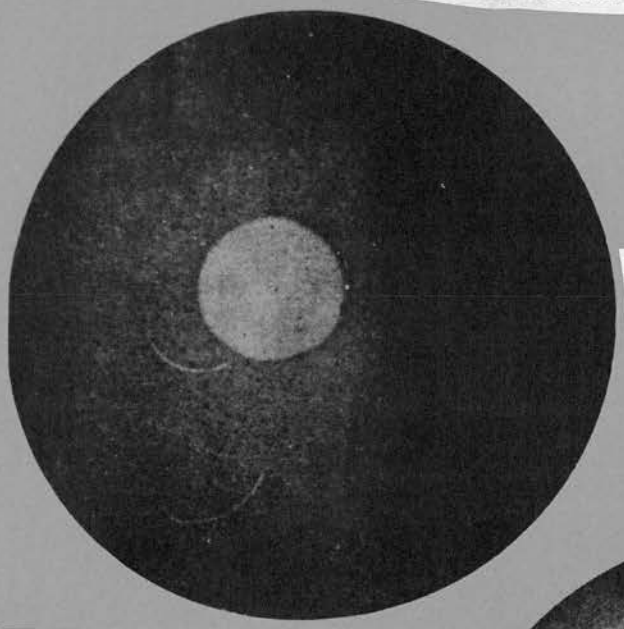

FIG. 4.—Blood-agar plate with non-hæmolytic colonies of *Str. agalactiæ*. It was incubated at 37° C. for 36 hours; one drop of toxin was added and the photograph taken one hour later. ×1.

FIG. 5.—Composite figure of two blood-agar plates. The right half is of a plate containing non-hæmolytic *viridans* colonies of non-group-B streptococci which had been incubated at 37° C. for 48 hours. One half (the upper) was then flooded with staphylococcal β toxin, the plate left at 37° C. overnight and the photograph taken. Some of the colonies in the area containing toxin simulate slightly hæmolytic *Str. agalactiæ* colonies. The left half is of a plate containing non-hæmolytic colonies of group-B streptococci which had been incubated at 37° C. for 48 hours. One half (the upper) was then flooded with toxin and the photograph taken one hour later. ×1.

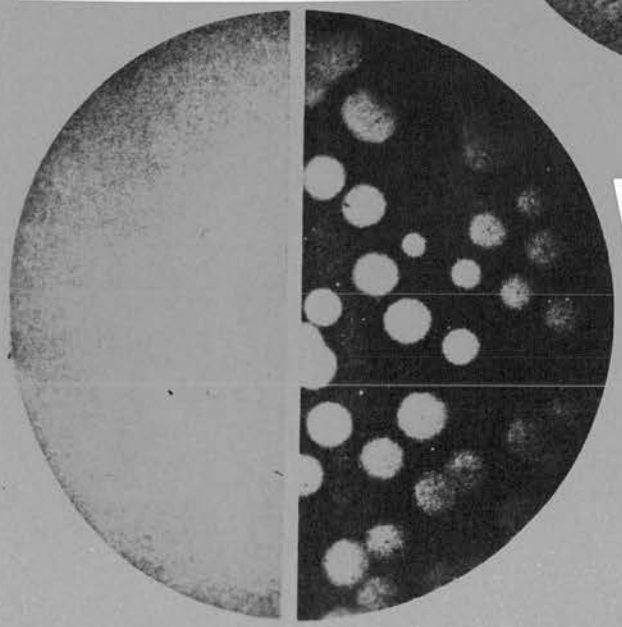

FIG. 6.—Composite figure of two blood-agar plates; the right half is of a plate to which one drop of staphylococcal β toxin had been added at the time of pouring. The photograph was taken after incubation at 37° C. overnight. The positions of the *Str. agalactiæ* colonies are clearly indicated by the presence of the lysed areas, although they are not themselves visible. The left half is of a control plate without the toxin; incubation was for 48 hours at 37° C. but colonies are not yet visible. ×1.

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TREATMENT OF THE NASAL CARRIER OF STAPHYLOCOCCUS AUREUS WITH FRAMYCETIN AND OTHER ANTIBACTERIALS

BRYAN STRATFORD M. B. Melb.

(Deputy Medical Superintendent, St. Vincent's Hospital, Melbourne.)

SYDNEY D. RUBBO M.D. Melb., Ph.D. Lond., Dip.Bact.

(Professor of Bacteriology in the University of Melbourne.)

R. CHRISTIE M.Sc. Melb.

(Bacteriologist, St. Vincent's Hospital, Melbourne).

SHIRLEY DIXSON

(Research Officer, School of Bacteriology, University of Melbourne).

There is general agreement that the healthy nasal carrier of Staphylococcus aureus constitutes one important reservoir of infection. Since many different phage-types are responsible for endemic staphylococcal disease in hospitals, it is impossible to distinguish between benign and dangerous carriers (Williams 1959). Accordingly, one is forced to regard

all carriers as potentially dangerous (Elek 1959). When an outbreak of staphylococcal infection is traceable to a particular phage-type - e.g., 80/81 - the detection of the dangerous disperser is greatly simplified; but this ideal epidemiological situation is seldom found in endemic hospital cross-infections affecting adults.

A reduction in the number of nasal carriers of staphylococci cannot be brought about by the use of antibacterial nasal toilet alone, as the nasal mucosae are continually exposed to reinfection by airborne organisms. The more widespread the environmental contamination, the higher the nasal carrier-rates among staff and patients. If, however, the endemic balance between primary and secondary sources of infection could be reduced, then the chances of acquiring clinical infection should be lower - assuming, of course, that there was no change in virulence of the organism and no lowering of resistance of the patient.

We are here concerned with the treatment of the nasal carrier. We have compared a number of recommended preparations - firstly as regards the rate of suppression of nasal carriage, and secondly, the success-rate after seven days of treatment. Recolonisation of the cleared carriers was sought, but this is as much an index of environmental exposure as a measure of the efficacy of nasal toilet.

Of the two methods of applying these preparations, we have found the aqueous spray generally more effective than the corresponding ointment (or cream). Doubtless the ointment remains longer in contact with the mucosal surface, but it seldom reaches sites of possible colonisation that are beyond the vestibule of the nose.

We have always used a "deep nasal swab" for detection of nasal carriers in preference to the commonly used "shallow swab" of the anterior nares. In a survey of 103 patients, deep swabbing yielded a higher proportion of positive carriers. As the success of any preparation depends on contact with the infecting organism, deeply and liberally applied sprays

must carry a greater chance of success than a cream applied to the anterior nares.

MATERIALS AND METHODS

Nasal Swabbing.

To have a sound basis for selecting nasal carriers, two types of swabs were taken in a group of 103 patients at the one visit. The first swab was made from the vestibule of each nostril, that is, an anterior nasal swab; and the other from the vestibule to a point just below the middle turbinate, that is, a deep swab (fig. 1). In each case the swab was rotated through three full turns during the operation of swabbing. The deep-swab technique should be done by a person familiar with the internal anatomy of the nasal cavity. All swabs were plated on blood-agar media, incubated for twenty-four to forty-eight hours at 37°C. Staph. aureus was identified on colony form, and on pigment and coagulase production.

TABLE I.

Comparison of Anterior and Deep Nasal Swabbing of 103 Patients in Hospital

Type of swabbing	Nasal carriers detected	
	Number	Carrier-rate
Anterior	35	34%
Deep (see text)	53	51%

Nasal Preparations.

The products used were:

(a) 'Neotracin' ointment containing neomycin 5 mg., polymyxin

5000 units, and bacitracin 500 units per g.

(b) 'Neomyxin' spray containing neomycin 2 mg. and polymyxin 5000 units per ml.

(c) 'Naseptin' ointment containing chlorhexidine hydrochloride ('Hibitane') 0.1% and neomycin 0.5%.

(d) Chlorhexidine-neomycin spray in saline containing the same concentration of active ingredients as naseptin.

(e) 'Soframycin' ointment containing framycetin 1.5% and gramicidin 0.005%.

(f) 'Soframycin' spray containing framycetin 1.25% and gramicidin 0.005% in isotonic solution containing phenylephrine hydrochloride 0.25% as a vasoconstrictor.

(g) Soframycin spray as (f) but without the vasoconstrictor.

The ointments were applied with a swab to the anterior nares. The sprays were instilled into the nasal cavity, under pressure, from plastic containers in such quantity that some of the liquid passed to the back of the patient's throat. All patients were treated individually by one or other of us. Each preparation was tested in 20 patients, each of whom was shown to have more than twenty colonies of Staph. aureus on plating of a deep swab. On every occasion, the patient was swabbed immediately before treatment, two hours after the first application, and afterwards daily for seven days. Medication then ceased, but daily swabbing was continued for at least twenty-one days, or for a shorter period if the patient was discharged from the hospital.

RESULTS

A significant difference in the number of detectable nasal carriers can be shown after shallow (anterior nasal) and deep (anterior and middle nasal) swabbing. Table I shows the results obtained on 103

patients, and emphasises the importance of taking a deep nasal swab.

Table II summarises the results of treatment of groups of nasal carriers with the different preparations.

In this table we have listed the results obtained twenty-four hours, and seven days respectively after the beginning of treatment. The soframycin spray was outstandingly more successful after twenty-four hours than any of the other preparations. The inclusion of a vasoconstrictor in this spray appeared to improve its antibacterial action over a period of seven days. The fluted nebuliser in which the soframycin spray is dispensed may contribute to its success. About 2 ml. of fluid is ejected, first as a coarse spray and then as a fine jet. On the figures recorded here, one application of soframycin spray suppressed nasal staphylococci in 85% of nasal carriers. In fact, approximately 80% of carriers are cleared within two hours of the first application of the spray. No other product in this series was so effective. Daily treatment for seven days with soframycin spray cleared all carriers in our series. Of the other preparations, chlorhexidine-neomycin spray appeared to be the next most efficacious, but was considerably less so than soframycin.

The remarkably rapid rate of clearance induced by soframycin in this series was investigated further using 85 nasal carriers, who were treated twice with the spray at twenty-four hour intervals. The results of this short treatment are shown in fig. 2.

It will be seen that 76% of the carriers were cleared within two hours after one application of the spray, and all were free of staphylococci after the second application. The antibiogram patterns of a number of staphylococci were done, and most of the strains were resistant to penicillin, streptomycin, and the tetracyclines; in other words, they were typical of the "hospital staphylococcus".

Reinfection of the nasal mucosae after clearance occurred with all preparations. Table III shows that approximately 50% of the

TABLE II.

Rate of Clearance of Staph. Aureus by Various Preparations

Preparation	Frequency of application	Number Treated	Number cleared (negative swab) after:	
			24 hours	7 days
Soframycin spray with vasoconstrictor	once daily	20	17(85%)	20(100%)
Soframycin spray without vasoconstrictor	once daily	20	17(85%)	17(85%)
Soframycin ointment	twice daily	20	5(25%)	18(90%)
Chlorhexidine-neomycin spray	once daily	20	5(25%)	15(75%)
Naseptin ointment (chlorhexidine-neomycin)	twice daily	20	1(5%)	9(45%)
Neomyxin spray	once daily	20	7(35%)	10(50%)
Neotracin ointment	thrice daily	20	4(20%)	13(65%)

cleared carriers reverted to the carrier state after cessation of treatment, but the time taken to revert after soframycin was clearly different from that found with the other preparations. With the latter, the staphylococcus free state lasted, on an average, two days, whereas after soframycin treatment an average of seven to fourteen days elapsed before reversion. Since all patients were exposed to the same degree of airborne infection, it would appear that the soframycin-treated group were reinfected from exogenous sources, whereas the reversion in other carriers was mainly endogenous. Elimination in the soframycin-treated group was bacteriologically complete, but incomplete with the other preparations. Unfortunately, phage-typing of isolates was not available to confirm this interpretation, which, if correct, provides a further reason for recommending soframycin spray for treatment of the nasal carrier.

DISCUSSION

A vast literature dealing with the nasal carriage of Staph. aureus has appeared during the past decade.

Some authors, e.g., Gould and Cruikshank (1957), Williams (1959) have suggested that acquired staphylococcal infections - as many as 80% of them according to Gould (1957) - might result from autoinfection. Williams et al. (1959) proved that "patients who were or became nasal carriers of staphylococci had three times as much staphylococcal sepsis as those who were never carriers". Further, it appeared "that self-infection is important in the aetiology of the septic lesions". In a study of postoperative surgical infections in tuberculous patients subjected to elective chest surgery, Weinstein (1959) reported that, of patients with positive nose-cultures for Staph. aureus, 37% exhibited postoperative infected complications, whereas among the group with negative nose cultures, 11% had infected complications.

These findings suggest that the suppression of nasal carriage would be a useful measure before elective surgery, particularly in patients

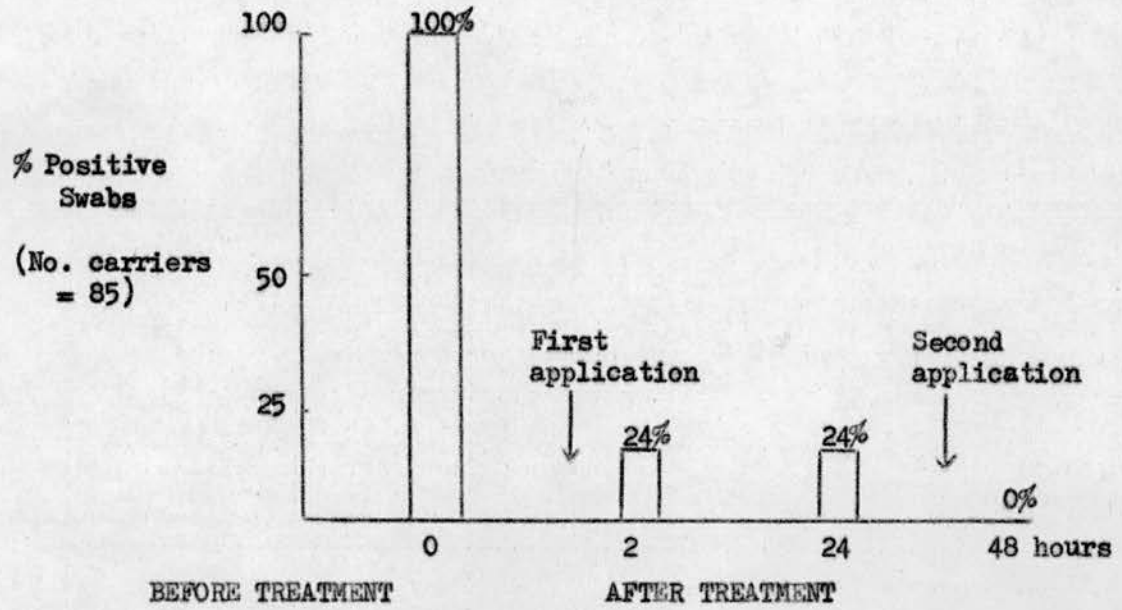

Fig. 2

Suppression of Nasal Carriage of Staph-aureus by Treatment with Soframycin Nasal Spray

who are admitted to hospital some time before operation. Recent work by Gillespie et al. (1959) indicates, however, that the suppression of nasal carriage will not necessarily reduce the overall incidence of cross-infection in general hospitals. Environmental contamination can arise from primary reservoirs other than the nose - for example, the perineum (Hare and Ridley 1958), skin and clean infected wounds (Gillespie et al. 1959), all of which maintain the staphylococcal infection cycle. Silent nasal colonisation and the hazard of autoinfection inevitably follow. Nevertheless, any plan for effective reduction of staphylococcal disease in hospitals must be broadly based, and the suppression of nasal carriers by topical chemotherapy is only one of a number of measures which must be considered in the epidemiological control of this disease.

The present study was designed to provide information on an administratively simple and clinically effective treatment of nasal carriers in hospital. Thus, in comparing various preparations, it is only fair to

TABLE III.

Recolonisation of Nasal Mucosae with Staph. Aureus After Successful Treatment

Preparation	Number of Patients	Number recolonised	Average time of recolonisation after cessation of treatment (days)
Soframycin spray with vasoconstrictor	20	10(50%)	14
Soframycin ointment	18	10(56%)	7
Chlorhexidine-neomycin spray	15	8(55%)	2
Naseptin ointment (chlorhexidine-neomycin	9	4(44%)	2
Neomyxin spray	10	5(50%)	2

state that we intentionally did not follow the manufacturer's directions if these were considered too complex for practical use. We were mainly concerned with discovering some preparation which could suppress nasal carriage in the shortest possible time. The test for suppression in this study was more rigorous than in most, since all observations were based on the deep swabbing technique (fig. 1). This method detected 17% more nasal carriers than swabbing the anterior nares (Table I).

Soframycin spray was outstandingly the most successful preparation (Table II). When a larger number of nasal carriers was treated, it was found that all carriers yielded negative cultures in

forty-eight hours - that is, after two applications of the spray (fig. 2). Furthermore, it appeared that clearance of nasal staphylococci by soframycin was more complete than by other methods of treatment, since recolonisation of the mucosae was significantly slower (Table III). The success of soframycin spray is attributable largely to its bactericidal action. Experiments, not reported here, showed that soframycin killed Staph. aureus, resistant to many antibiotics, in less than a minute in the presence of 50% serum.

This work is now being extended to determine whether intermittent use of soframycin spray will decrease the nasal-carriage rate, and thus indirectly lower the environmental contamination. We hope that this proposed extension of the present study will provide a basis for control of the nasal carrier which does not impose too many demands on the nurses.

SUMMARY

The treatment of the nasal carrier of Staphylococcus aureus by chemotherapeutic agents was investigated. The emphasis in this study was on the rate of clearance as well as the success after seven days' treatment.

The results indicate that, in general, atomised spray preparations are more effective than the corresponding ointments.

Of the preparations compared, 'Soframycin' spray, containing framycetin and gramicidin as antibacterial agents, was the most effective. With this spray, approximately 80% of nasal carriers were cleared in twenty-four hours (that is, by one application only), and 100% after two days' treatment.

The importance of deep nasal swabbing for detecting nasal carriers has been demonstrated.

ACKNOWLEDGEMENTS

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EXPERIMENTAL PERTUSSIS IN THE MONKEY
(MACACA MULATTA)

by

E. A. NORTH, E. V. KEOGH, R. CHRISTIE and G. ANDERSON

(From the Commonwealth Serum Laboratories, Melbourne).

(Submitted for publication 24th April, 1940.)

Reports of different workers are at variance concerning the ease with which the monkey may be infected with H. pertussis. Sauer and Hambrecht (1929) infected two ringtail monkeys intratracheally and one macaque intranasally, but the strength of the infecting doses is not mentioned. Sprunt et al. (1938) quote the same workers as having infected five cebus monkeys and three macaques by the intratracheal or intranasal routes. They give no details of dosage or of the criteria of infection.

Culotta, Harvey and Gordon (1935) used a dose of 2 to 5 c.c. of the 48 - 72-hour growth from three Bordet-Gengou plates (15 cm. diam.) suspended in 10 c.c. saline. By the intratracheal route they infected two of two cebus and one of sixteen rhesus monkeys. By the intranasal route one of two cebus and none of five macaques were infected. They concluded that the latter species of monkey is refractory to infection with H. pertussis.

Sprunt, Martin and McDearman (1938) attempted to infect erythrocebus monkeys intratracheally. They used enormous doses, the 48-hour growth of from two to twenty-four Bordet-Gengou slopes, but were successful in only two of nine attempts. One of their successes was an animal given initially the growth from twelve slopes and ten

days later the same dose. When killed ten days later, twenty-five colonies were obtained on a plate inoculated from the lungs. They state "That the Bordet-Gengou bacilli failed except in two instances to multiply in the lungs of the monkeys whereas they are found in large numbers in human pertussis was attributed to the higher susceptibility of man".

Using much smaller doses than these authors, it has been found easy to infect the rhesus monkey by the intranasal and intratracheal routes. Since practically all workers have used the intratracheal route almost exclusively, while in our hands the intranasal method of inoculation has proved uniformly successful, these results seem worth recording.

METHODS

1. Preparation of bacterial suspensions. The growth from a 24-hour culture of Phase I H. pertussis on a Bordet-Gengou slope was emulsified in nutrient broth and the bacterial content estimated by standard opacity tubes. The requisite dilutions were also made in nutrient broth, the final strength of the suspensions being 50,000,000 organisms per c.c. For intratracheal inoculations, the dose was 1 c.c. of this suspension. Intranasally, 2 c.c. were used.

2. Method of inoculation. The monkeys were anaesthetized with open aether. For the intratracheal inoculation the neck was extended and the needle inserted into the lumen of the trachea in the mid-line. The skin was not incised. For intranasal inoculation, the bacterial suspension was dropped into the nostrils from a Pasteur pipette.

3. Autopsy. Before sacrificing with chloroform, blood was withdrawn by heart puncture under aether anaesthesia. The thorax was opened aseptically, and the lungs and air-passages as high as

the larynx removed. A small piece of lung tissue from each lobe was excised and the cut surface rubbed over a Bordet-Gengou plate. The trachea was divided transversely at three points and Bordet-Gengou plates inoculated from the exudate in the lumen. The root of the lungs was similarly dealt with. Specimens were selected for histological examination. In the series of monkeys infected intranasally, the autopsy included dissection and culture of the mucous membrane of the accessory nasal sinuses.

RESULTS OF INTRATRACHEAL INOCULATION

In a preliminary trial two monkeys were inoculated. Neither developed any obvious symptoms of infection. One was killed on the seventh day after inoculation. The cut surfaces of the lungs exuded fluid, but there were no other gross macroscopic changes. A confluent growth of H. pertussis was obtained on plates inoculated from all lobes of the lungs, and from the tracheal exudate. The other animal was killed on the fourteenth day. The lungs, trachea, and bronchi appeared normal, and H. Pertussis was not isolated.

A larger experiment was then undertaken to ascertain what proportion of animals could be infected by this method and to determine the progress of the infection. Twelve monkeys were inoculated intratracheally. One was killed immediately after inoculation to determine whether sufficient organisms had reached the lower respiratory tract to be demonstrable on plating. H. Pertussis was not recovered. Bordet-Gengou plates inoculated from various sites in the lungs contained numerous other microorganisms, presumably washed down from the site of inoculation at the upper portion of the trachea. Two animals were sacrificed twenty-four hours later with similar findings. The lungs were congested. There was an abundant growth of a mixed flora, similar to that of saliva, but H. pertussis was not isolated. Two more animals were killed on the fourth day. The lungs of both animals were congested. Platings of the lungs were sterile in one case; in the other a few colonies of organisms

regarded as contaminants from the upper respiratory tract were found. H. pertussis was not isolated. Another pair were killed on the sixth day; the lungs appeared normal and were sterile. Another pair were killed on the eighth day. The lungs were congested, there was a sticky exudate in the trachea and large bronchi, and H. pertussis was isolated, though not in large numbers, in cultures of the tracheal exudate and right lung in one animal, and in more profuse culture from the trachea and both lungs of the other. A final pair were killed on the eleventh day after inoculation. The lungs of both were pale and collapsed. There was abundant exudate in the trachea. A confluent growth of H. pertussis in pure culture was obtained from the tracheal secretion and from both lungs. These findings are summarized in Table I.

TABLE 1.

Pathological Appearances and Bacteriological Findings in Lungs of Monkey with Experimental Pertussis.

(a) Following Intratracheal Inoculation.

Time of Autopsy	*Lung Congestion	† H. pertussis	ƒ Other Organisms
Immediately after Inoculation	-	-	+++
1st day	+,+	-,-	+++,+++
4th "	+,+	-,-	+,-
6th "	-,-	-,-	-,-
7th "	+	++++	-
8th "	+,+	+,+++	-,-
11th "	-,-	++++,++++	-,-

(b) Following Intranasal Infection.

3rd day	+,+	++++,++	-,-
7th "	+,+,-	++++,+++,-	-,-,-
10th "	+	+++	-
13th "	+,+	-,+	-,-
17th "	-,+	+++,+++	-,-
21st "	±	-	-

* + = congestion.
± = slight congestion.
- = normal appearance.

† ++++ = confluent growth on Bordet-Gengou plates.
+++ = semi-confluent growth.
++ = numerous discrete colonies.
+ = occasional discrete colonies.
- = no growth.

The findings in the duplicate animals of this series were consistent and enable a plausible reconstruction of the course of events. The injection, under anaesthesia, of 1 c.c. of fluid into the monkey's trachea at the level of the cricoid cartilage, results in the washing into the lungs of material from the upper respiratory tract (possibly gaining access thereto from the pharynx during anaesthesia) which contains numerous bacteria. The lung responds to this insult, and inflammatory changes ensue. During this period, from the first to the fourth day following inoculation, H. pertussis cannot be isolated, but other organisms are present throughout the lungs, decreasing in numbers later. The lungs become normal in appearance, and the cultures become sterile on about the sixth day. The inflammatory changes re-appear, and H. pertussis is recoverable in pure culture, in greater numbers about the eleventh day.

RESULTS OF INTRANASAL INOCULATION

A trial animal was inoculated intranasally and killed seven days later. H. pertussis in practically pure culture grew abundantly from all parts of the respiratory tract from the trachea downwards. Ten monkeys were therefore inoculated intranasally and killed at intervals of three or four days from the third day to the twenty-first day. The details are shown in Table 1. H. pertussis was recovered in almost pure culture from all but three animals killed on the seventh, thirteenth and twenty-first days, respectively. This method therefore certainly infected eight of eleven monkeys. It is possible that the monkeys from which H. pertussis was not recovered on the thirteenth and twenty-first days might have been infected at an earlier stage.

In this series, culture was also attempted from the maxillary antra and nasal mucosa. H. pertussis was recovered from the nose of only one monkey killed on the seventh day, and was never isolated from the antra. The findings did not support the possibility that H. pertussis first establishes itself in the upper air passages and that the bronchial

infection develops later.

PATHOLOGICAL CHANGES IN LUNGS

No gross macroscopic changes were found in the lungs of any of the animals. Some appeared more deeply pink than others, but the depth of colour did not bear any relation to the degree of histological abnormality. Regarding the histological changes, the sequence of events was similar in the intratracheally and intranasally inoculated monkeys with one exception, namely, in the bronchi.

In both series, on the third and fourth days after infection a fibrinous and cellular infiltration, both alveolar and interstitial, was seen. At the end of the first week vascular congestion was prominent, whilst the cellular infiltration tended to become mostly mononuclear. The congestion was not seen in monkeys killed from the tenth day onwards, and the infiltration was then mainly interstitial. A patchy interstitial pneumonia was still evident after three weeks.

In one monkey only (killed at thirteen days) was neither histological nor bacteriological evidence of infection found.

The bronchial and bronchiolar epithelium in the intratracheally infected monkeys showed polymorphonuclear and mononuclear infiltration from the third day onwards, whilst this change was not detected in any instance in those infected intranasally. In both series the lumina of some bronchioles were filled with cells and debris, apparently deriving in part at least from desquamated bronchial epithelium.

LACK OF AGGLUTININS AND PROTECTIVE ANTIBODY

The sera of monkeys killed at the times after inoculation shown in Table 1 were tested for agglutinins, which were not detected except in one monkey killed seven days after intranasal inoculation,

from whose lungs H. pertussis was not isolated. This serum agglutinated H. pertussis (Phase I) fully at 1:5 and slightly at 1:20. The sera of two monkeys, which were inoculated intratracheally, were tested at intervals. Both were negative until three months after inoculation, when both weakly agglutinated a Phase I suspension (partially 1:5 and a trace to 1:40).

The sera were tested for protective antibody in mice by the methods described by North, Keogh, Anderson and Williams (1939). No protective antibody was detected in any sample.

DISCUSSION

In contrast to previous workers, we have found it easy to infect the rhesus monkey with H. pertussis by the intranasal and intratracheal routes. In these experiments, infection was proved by recovery of the specific organism from the lungs of the animals. Had the criteria of infection consisted of the occurrence of spasmodic cough and the recovery of H. pertussis from the sputum, we would have had to report the same lack of success as previous workers, for none of our animals developed a cough, and consequently sputum was not available for culture in this series. H. pertussis was not, however, recovered from the mucous epithelium of the nose or accessory sinuses. Culotta et al. noted that H. pertussis was recoverable only from the sputa of animals with a spasmodic cough. Pertussis in the rhesus monkey is evidently usually a very mild infection, and only an occasional animal develops spasmodic cough. The association of the lack of symptoms with failure of development of agglutinins or protective antibody suggests that the rhesus monkey is capable of dealing with pertussis infection by a local cellular response in the respiratory organs. It does not seem to be a suitable animal for use in the study of experimental pertussis.

SUMMARY

Rhesus monkeys may be readily infected with H. pertussis

by either the intranasal or intratracheal routes. The resultant infection is mild, and spasmodic cough was not noted. Agglutinins and protective antibodies could not be detected in the sera of infected animals.

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PENICILLIN SENSITIVITY OF STREPTOCOCCI MOSTLY OF GROUPS A, B, C AND G

By R. T. SIMMONS and R. CHRISTIE

(From the Commonwealth Serum Laboratories, Melbourne).

The sensitivity of haemolytic streptococci to penicillin was first demonstrated by Fleming (1929), who tested the sensitivity of various organisms by the "gutter-plate" technique.

Tests for penicillin sensitivity of Streptococcus pyyogenes (three strains) were made by Abraham et alii (1941) by the tube technique, and the strains were found to be sensitive. Hobby et alii (1942) reported that haemolytic streptococci amongst other organisms were sensitive to penicillin, but no details as to the number of strains tested or the Lancefield groups were given.

McKee et alii (1944) tested various organisms for penicillin sensitivity, and among these were five strains of Streptococcus pyogenes which were found to be sensitive.

Dawson et alii (1944) reported having tested 50 strains of non-haemolytic streptococci isolated from cases of subacute bacterial endocarditis; 10 of these strains were found to fall into Lancefield's groups B, C, D, F and H, and all except one strain were found to be approximately as sensitive as a standard streptococcus used for

comparison. The tests were made by a tube technique.

Watson (1944) stated that in general the effectiveness of penicillin in the treatment of infections in vivo is closely paralleled by its activity against the same infectious agents in vitro. He tested the penicillin sensitivity of approximately 50 strains of streptococci of Lancefield groups A to N, isolated from human and other sources, by the tube dilution method described by Rammelkamp (1942), and found that there was little variation in sensitivity among the strains within any single group, and that the strains of all groups tested were penicillin-sensitive except those of group D. Strains of groups B, E, F, K and N were slightly less sensitive than those of groups A, C, G, H, L and M. The series included only 13 strains of group A streptococci and a few strains of groups B, C and G from human sources.

Rank (1944) has found that haemolytic streptococci are the most rapidly eliminated of all organisms from wounds during penicillin treatment, and that they usually disappear in two or three days.

Various modifications of technique by means of tubes or plate tests have been suggested for determining the degree of sensitivity to penicillin of different organisms, but no large collection of β-haemolytic streptococci isolated from human sources appears to have been tested to date.

The general opinion on streptococcus sensitivity to penicillin, as shown in the literature, is that strains of groups A, C and G are sensitive, strains of group B slightly less so and strains of group D relatively resistant. The purpose of the work reported in this paper was to examine the reliability of this generalization when a large number of streptococci was tested. Tests have been carried out on 721 strains of streptococci, mostly of groups A, B, C and G. The majority of these strains had been isolated prior to the advent of penicillin treatment, but a few isolated during penicillin treatment

were included in the series. One hundred and thirty-five of these strains were from sources other than human.

The technique employed for testing the penicillin sensitivity of the cultures in this series was similar to that used by Dr. E. A. North of these laboratories in tests on the penicillin sensitivity of staphylococci. Various concentrations of penicillin were incorporated in horse-blood agar plates, and the plates, after being cooled and dried, were inoculated with broth cultures of the streptococci to be tested. The results were read after incubation overnight at 37°C.

This technique[1] is simpler than either the "gutter-plate" or the tube method. It does not necessarily give results accurately comparable with either of these two methods but it was considered suitable, since we were more concerned with variations in sensitivity within the groups than with the obtaining of absolute figures for penicillin sensitivity. It was soon found that when streptococci had been grouped by the precipitation test their sensitivity could be forecast with a fair degree of accuracy, and that only a limited range of tests was necessary to serve as a check.

MATERIALS AND METHODS

Strains Tested.

The following β-haemolytic streptococci of human origin were tested:

Group A: 288 strains, representative of the 26 serological types of Griffith (1934) and the 12 Australian types, described by Rudd, White and Ward (1939) and by Keogh, Simmons and Wilson (1941).

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1 Since preparing this paper for publication we have noticed that Cowan (1945) has reported using a similar technique for testing the penicillin sensitivity of staphylococci.

Group B: 102 strains.

Group C: 118 strains.

Group G: 72 strains.

The physiological characters and serological types of the B, C and G streptococci were described by Simmons and Keogh (1940).

The following miscellaneous strains were tested:

Group B: 120 strains of haemolytic and non-haemolytic streptococci (Streptococcus agalactioe) isolated from bovine sources. These strains included the serological types isolated by Stableforth (1937) and five serological types isolated by Mr. D. Murnane from Victorian dairy herds (about 1940). A group of recently isolated strains before and after penicillin treatment were kindly supplied to us by Mr. D. Murnane and Mr. E. Munch-Petersen, of the Animal Health Research Laboratory, Council for Scientific and Industrial Research, Melbourne.

Group C: 12 strains of equine streptococci representative of the serological types described by Bazeley and Battle (1940).

Group D: Five strains.

Group E: Two strains.

Group F: One strain.

Group H: One strain.

The Penicillin Used for Testing.

The calcium salt of penicillin II was used throughout the survey. Penicillin II is the type of penicillin in general use for treatment today. The standard samples were kindly supplied to us by Mrs. S. E. R. Baldwin, of the Penicillin Assay Department of these Laboratories.

Representative strains of the various groups of streptococci A to H were also tested on blood-agar plates containing varying amounts of penicillin I and also penicillin III; the results obtained did not differ from those found with penicillin II.

Preparation of 6% Horse-Blood Agar Plates Containing Penicillin.

As an example details are given for the preparation of blood-agar plates containing one-twentieth of a unit of penicillin per millilitre.

Nutrient agar in quantities of 150 millilitres in flasks was melted and cooled at 46°C. To nine millilitres of sterile citrated horse blood in test tubes measuring six inches by three-quarters of an inch was added 0.8 millilitre of a freshly-prepared buffered-saline solution containing 10.0 units of penicillin per millilitre. The blood and penicillin were thoroughly mixed, warmed to 46°C. and added to the agar, the tube being then washed out with some of the mixture. The contents of the flask were thoroughly mixed and poured into four-inch Petri dishes (approximately 15 millilitres per dish).

Control Blood Agar Plates.

Blood agar plates without penicillin were prepared as described above and used throughout the tests in parallel with the penicillin plates to control both growth and purity of the cultures tested.

Control Cultures of Streptococci.

Throughout the entire survey, with each lot of cultures tested, we included three control strains. These were as follows:

(i) a known penicillin-sensitive strain of streptococcus group A (Rammelkamp, 1942); this strain was obtained by Major P. L. Bazeley of these laboratories from Dr. C. H. Rammelkamp;

(ii) a locally-isolated, penicillin-resistant β-haemolytic streptococcus,

not of groups A, B, C or G;

(iii) an α-haemolytic streptococcus which was partially resistant to penicillin. The second and third strains were amongst cultures kindly supplied to us by Major John Perry.

Inoculation of Plates.

The blood-agar plates were dried in the incubator at 37°C. for one hour. Four cultures were tested on each plate in areas marked off with grease pencil. The inoculations were made from eighteen-hour broth cultures with a small loop of number 30 gauge platinum wire over the available medium, the bulk of the inoculum being left in one area; the loop was then stroked over the remaining surface to give single colonies. Throughout the series we have used 20% horse-serum in broth as the culture medium, as many of the strains were revived from the dried state. The only disadvantage of using a serum-broth culture instead of plain broth is that, where the inoculum is heaviest, there may appear with many cultures a small area of haemolysis due to transferred serum-streptolysin, although there is no visible growth. This area of haemolysis has no significance in these tests and can be disregarded when the results are being read.

Reading of Results.

The plates were examined after eighteen to twenty-four hours' incubation at 37°C. for evidence of colony growth, and the degrees of growth from "+++" (equal to the control plate) to "-" were recorded.

In some tests the incubation was continued for forty-eight to seventy-two hours. It was found,

(i) that when growth was absent at eighteen hours it did not appear on subsequent incubation, and

(ii) that when growth was visible at eighteen hours, both growth and haemolysis increased on further incubation.

Stability of Penicillin in Blood-Agar Plates.

Blood-agar plates containing one-twentieth of a unit of penicillin per millilitre showed no detectable deterioration after storage at 5°C. for twenty-one days or at room temperature (approximately 20°C.) for seven days. Throughout this survey we have used penicillin-blood-agar plates which had been prepared on the day prior to the tests and which had been stored overnight at 5°C.

RESULTS

On the basis of preliminary sensitivity tests on penicillin-blood-agar plates made with representative cultures of streptococci of groups A to H and the control strains, different concentrations of penicillin for the various groups of streptococci were chosen when the collection of strains was being tested. The sensitivity of the various groups of streptococci together with the number of strains tested and the source of the strains are shown in Table I.

Most strains had been isolated prior to the advent of penicillin treatment, but a few strains which, on being tested, did not differ in penicillin sensitivity from other members of the same group, were isolated during penicillin treatment. These strains were mostly of group A, from human sources (war wounds) and of group B, from bovine sources (mastitis).

In all, 721 strains of streptococci of groups A to H were tested for penicillin sensitivity.

It was found (Table I) that all streptococci of groups A, C (human and equine) and G were sensitive to penicillin, and that growth was uniformly inhibited on blood-agar plates containing one-fortieth of a unit of penicillin per millilitre. When plates containing one-eightieth of a unit of penicillin per millitlitre were used, as was done with a small series of representative strains, variations in sensitivity were detected, some strains being completely and the rest

partially inhibited.

Streptococci of group B from human and bovine sources were all sensitive to penicillin, but less so than strains of groups A, C and G. They varied slightly in their penicillin sensitivity, but the growth of all was inhibited by one-fifth of a unit of penicillin per millilitre, and that of most by one-tenth of a unit.

One representative strain of group H behaved as did those of groups A, C and G.

Streptococci of groups E and F (three only were tested) were sensitive to penicillin, but less so than those of groups A, C and G, and their sensitivity was of the same order as in group B.

Streptococci of group D (not pathogens, as indicated by the source) of human origin, and one from cheese, were resistant to penicillin as compared with strains of streptococci from other groups. The group D strains were completely resistant to 1.0 unit of penicillin and partially resistant to 2.5 units, but growth was inhibited by 5.0 units of penicillin per millilitre. In a large collection of streptococci from pathogenic conditions, previously isolated in this laboratory, no strain of group D which could be regarded as the causal organism of the condition was encountered. Wheeler and Foley (1943), however, report that they isolated nine strains of group D streptococci from human sources amongst several thousand strains of haemolytic streptococci, and that six strains were associated with fatal cases of peritonitis, septicaemia, meningitis and subacute bacterial endocarditis. Fleming (1929) tested 11 strains of Streptococcus faecalis and found them to be resistant to penicillin. Bornstein (1940) tested 27 strains of enterococci for penicillin sensitivity, and all were resistant. These strains were classified as Streptococcus faecalis, Streptococcus liquefaciens, Streptococcus zymogenes and Streptococcus durans, and according to Sherman (1937), members of these species would fall into Lancefield's group D. Group D strains (a few were tested) have also

been shown to be highly resistant to sulphathiazole (Long and Bliss, 1940; Hamilton and Hamilton, 1944). The test strain of the last-mentioned workers was the Lancefield group D strain isolated from cheese. On the basis of penicillin tests, the group D strains from human sources and cheese, and strains of the species classed as enterococci, behave in an identical manner. This evidence, in addition to that of heat resistance and other known physiological characteristics, supports the contention that enterococci are of group D or are closely related streptococci.

(see attached Table I)

TABLE I.

Results of Tests of Growth of Streptococci on Blood-Agar Containing Varied Quantities of Penicillin

Streptococcus Group	Source	Number of Strains Tested (Total 721)	Units of Penicillin per Millilitre.							
			0	1/40	1/20	1/10	1/5	1.0	2.5	5.0
A	Human	288	+++[1]	-[1]	-					
C	Human	118	+++	-	-					
C	Equine	12	+++	-	-					
G	Human	72	+++	-	-					
H	Human	1	+++	-	-					
B	Human	102	+++		++[1] to -	+ to -	-			
B	Bovine	120	+++		++ to -	+ to -	-			
E	Milk	2	+++		+[1]	±[1]	-			
F	Human	1	+++		+	±	-			
D	Human	4	+++		+++	+++	+++	+++	+	-
	Cheese	1	+++		+++	+++	+++	+++	+	-

1 "+++" means normal growth; "++" means growth with slight inhibition; "+" means growth with considerable inhibition; "±" means a trace of growth only; "-" means no growth.

SUMMARY

1. Streptococci (721 strains), mostly from human sources, of groups A to H, have been tested for penicillin sensitivity by a simple penicillin-blood-agar plate technique. Strains of groups A, C, G and H from human sources were all sensitive to penicillin, growth being uniformly inhibited on blood-agar plates containing one-fortieth of a unit of penicillin per millilitre. Representative strains of the serological types of equine streptococci (group C) behaved as did those of group C from human sources. Streptococci of group B from human and bovine sources were all sensitive to penicillin, but less so than those of group A. They varied slightly in their penicillin sensitivity, but the growth of all was inhibited by one-fifth of a unit of penicillin per millilitre, and that of most by one-tenth of a unit per millilitre. Streptococci of groups E and F were of the same order of sensitivity as those of group B. Group D streptococci were resistant to penicillin when compared with strains from other groups. They were partially resistant to 2.5 units of penicillin per millilitre.

2. The penicillin used throughout the present survey was the calcium salt of penicillin II. A few tests were made using penicillin I and penicillin III, and the results obtained did not differ from those found with penicillin II.

3. The method for preparing penicillin-blood-agar plates is described. It was found that these plates showed no detectable deterioration after storage for twenty-one days at 5°C. or seven days at 20°C.

4. This method of testing for penicillin sensitivity is simple, and could be used in any laboratory as a routine test.

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MENINGITIS: ISOLATION OF AN ORGANISM RESEMBLING NEISSERIA CATARRHALIS FROM CEREBRO-SPINAL FLUID: REPORT OF CASE

by W. J. NEWING and R. CHRISTIE

(St. Vincent's Hospital, Melbourne)

Although in the majority of cases of purulent meningitis the organism found is Neisseria meningitidis, Mycobacterium tuberculosis, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophilus influenzae or Staphylococcus pyogenes, about fifty different organisms have at various times been found. In a series of 3,178 cases reported by J. B. Neil in 1935 Neisseria catarrhalis was responsible on two occasions. In the case now being reported the organism found was a Neisseria resembling Neisseria catarrhalis morphologically and biochemically, but possessing a well-marked capsule.

CLINICAL RECORD

The patient, Mrs. M. M., aged forty-five years, was admitted to hospital at 5.00 p.m. on June 23, 1946. The following history was obtained from her relatives. Six days previously she caught a heavy cold and went to bed. She had a severe cough but little sputum. This "cold" had not improved. On June 22 she was drowsy all day and continually fell asleep. She vomited frequently during the day. On the morning of June 23 she showed no signs of recognizing her relatives, was semi-comatose and

became violent if interfered with.

Examination showed the patient to be a heavily built woman, semi-comatose. She screamed and struggled when moved and lay on her side with her head fixed. Her temperature was not recorded because of her resistance. Her pulse rate was 100 per minute and the respirations numbered 40 per minute; they were shallow. Some crepitations were heard at the base of the left lung. Tests for neck stiffness could not be made. The rest of the physical examination revealed no abnormality.

At 6.20 p.m. on June 23 a lumbar puncture was performed and yellow, cloudy cerebro-spinal fluid was obtained at a pressure of 200 millimetres. "Sodium Gardenal", three grains, was given and 15,000 units of penicillin were administered intramuscularly every three hours. At 8.45 p.m. lumbar puncture was repeated and 10,000 units of penicillin were introduced into the theca. The respirations at this time numbered 62 per minute. The intravenous injection of saline solution was commenced. Death occurred at 12.45 a.m. on June 24, seven hours after the patient's admission to hospital.

POST-MORTEM FINDINGS

Post-mortem examination revealed the following findings. The meninges were congested. The subarachnoid space was filled with purulent fluid. The pontine cistern contained frank pus. Dense adhesions were present in the subarachnoid space in the region of the optic chiasm and the circle of Willis. The foramina of Magendie and Luschka were patent. The ventricles were not dilated. The chorioid plexus was somewhat congested. The brain substance was soft, but showed no gross macroscopic change. Examination of microscopic sections revealed a considerable amount of nuclear degeneration. The ethmoid, sphenoid, frontal and mastoid sinuses and the middle ear were normal in appearance. Both lungs were congested, and patchy areas of bronchopneumonia were present on the lower lobe of the left lung; they were of recent origin. The spleen

weighed eight ounces and was soft and congested. No gross macroscopic abnormalities were detected in the other organs.

BACTERIOLOGICAL INVESTIGATION

Both samples of cerebro-spinal fluid, examined immediately after collection contained numerous polymorpho-nuclear leucocytes and diplococci. The latter were so numerous that several were visible in each oil immersion field of a stained specimen prepared without centrifugation. They were intermediate in their reaction to the Gram stain. Mice inoculated intraperitoneally with one millilitre samples of the fluid were unaffected.

The organism, although first isolated on blood agar in an atmosphere with increased carbon dioxide pressure, grew well on nutrient agar and gave as heavy a growth at 22°C. as at 37°C. When first isolated it was still intermediate in its reaction to the Gram stain, but it became "negative" after several subcultures. Colonies on blood sugar were fairly large, of a mucoid consistency and non-pigmented; they failed to react to the oxidase test. Stable suspensions were formed in saline solution. Lactose, dextrose, saccharose and maltose were not fermented. Well-formed capsules were easily demonstrated. Growth in nutrient broth produced uniform turbidity; involution forms were found in cultures after twenty-four hours' incubation. Very little growth occurred under anaerobic conditions.

The organism was only slightly pathogenic to mice and guinea-pigs when inoculated intraperitoneally. Doses of 4,000,000,000 organisms and over were required to cause death in mice, while in the case of guinea-pigs 16,000,000,000 organisms and over were required. Post-mortem examination of the guinea-pigs revealed no changes except considerable vascular congestion of the peritoneum; the organism was recovered from the peritoneal fluid but not from heart blood, spleen or kidneys.

The organism was relatively resistant to penicillin in vitro. On blood agar containing varying quantities of penicillin it grew well when the unitage was as high as one per millilitre, but was inhibited by two units per millilitre. Its resistance to penicillin in nutrient broth was of the same order. A freshly isolated culture of Neisseria meningitidis, tested at the same time on blood agar, was inhibited by one-tenth of a unit of penicillin per millilitre, while a standard staphylococcus strain (F.D.A. 209) was inhibited by one-twentieth of a unit per millilitre.

When tested for sulphonamide sensitivity by Harper and Cawston's (1945) method, the organism was found to be sensitive to sulphanilamide, sulphadiazine, sulphathiazole and sulphapyridine. It was not agglutinated by anti-meningococcus antisera of group I, II and IV.

COMMENT

These results indicate that the organism except for its capsule resembles Neisseria catarrhalis more closely than any of the commoner members of the genus Neisseria. It was considered advisable to report these findings in view of the fact that the organism is not commonly found in cerebro-spinal fluid and also in view of its reactions to penicillin and the sulphonamides.

SUMMARY

A case of purulent meningitis is reported, in which an organism resembling Neisseria catarrhalis was isolated. This organism was relatively resistant to penicillin but sensitive to sulphonamides.

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OBSERVATIONS ON THE BIOCHEMICAL AND SEROLOGICAL CHARACTERISTICS OF PSEUDOMONAS PYOCYANEA

by R. CHRISTIE

(From St. Vincent's Hospital, Melbourne).

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The genus Pseudomonas includes bacilli which are gram-negative, non-sporing, aerobic, ammonia-forming and motile by means of polar flagella. They show little ability to ferment carbohydrates but frequently liquefy gelatin. They mostly produce a water-soluble pigment which may be blue, green, yellow, purple or brown in colour as well as a yellow, fluorescent, water-soluble substance. They are common in soil and water, many are plant pathogens and some are connected with pathological conditions in man. The pigments are derivatives of phenazine and Tobie (1945) has suggested that the genus Pseudomonas should comprise those bacilli which produce phenazine derivatives or water-soluble fluorescent pigments or both. The relationship, if any, between the phenazine and the fluorescent pigments is unknown.

The species, Ps. pyocyanea, first isolated by Gessard in 1882, is widely distributed in nature being found in water, sewage and sometimes on the normal skin, particularly of the axilla and perineum (Ruzicka, 1898). It is not infrequently found in wounds, where it gives

rise to the blue pus from which it has received its name. It may invade the middle ear, meninges, bronchi and other organs and even invade the blood-stream (Williams and Cameron, 1896). According to Pons (1927), it is especially pathogenic in the tropics where it may give rise to typhoid-like infections and abscess of the liver. It is otherwise regarded as an organism of low-grade pathogenicity and is usually associated in wounds with other organisms, of which Staphylococcus pyogenes is common. It is pathogenic for animals if injected in sufficiently large doses and may cause death within 24-48 hours. Different strains vary in virulence.

In culture, Ps. pyocyanea produces a bluish-green pigment which diffuses through the medium. Two substances are connected with this pigment, one, pyocyanin, is green in colour, non-fluorescent and soluble in chloroform or water, the other, fluorescin, being yellow, fluorescent, soluble in water but insoluble in chloroform. In old cultures a brown or black pigment forms from the oxidation of the green pigment. Jordan (1899) divided his series into four varieties:

(1) Those producing both pigments;

(2) Those producing pyocyanin only;

(3) Those producing fluorescin only; and

(4) Those producing neither.

Gessard (1892), working with strains producing both pigments, was able to produce varieties giving pyocyanin or fluorescin only as well as some which produced neither.

Strains isolated from water and closely related to the species Ps. pyocyanea but with a lower optimum temperature and less pathogenicity for animals have been regarded as of a separate species, Ps. fluorescens. With some strains difficulty has been found in deciding to which species they belong and Sandiford (1937) was inclined to regard strains with the characteristics of the species Ps. fluorescens as members of the other which had become adapted to a saprophytic existence. Strains isolated

from water could not be distinguished from those of human origin by means of the cultural characteristics which Bergey (1934) employed to differentiate the two species.

Efforts to sub-divide strains on the basis of biochemical tests have given a large number of groups. Tanner (1918) was able to divide 42 strains into 27 groups. Seleen and Stark (1943) in a study of 199 strains, were able on the grounds of ability to liquefy gelatin, affect milk or reduce nitrates, to form 14 groups, with further sub-groups on ability to produce pyocyanin and to utilize sucrose, acetic acid, lactic acid or tartaric acid as sole source of carbon.

Reports on the serological identity or otherwise of strains of Ps. pyocyanea do not agree. Meader, Robinson and Leonard (1925) concluded from the results of absorption tests that the organisms were serologically uniform. Aoki (1926) on the other hand, found 22 types in an examination of 50 strains. Elrod and Braun (1942) and Munoz, Scherago and Weaver (1945) also found the group to be serologically heterologous, Gaby (1946), who made an intensive study of the variants from seven strains, found that there were three basic colony forms, that the flagellar agglutinogens of identical colony forms were alike but that they were different from those of dissimilar colony form, all forms having a common somatic antigen.

The investigation which forms the subject matter of the present report was undertaken with two practical purposes in view. The first purpose was to obtain an anti-serum or mixture of anti-sera which could be used for the rapid identification of strains of Ps. pyocyanea, preferably by slide-agglutination; this would be particularly helpful with strains which produce little pigment on agar or which are isolated on media such as blood agar or MacConkey's agar where the characteristic pigment is not easily recognized. The second purpose was to provide,

if possible, typing sera, to attempt to trace the origin of the organisms which invade damaged tissues and to assist in deciding whether these organisms came from the patient or were the result of cross-infection. Both of these purposes have been, to a certain degree, achieved.

MATERIAL AND METHODS

Strains Used.

One hundred and thirty-eight strains, isolated from faeces, urines, suppurative conditions, bile, sewage, water and contaminated media were examined. Ten stock strains were of unknown but probably human origin. Organisms with the biochemical characteristics of Ps. pyocyanea but producing no typical green or yellow pigment were not included. Strains were numbered serially as collected, P1, P2, P3, etc.

Preparation of Anti-sera.

The organisms were incubated for 18 hours on nutrient agar slopes, the temperature of incubation being 37°C. for the strains of human origin and 25°C. for those which failed to grow at 37°C. The organisms were then suspended in normal saline containing 0.2 p.c. formalin, to give a concentration of approximately 8,000 million per ml., incubated for 4 hours at 37°C. and stored at 4°C. Rabbits were inoculated intravenously twice weekly for three weeks, the volumes of the successive inocula being 0.1, 0.2, 0.4, 0.7, 1.0 and 1.0 ml. Four days after the last injection, blood was withdrawn by heart puncture. The serum was separated, preserved by the addition of chloroform and stored at 4°C.

Preparation of Suspensions for Agglutination.

Suspensions of living organisms were prepared by washing off the organisms from an overnight agar slope culture with 1.5 ml. of saline, the resulting concentration being approximately 20,000 million per ml. Suspensions for tests for somatic antigens were prepared from these by suspending the organisms, separated by centrifugalization, in 1.5 ml.

of absolute alcohol, maintaining them at 45°C. for 30 minutes and re-suspending them, after further separation by centrifugalization, in the original volume of saline.

Technique of Slide Agglutination.

With the aid of a Pasteur pipette, a drop of suspension was placed on a slide and mixed with an equal-sized drop of diluted serum. The slide was then rocked for two minutes and the result read with a hand lens.

Haemolysis.

This was detected on nutrient agar plates to which 5 p.c. sheep blood had been added. Horse blood was found to be less suitable than sheep blood since incubation for several days was necessary for haemolysis to show by which time the horse red-cells had begun to lyse throughout the whole of the medium. The various strains were "spot-inoculated" on the medium, six strains being applied to each plate. Incubation was carried out at 25°C., since some strains failed to grow at 37°C. and it was desired to carry out the tests at the same temperature for all strains for comparative purposes.

Absorption of Anti-sera.

The organisms to be used for absorption were grown on agar slopes for 18 hours, washed off with saline and separated by centrifugalization. The serum was diluted 1:5 with saline and 0.5 ml. added to the organisms from 1, 2 and 4 slopes in separate tubes. After being allowed to stand for one hour at 37°C., the organisms were removed by centrifugalisation.

RESULTS

BIOCHEMICAL TESTS.

All strains from human sources grew well at 37°C. and at 25°C.

Of the twelve strains from non-human sources, e.g. sewage, water and contaminated media, all grew well at 25°C. but very poorly or not at all at 37°C. with two exceptions. These exceptional strains were isolated from water samples.

Most of the strains were haemolytic. Two kinds of haemolysis were noticed, one resembling the β haemolysis and the other the α prime haemolysis of the streptococci. In most instances, the haemolysis appeared in two or three days. Further incubation produced extension of the zones, some of the strains which at first showed only partial haemolysis, developing inner zones of complete haemolysis while others showed merely extension of the zones of incomplete lysis. Ten strains showed no haemolysis after seven days. These were the ten strains of non-human origin which failed to grow well at 37°C.

Attempts were made with one of the markedly haemolytic strains to produce a cell-free filtrate containing haemolysin. The organisms were grown at 37°C. and 25°C. in nutrient broth with and without 0.1 p.c. agar and with and without increased CO_2 pressure. Incubation for 1, 3 and 7 days was allowed. The liquids were then clarified by centrifugalization and filtration through a Seitz EK filter and the filtrates tested against washed sheep red-cells at 37°C. No haemolysis was detected.

Pigment, blue-green or greenish-yellow, formed on all agar slope cultures, to a degree which varied from strain to strain. After a few days at room temperature, the colour changed to yellow and then to brown. Two strains formed a brownish-black pigment, apparent within 24 hours. In the deeper parts of the medium on which these two strains had grown, blue-green pigment was visible below the darker surface layer, suggesting that the pigment system in these two strains was similar to that in the others but that the oxidation process by which the brown pigment is produced from the blue one was unusually rapid.

The presence of fluorescin was detected by holding tubes con-

taining broth cultures, in the rays of a mercury lamp and viewing the tubes against a dark background. Some strains gave a strong fluorescence within 24 hours, some showed very little after a week's incubation. Varying degrees of reaction between these extremes were found. There was no fading of the reaction once it appeared, as with the blue pigment. Formation of pyocyanin did not parallel that of fluorescin.

All strains, with one exception, were actively motile when first isolated. After 6 months, one strain originally motile was found to be non-motile and no longer agglutinable with a flagellar anti-serum (see below) although it had been originally agglutinable. A sub-culture of the stock strain which had been preserved under paraffin was found to be still motile and agglutinable. The motile form possessed a single well-formed flagellum. No flagella could be demonstrated on the other form. Both forms of the organisms produced pyocyanin to the same degree and showed no differences in their other biochemical properties.

A second strain was non-motile, even on primary isolation. It gave very mucoid colonies, resembling those of Friedländer's bacillus. After several sub-cultures, the mucoid nature of the growth became much less marked. A third strain, on the primary isolation plate, showed two colony forms, one motile and the other non-motile. That the two were related and not merely two forms accidentally occurring together, was supported by the fact that both produced dark-brown pigment within 24 hours on nutrient agar, an appearance shown by only one of the other strains in the collection.

In litmus milk, most strains produced some clotting with subsequent solution, with little differences other than in degree and in rate of reaction. Three strains produced peptonization without previous clotting as shown by a gradual clearing of the medium.

Lactose was not fermented by any strain.

Since Ps. pyocyanea is an active producer of ammonia resulting

in an alkaline reaction to broth in which it has grown, it was possible that lactose fermentation, if it occurred, would be masked by the simultaneous production of alkali, since the method used to detect fermentation was the noting of colour-change in an acid-base indicator in the medium. To examine this possibility, tubes of peptone water containing 1 p.c. lactose were inoculated with a strain of the organisms and incubated for varying times. Seitz filtrates of the cultures were then examined for reducing sugar by the Hagedorn and Jensen technique as used for estimating sugar in blood. The titrations showed that, taking the titre for the uninoculated controls to represent 1.00 p.c. the figures for 1, 3 and 7 day cultures were 0.903, 0.927 and 0.988 p.c. respectively. To reduce ammonia-formation and complications due to the presence of peptone, lactose broth was prepared using Koser's citrate medium instead of peptone water as base. Again taking the titration figure for the controls to be 1.00 p.c., the corresponding figures for two 7-day cultures were 1.00 and 1.05 p.c. A parallel set of tubes of this medium with indicator added showed no colour change indicative of acid formation. As a result of these tests, it was concluded that lactose was not fermented.

No strains produced indole within 5 days when tested by the method of Holman and Gonzales (1923). Atypical positive reactions were obtained when Ehrlich's indole reagent was used; the reactions showed more in the medium than in the layer of reagent on top. This has been attributed by Sandiford to the action of the acid in the reagent on the pigment of the organism and not to indole.

All strains formed catalase and were able to utilize citrate as their sole source of carbon. All produced ammonia in peptone water. On potato slopes, some strains produced much blue-green pigment and some very little. After incubation for a week at room-temperature, a brownish, slimy growth had formed from all strains with varying amounts of blue pigment still evident.

Fibrinolytic activity, as shown by clearing of heat-precipitated

fibrinogen on fibrinogen agar plates (Christie and Wilson, 1941), was shown by all strains. Some produced zones of faint opacity as well. No such zones were produced on serum agar. The lysis was not very marked or distinct as it is with staphylococci but resembled more the weak reactions given by fibrinolytic streptococci. Attempts were made to obtain cell-free culture filtrates containing fibrinolysin as has been done with staphylococci (Christie, Graydon and Woods, 1945) but only very weak reactions were obtained.

No lipolytic activity was shown when strains were grown on butter-fat agar plates.

Dextrose was fermented by 59 strains in 1 day, by 27 strains in 2 days, by 15 strains in 3 days, by 12 strains in 4 days and by 1 strain in 6 days. No fermentation was given by 24 strains in 14 days. The tests were repeated with the non-fermenters and the slow fermenters using Koser's citrate medium as base instead of peptone water. Similar results were obtained except that fermentation was less rapid.

Fifteen strains of human origin and six strains of non-human origin were inoculated from 24-hour agar slope cultures into oxalated human plasma diluted 1:5 with saline. The tubes were incubated at 37°C. and 22°C. for 24 hours. No coagulation was detected.

Gelatin stabs were inoculated from 85 strains and incubated at 37°C. and 22°C. Tests for liquefaction were carried out by placing the tubes at 4°C. for one hour and then inverting. Some strains caused rapid liquefaction, others required incubation for periods varying from two to fourteen days, while twelve strains failed to cause liquefaction within fourteen days. The percentage of non-liquefiers was higher amongst the strains of non-human origin than amongst the others. The tests, repeated with two other batches of medium, gave results which were approximately but not strictly parallel.

Flagellar staining showed that most strains possessed one

flagellum but the results were not always straightforward. Preparations of strains showing predominantly one large well-formed flagellum not infrequently showed occasional bacilli with several apparent flagella bunched at one pole. These, however, were variable in size and number with as many as 14 per bacillus; they were finer, shorter and less regular than the single flagellum shown on other bacilli in the same preparation. An occasional bacillus showed a definite envelope, unstained but with a stained outline and with the flagellum attached to the outside of this envelope and showing no sign of being attached directly to the inner cell. The appearances agreed with Pijper's (1946) suggestion that with flagellated bacilli there is a surrounding capsule-like material of which the flagellum is an extrusion and that on drying artefacts with the appearance of flagella can originate from disruption of this substance. Where more than one flagellum appeared, it was usually one of two or more attached to one pole and in such instances was smaller and finer than the single flagellum shown on neighbouring bacilli. It was concluded that normally strains possess one flagellum only.

No flagella were seen in stained preparations of the three non-motile strains in the collection.

SEROLOGICAL TESTS.

Thirty-five antisera were prepared, each being given the number of the strain from which it was prepared. No animal failed to give a good response except in cases where there was reason to suspect that degenerate strains were used. The average end-point in titration of sera by slide-agglutination was about 1:160 and in titration by the tube method, in a trial series, about ten times greater. All results given below were obtained by slide-agglutination.

Serum 1 agglutinated eight of the first eleven strains to an appreciable but not identical titre, two strains (P3 and P4) to a low titre and strain P2 not at all. The lowest dilution used was 1:10. When the serum was absorbed with a suitable dose of organisms of strain

P4, its agglutinins for strains P3 and P4 were simultaneously removed. These tests were carried out using suspensions of living organisms in saline. When carried out with alcoholized organisms, the agglutination of strains P3 and P4, using unabsorbed serum, did not occur, suggesting that this had been due to common flagellar antigen.

Serum 3 agglutinated all eleven strains except strain P2 but gave a much higher titre against organisms of strain P3 than against any of the others, when living organisms were used. When tested against organisms in the O phase, the serum was specific for strain P3.

Similar results were obtained with serum 4 and strain P4.

Serum 2 was highly specific. It agglutinated strain P2 to a titre of 1:160 with the organisms in the O or O-H phase but none of the other eleven strains in either phase.

Strain P15 reacted less strongly with serum 3 than did strain P3. Serum 15 reacted in the reverse manner so that by using each serum suitably diluted (1:40) they were made to agglutinate the homologous strain only. It was at first thought that the two strains had each two antigens, one specific and one common but as more strains related to these two were encountered, it was found that they did not separate into two clear-cut groups but that gradation in quantity of either antigen occurred. These strains have, therefore, been classed as one group.

Serum 13 agglutinated strain P13 but not strain P1. Serum 1, on the other hand, agglutinated both strains. A month later serum 1 also failed to agglutinate strain P1. When strain P1 was sub-cultured from the culture of the original strain preserved under paraffin, it reacted equally well with both sera. This was evidently another instance of the sharing of antigens, strain P1 having degenerated, losing first one and then the other antigen, while the antisera varied in their content of the two agglutinins. Further evidence to support this explanation on the grounds of degeneration or variation was found when colony differences and differ-

ences in pathogenicity were examined, as described below.

Strain P16 was agglutinated weakly by serum 1 when in the living phase but by none of the sera when in the O phase. An antiserum prepared from it agglutinated many strains strongly when these were in the living phase but none at all when they were in the O phase. A second serum prepared from it, using larger inocula, reacted similarly. Such O anti-bodies as were detected in this second serum suggested that it had an antibody similar to one of those in serum 1. It was found possible to absorb serum 1 with organisms P16 and to remove the agglutinins for strain P16 while leaving a considerable titre of agglutinins for strain P1. On account of the high titre of flagellar antibodies in serum 16 and its almost negligible somatic antibody content, this serum was used in high dilution (1:80) to test for common flagellar agglutinogens in the other strains, using suspensions of untreated organisms. Sixty-eight strains were agglutinated.

Strain P17, which was agglutinated weakly by serum 15 but by none of the others yielded an antiserum low in somatic antibodies but high in flagellar antibodies. When tested in high dilution (1:160) against living suspensions of all the strains, 37 were agglutinated. None of these 37 had reacted with the other flagellar serum.

Serum 13 had agglutinated strain 2 in the living condition to an appreciable titre but not at all in the O phase, suggesting that P13 and P2 had a common flagellar antigen distinct from the other two. It was found possible to absorb out the agglutinins for P2 while leaving the serum still capable of agglutinating the O form of strain P13. Absorption of the serum with a suitable organism with the same O but a different H antigen did not give a residue sufficiently rich in H antibody to allow this to be used as a flagellar antiserum. However, it was possible to show that many strains did not have the same H antigen as P13 since these strains were not agglutinated in the living condition by the unabsorbed serum in low dilution.

Strain P8 was agglutinated by serum 1 to a titre appreciably less than that obtained with strain P1. A serum made from strain P8, on the other hand, agglutinated both strains almost equally strongly. Other strains which agglutinated weakly with serum 1, reacted strongly with serum 8. It seemed again as if there were specific and common antigens. This was borne out by precipitin reactions. Preliminary tests on precipitin reactions with one strain (p13) had shown that a substance could be extracted from the organisms either by the formamide or the HCl method which gave a precipitin ring with anti-sera. It was later found that the supernatant fluid from centrifugalized 48 hours' broth cultures gave the same reaction. When the P1, P8 strains were tested by the precipitin method against sera 1 and 8, they could be divided into two clear-cut groups, the specific substance being apparently of a polysaccharide nature while the common substances were probably protein.

It seemed possible that the P3, P15 series might be separated in a similar manner but with these strains, there was cross-agglutination by the precipitin as well as by the agglutination technique.

Two strains P36 and P50 gave specific antisera which agglutinated the parent strains and some others, none of these strains having been agglutinated by any of the sera previously prepared.

TABLE 1.

Results of Somatic Agglutination Tests, Showing Number of Strains In Each Group.

Origin of Strains	Human								Non-human						
No. of anti-serum	8	1	15	2	4	50	36	X	23	24	25	46	51	60	X
No. of strains	41	21	20	12	7	6	2	17	2	1	2	2	1	1	1

Strains in Column X were not agglutinated by any of the anti-sera.
Two strains isolated from water but showing the characteristics of the human group are not entered in the table. They were agglutinated by anti-sera Nos. 8 and 1 respectively.

By means of all these sera, it was possible to type the two water-borne strains which grew well at 37°C. and all of the human strains except 17. Sera prepared from eleven of these showed no specific antibodies and it is possible that these strains were degenerate forms such as the one mentioned above in connection with strain P1.

The ten strains of non-human origin, which grew poorly or not at all at 37°C., were not agglutinated by any of the sera so far prepared. By making anti-sera from these, it was found that there were six groups. No cross-agglutination occurred with these sera, using suspensions of organisms on the O phase and very little in the living phase. Precipitin reactions were also specific. One strain, however, P46, gave a serum which agglutinated twelve of the human strains, ten of them fairly strongly, although P46 itself gave no agglutination or precipitin reaction with any of the sera prepared from human strains. The reacting human strains, however, gave very weak precipitin reactions with serum 46, whereas strain P46 gave a very strong one. It was found possible to remove the common agglutinins by absorption and it is the results found after absorption that are entered in Table 1.

Another example of a connection between the non-human and the human strains was found with a serum from the human strain P19. A serum prepared from a non-motile variant of this strain was found to be relatively poor in antibody content. A second serum prepared with larger inocula including living organisms reacted with strain P19 in low dilutions only. This serum agglutinated many of the human strains as well as the non-human strain, P25, in a manner which did not parallel any of the specific reactions already described. It did not agglutinate any of the strains agglutinated by serum 46, and gave no precipitin reactions even with the parent strain.

PATHOGENICITY OF STRAINS

The pathogenicity of strains was tested by inoculating mice,

TABLE 2.

Results of Mouse Pathogenicity Tests With Strains of Ps. pyocyanea

	Human Strains										Non-human Strains										
Serial No. of Strain	2	3	4	8	13	15	16	17	36	50	1	1A	76	22	23	24	25	45	46	47	51	60
Dose in millions of organisms																						
2,000	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+2	+1	-	+1	-	+2	+1
1,000	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	-	-	-	-	-	-	-	+1
500	+1	+1	+1	+1	+2	+1	+1	+1	+2	+1	+1	+1	+1	+2	-	-	-	-	-	-	-	+2
250	+1	+1	+1	+1	+1	+1	+1	+1	+2	-	+1	+2	-	+2	-	-	-	-	-	-	-	-
125	+1	+1	+1	+1	+1	+1	+1	-	-	-	+1	-	-	-	-	-	-	-	-	-	-	-
62.5	-	-	-	-	-	+1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+1 means death within 24 hours. +2 means death within 48 hours. - means survival.

weighing 30 gm., by the intra-peritoneal route, the volume of the inoculum being 1 ml. Organisms were grown for eighteen hours on nutrient agar slopes, at 37°C. for the human strains and at 25°C. for the others. They were then washed off with saline and the concentrations estimated with Burroughs Wellcome standard opacity tubes. Serial doubling dilutions were prepared commencing with suspensions containing 2,000 million organisms per ml.

Where death occurred, this happened on one or two days. The survivors were still alive and apparently normal after fourteen days.

The results are shown in Table 2. It will be seen that the lethal dose for the human strains was much smaller than that for most of the non-human strains but that there were strains intermediate in killing power in both groups.

Strain P1 showed the normal killing power for a human strain but strain P1A, an inagglutinable variant of this strain, had less pathogenicity. Strain P76, a non-mucoid variant of a strain originally giving very mucoid colonies, also shows lowered pathogenicity.

The greater lethal power of the strains of human origin may have been partly or entirely due to their ability to grow at 37°C. To test this, suspensions of strain P2, a human strain (see Table 2), and of P24, a non-human strain, were killed by exposure to a temperature of 55°C. for one hour and mice were inoculated with graded doses. The lethal dose for strain P2 was 2,000 million organisms and for strain P24 4,000 million. The difference in lethal power between the two organisms had thus been greatly reduced, suggesting that the difference in optimum temperature for growth had played a large part in deciding their pathogenicity when in the living condition.

No toxin was detected in broth cultures. Strain P2 was grown in nutrient broth for one and four days at 37°C., the broth rendered clear by centrifugalization and the mice inoculated, intraperitoneally with 1 ml.

quantities. None showed ill effects.

VARIABILITY OF STRAINS.

Strain P1, after repeated sub-culture, became so altered that it was no longer agglutinated with serum 1. That the serum had not altered was shown by the fact that it still agglutinated other strains and that it still agglutinated strain P1 when this was revived from the original culture preserved under paraffin. Both forms of P1 produced abundance of pyocyanin and both were motile but the agglutinable form gave a less homogeneous suspension in saline than the other and the two forms gave different colony forms when plated out on nutrient agar. After three days' incubation the colonies of the inagglutinable form were smooth and uniform whereas the others had an irregular edge and a somewhat pitted surface. Attempts to produce agglutinable organisms from the others by treble passage through mice were unsuccessful. The degeneration had taken place in at least two stages, the first when the organisms would agglutinate with serum 1 but not with serum 13, and the second when they would agglutinate with neither although they originally agglutinated with both. The inagglutinable form was less lethal for mice than the other (see below).

The loss of motility shown by strain P19 is another form of variation that can occur in vitro. That this can also occur in vivo was suggested by the fact that when strain P53 was isolated, a motile and a non-motile form were obtained from the primary isolation plate. That they were related to each other was extremely probable since both produced, within 24 hours, dark-brown pigment, a reaction shown only by one other strain in the series.

The degeneration shown by strain P1 was shown by some other strains, originally normal, which later gave suspensions showing partial or complete auto-agglutinability and inagglutinability with antisera. Sub-cultures of these from paraffin-covered stock cultures behaved normally. A fairly large number of strains (18) was not agglutinated by any of the

sera. Some of these strains could not be tested on isolation as they were received from other laboratories but this could not be said for all strains. The untyped strains gave mostly non-homogeneous suspensions in saline and were probably degenerate forms comparable with strain P1A. On two occasions agglutinable and inagglutinable forms were found together on the primary isolation plate, suggesting that the degeneration can occur in vivo as well as in vitro. Antisera prepared from eleven of these un-agglutinated strains were unsatisfactory as compared with those from normal strains, in that they were all of low titre and showed little or no specificity.

DISCUSSION

With the exception of haemolysis and optimum temperature for growth, none of the usual biochemical tests was helpful in grouping strains. Tests for fermentation of dextrose, liquefaction of gelatin and action on milk showed differences between strains, but these tended to be in degree rather than in kind and in no way agreed with serological type or source. It is questionable if these differences have any significance. Liquefaction of gelatin has been given some significance in the past, but the results obtained in this survey indicate that its value as a differential test is very limited, since the results vary slightly with the batch of medium used, there is a broad range between the rapid liquefiers and the non-liquefiers so that a lengthy time of incubation must be allowed and, finally, the division obtained means little since it correlates with no other characteristic.

Haemolysis, on the other hand, correlates with optimum temperature for growth and with source. All non-haemolytic strains were of non-human origin and unable to grow well at 37°C. The test for haemolysis is a clear-cut one. All strains from pathological conditions as well as all strains which grew well at 37°C. were haemolytic. Salvin and Lewis (1946) found that all strains of Ps. pyocyanea (aeruginosa) were haemolytic on ox blood agar, while strains of Ps. fluorescens were non-haemolytic.

The lesser pathogenicity of the non-human strains as compared with the others is of reduced significance when it is borne in mind that it may be largely the result of their lower optimum temperature for growth. The tests lose further value on account of the fact that, like fermentation of dextrose and liquefaction of gelatin, they give intermediate results with strains of both groups.

The non-human strains tested in this survey are apparently similar to those referred to by other workers as of the species Ps. fluorescens, but, as is generally stated, the distinguishing tests for this species are at present inadequate. The members may be regarded as saprophytic forms of Ps. pyocyanea, but if they are considered to have properties as a group, sufficiently distinct from those of the group found in lesions, to warrant being placed in a separate species, the results found above suggest that the distinguishing tests be inability to haemolyze sheep red cells and to grow well at 37°C.

The ability to produce pyocyanin can vary from strain to strain as can the ability to produce fluorescin, so that the apparent inability to produce either may be merely an indication that the test to which the strain is submitted is not sufficiently sensitive to detect weak reactors, or that the strains found negative in either test differ from normal strains in a manner which will cause them to be regarded merely as different varieties and not necessarily as members of a different species or sub-species.

The Society of American Bacteriologists uses the species name aeruginosa on priority grounds instead of pyocyanea as used by English writers. Since it is evident that there is a pathogenic group which is capable of causing the formation of "blue pus" and a saprophytic group incapable of doing so, it might be a rational procedure to use the name pyocyanea for the former and the name aeruginosa for the latter, the use of the name fluorescens being discontinued.

Further differentiation of strains is possible, as has been shown, by serological tests, using slide-agglutination with the organisms in the O phase or the precipitin test with the supernatant fluid from broth cultures. In preparing antisera, excessive dosage of the animals should be avoided, since this is liable to produce antibodies which detect nonspecific antigens.

Apart from the divisions thus obtained, further subdivisions can be made according to which flagellar antigen the organism possesses. At least two common flagellar antigens are detectable, a reacting strain possessing either independently of its somatic antigen. There is some indication of a third less common flagellar antigen possessed by a few of the strains showing neither of the two common ones, while a fourth is postulated to accommodate those strains possessing none of these three. No antiserum has yet shown any antibodies capable of detecting H antigens, with water-borne strains.

The number of groups shown in Table 1 is much smaller than the number (22) found by Aoki, but if it is remembered that each group shown can be divided into two or more types on the basis of flagellar antigen, closer agreement is apparent. Gaby's finding, that there is a common somatic antigen and one of three flagellar antigens possessed by each strain, is explainable on the assumption that, since he worked with a small number of strains (7), they all belonged to the commonest group shown in Table 1.

The number of groups found amongst the water-borne strains is unexpectedly high considering the small number of strains examined, and the probability is that there are still more to be found. If the six groups found were the only ones, it is most unlikely that they would all have been found in the first ten strains tested.

An effort was made to detect the source of strains which invade wounds. Swabs were taken from the ear, nose, throat, axilla,

perineum and rectum of 12 patients with no history of infection. Examination failed to reveal any strains of Ps. pyocyanea, as did also an examination of 12 samples of normal faeces. A series of cases occurred however, where it was possible to show that three patients with wounds had become infected from a fourth in the same ward. All four strains had the same flagellar antigen and the same somatic antigen (antiserum 4). Analysis of the distribution and numbers of strains in each group showed that the probability of four strains of this kind occurring by chance in the same ward was negligible (P=0.0003).

The presence of Ps. pyocyanea in water samples is of some significance to Public Health Officials, especially in tropical areas. Strains found in routine analysis, however, can be disregarded if they are non-haemolytic on sheep blood agar.

SUMMARY

An examination of 138 strains of Ps. pyocyanea has shown that they can be divided into a pathogenic and a saprophytic group.

The differentiating tests are ability or otherwise to haemolyze sheep red cells and to grow well at 37°C.

It is suggested that the species name aeruginosa be used for the saprophytic group.

The organisms may be further sub-divided into groups by slide-agglutination on the basis of somatic antigen. There are at least 7 groups in the pathogenic and 6 in the saprophytic group. There is also evidence that the strains may further differ in their flagellar antigens.

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THE DETECTION OF CANDIDA ALBICANS

by R. CHRISTIE and MARY M. MORTON

(Pathology Department, St. Vincent's Hospital, Melbourne)

Yeast-like fungi are frequently found in specimens from human beings in normal and pathological states. In low-grade infections, when such an organism is recognized as the cause of the disease, it is usually found to be Candida (Monilia) albicans. The diseases attributed to this organism include thrush, perleche, vulvo-vaginitis, paronychia, onychia, intertrigo and generalized cutaneous moniliasis. It is commonly found in sputum as a secondary invader, although cases of bronchopulmonary and pulmonary moniliasis are not unknown.

C. albicans is differentiated from lower forms of yeast-like organisms in that it forms hyphae and from other species of Candida in that it forms chlamydo-spores. A further differentiating test is its ability to cause death in rabbits if given intravenously and to cause an inflammatory reaction in rabbits and guinea-pigs if rubbed into the abraded skin (Benham, 1931).

Benham found that agglutination by an anti-serum was the most satisfactory method of identifying the organism, but that a special agitation technique of performing the test was necessary to minimize the

confusing effect of the instability of suspensions. Stone and Garrod (1931) showed that all Candida from cases of thrush were identical in complement-fixation and precipitin reactions and in pathogenicity for the rabbit. Anderson (1946) showed that corn-meal extract containing Congo red was a useful culture medium for detecting the formation of hyphae and chlamydospores, but found that the instability of suspensions made agglutination tests unsatisfactory.

Weld (1952, 1953) has shown that cultivation on eosin-methylene blue agar in an atmosphere with increased carbon dioxide tension gives colonies of C. albicans which are readily recognized as typical of this organism.

In present-day routine bacteriological examination of specimens, C. albicans is more often found than was the case before the widespread use of antibiotics. The possibility of the organism occupying a more pathogenic role than formerly, when present in damaged tissues where the primary organism or organisms have been suppressed, has been recently mentioned (Harris, 1950; Tomaszewski, 1951; Woods et al., 1951).

During the examination of specimens in this hospital, particularly those required by the dermatologists, it was found that a considerable number of organisms, assumed at first to be C. albicans, failed when further examined to meet the requirements of this organism. This led to the collecting and testing of a large number of yeast-like organisms with a view to deciding on a reliable and reasonably rapid method of identifying C. albicans.

In the following report the word "pathogen" is used, for convenience, to signify C. albicans, except where otherwise indicated by the context.

MATERIAL AND METHODS

Source of Strains.

Seventy-eight strains of yeast-like organisms were isolated on Sabouraud and blood agar and numbered serially as received. The sources were skin (14), urine (11), nails (19), faeces (4), sputum (16), throat and mouth swabs (4), vaginal swabs (5), pus (2), cerebro-spinal fluid (1). A brewer's yeast and a standard culture of C. albicans (strain Windisch) were also included. Strains were maintained on nutrient agar. Incubation, except where otherwise stated, was carried out at 28°C.-30°C.

Fermentation Reactions.

The sugars used were dextrose, maltose, saccharose and lactose. They were made up in concentration of 3% in peptone water with brom-cresol-purple as indicator. Readings were made daily for 28 days.

Preparation of Antisera.

Suspensions of the organisms in normal (0.85%) saline, of density equal to that of a Burroughs Wellcome standard opacity tube No. 6, previously killed by the addition of 0.4% formalin, were given intra-venously to rabbits in six 1 ml. quantities over a period of three weeks. The animals were bled from the heart at the end of the fourth week and the separated sera preserved by the addition of chloroform and stored at -20°C.

Absorptions were carried out by adding varying quantities of the absorbing organisms to 1 ml. quantities of 1:5 dilution of the serum, allowing the mixtures to stand at 37°C. for one hour and then clarifying by centrifugalization. Absorbed sera were stored at 0-4°C.

Technique of Agglutination Tests.

The suspensions used in agglutination tests were prepared by washing the organisms from an overnight culture on nutrient agar with 1 ml. of normal saline. A drop of this suspension was added to a drop

of the serum, suitably diluted, on a slide; the mixture was rocked for one minute and readings were made with the aid of a hand lens. A control test was carried out at the same time, using a drop of saline in place of the serum.

Detection of Hyphae and Chlamydospores.

Corn-meal extract was prepared as described by Benham and tubed in 5 ml. quantities. 0.05 ml. of sterile 1% aqueous solution of congo red was added to each tube when required (Anderson). After two and five days' incubation with organisms, a drop of the sediment was withdrawn with a sterile Pasteur pipette and examined microscopically under a cover-slip.

Animal Inoculations for Pathogenicity.

Suspensions of the organisms of the same strength as that used for immunization were inoculated intravenously in 1 ml. quantities into rabbits, subcutaneously in 0.2 ml. quantities under the clipped skin of the abdomen in guinea-pigs, and subcutaneously in 0.5 ml. quantities under the skin of the back in mice. The animals were examined daily.

Eosin-Methylene Blue Agar Tests.

The medium was prepared as described in "Approved Laboratory Technique" (Kolmer and Boerner, 1945, p. 354). Ten milligrammes of crystalline aureomycin hydrochloride dissolved in 1 ml. distilled water were added to each 100 ml. of liquid medium at 45°C., before pouring into plates. The organisms were streaked on the medium as described by Weld, and also "spot-inoculated" with nine strains per plate. The plates were then placed in a McIntosh and Fildes' jar and approximately 10% carbon dioxide added. Examination of the plates was made after two, three, four and five days' incubation at 37°C.

RESULTS

Fermentation Reactions.

All strains of C. albicans produced acid in dextrose within 24 hours, and gas within 48 hours. All gave acid in maltose within 24 hours, usually but not always with gas formation on the second day. Fermentation of saccharose occurred more slowly, usually in five to eight days; gas formed in six of the 50 strains. No strains of the whole collection of 78 fermented lactose, although incubation for 28 days was allowed before the tubes were discarded.

Results with Corn-Meal Extract.

Most strains of C. albicans, when grown for two days in this medium, showed numerous hyphae and the thick-walled red-stained chlamydospores as described by Anderson. With some strains hyphae were not detected until later, but none failed to give hyphae within five days.

All pathogens showed chlamydospores, but some needed more than two days' incubation before they appeared positive. Some strains, not C. albicans, showed large forms resembling the chlamydospores of C. albicans, but without the typical thick-walled appearance and usually with less intense staining. Occasionally it was necessary to refer to the results of the other tests before a decision could be made as to whether the strain were a pathogen or not. A stock strain of T. histolytica, for instance, which forms no hyphae, gives within two days large deeply-stained forms which are difficult to distinguish from those given by C. albicans.

Pathogenicity Tests.

Four strains of C. albicans injected intravenously into rabbits caused death of the animals within five days. From three of the animals the organisms were recovered from the kidneys. The test repeated with strain Y15 after this had been maintained on nutrient agar with

monthly subculture for one year caused obvious distress to the rabbit, although it recovered within a fortnight. Strain Windisch had no obvious affect on a rabbit.

Two strains of yeast-like organisms, not C. albicans, showed no pathogenicity for rabbits.

A rabbit immunized against C. albicans during the preparation of an anti-serum was unaffected by an intravenous injection of living organisms such as had previously caused the death of an unimmunized rabbit.

It was not practical to test all strains against rabbits, but it was found that guinea-pigs and white mice could be used instead.

Guinea-pigs injected intraperitoneally showed no ill effects, but when injected subcutaneously under the clipped skin of the abdomen they developed, in two to three days, an easily palpable swelling. This persisted for one to two weeks in some cases, but longer in others, one animal being positive for 34 days. No animal died. Animals used after an interval of two weeks from the disappearance of the swelling failed to show any immunity when given a second injection of the same organisms. The animals injected intraperitoneally also failed to show immunity to a subsequent subcutaneous injection.

Of the 50 strains judged to be C. albicans, only three failed to show the normal pathogenicity reaction in guinea-pigs. One of these strains (Windisch) which failed to show pathogenicity for a rabbit affected two only of four mice injected; it showed typical hyphae and chlamydospores and was agglutinated by C. albicans antiserum but failed to give characteristic colonies on eosin-methylene blue agar. The second strain (Y65) failed to affect guinea-pigs on two occasions and affected two only of four mice injected; otherwise it gave positive results with the other tests. The third strain (Y54), C. albicans by all in vitro tests, affected

TABLE I.

Results of Various Tests on 78 Strains of Yeast-like Organisms

1. Agglutination	+	+	+	-	-	-	-	-	-
2. Formation of hyphae	+	+	-	+	+	+	-	-	-
3. Formation of chlamydospores	+	+	-	-	-	-	-	-	-
4. Animal pathogenicity	+	+	-	-	-	-	-	-	-
5. Fermentation of dextrose	AG	AG	AG	AG	AG	A	AG	A	A
6. Fermentation of maltose	A(G)	A(G)	A(G)	A(G)	A	A	A(G)	A	-
7. Eosin-methylene blue agar test	+	-	-	-	+	-	-	-	-
Number of Strains	44	6	1	9	1	2	5	8	2

Incubation time for tests 2, 3 and 7 extended to five days.
Incubation time for tests 5 and 6 extended to 28 days.
AG means acid and gas.
A(G) means acid with or without gas.
In test 4, + means that at least one of the animals used gave a positive reaction.

one of three guinea-pigs and one of four mice tested.

The results with mice were similar to those with guinea-pigs in most cases. The typical result with a pathogenic strain was a definite swelling in two to three days persisting for one to two weeks. However, six strains of C. albicans gave the reaction in only one of two mice injected, four strains affected only one of three mice, one strain affected one of four mice, and three strains affected two of four mice.

No organism not C. albicans affected mice or guinea-pigs.

When one animal only is being used, the guinea-pig is more reliable than the mouse.

Agglutination Tests.

For the preparation of an anti-serum, a strain of C. albicans (Y15) giving typical reactions of the species was chosen. It formed hyphae and chlamydospores within 48 hours in corn-meal extract, formed acid and gas in dextrose and maltose, acid in saccharose with no change in lactose, was pathogenic for the rabbit, guinea-pig and mouse and gave characteristic colony appearance on eosin-methylene blue agar.

The anti-serum agglutinated the homologous organisms to a titre of 1:80. When all 78 strains were tested (using serum in dilution 1:20) all strains judged to be C. albicans by the other tests were agglutinated, as well as a few others. Absorption of the serum with one of the latter (Y9) removed agglutinins for all the organisms which were not C. albicans, with one exception (Y78). It was not found possible to remove the agglutinins for this strain without removing those for the homologous strain. Microscopically strain Y78 was easily distinguishable from C. albicans in that the cells were only half the size of those of the pathogens.

TABLE II.

Results of Tests on 50 Strains of C. albicans

1. Agglutination	+	+	+	+	+	+	+	+	+	+
2. Formation of hyphae	+	+	-	+	+	+	-	-	-	-
3. Formation of chlamydospores	+	+	+	+	-	+	+	+	+	-
4. Animal pathogenicity	+	+	+	±	+	±	±	+	±	+
5. Eosin-methylene blue agar test	+	-	+	+	+	-	+	-	+	+
Number of Strains	19	6	6	8	1	3	1	4	1	1

Incubation time for tests 2, 3 and 5 was limited to 2 days.
± in test 4 means that more than one animal was used and not all were positive.
All strains gave acid and gas in dextrose and acid in maltose, with or without gas, within 48 hours.

The results given in Tables I and II are those obtained using anti-serum Y15, in dilution 1:10, after absorption with strain Y9.

A strain of Candida (Y53), isolated from a finger-nail lesion, was given special examination since it was isolated in pure culture on three occasions from the same patient at three months intervals. The condition suggested pathogenicity in the organism, but although it produced hyphae it failed to produce chlamydospores, was non-pathogenic for the rabbit, guinea-pig and mouse, did not give the typical colonies on eosin-methylene blue agar, and was not agglutinated by anti-serum Y15. A serum prepared with this organism gave a titre of 1:40 only and agglutinated C. albicans to the same titre. Attempts at absorption with varying quantities of pathogenic organisms, reduced and finally removed the agglutinins for the homologous organisms simultaneously with those for the absorbing organisms. No further use was made of this serum.

On account of the rapidity of the tests, instability of suspensions caused no difficulties in reading results.

Eosin-Methylene Blue Agar Tests.

Most strains of C. albicans grown on this medium gave colonies within 24-48 hours with the typical feathery or spidery appearance at the periphery as described by Weld. The reaction, however, was one of degree as well as quality, some strains being strongly while others were weakly positive. When some strains were streaked out on a plate, it was not uncommon to find only one or two colonies giving the positive effect, while a hundred or more were negative. This may explain the fact that six strains of C. albicans failed to give the reaction at all in five days, while one strain negative by the plating-out method showed a positive effect, but only at one part of the periphery of a mass-colony produced by "spot-inoculation". Two strains of C. albicans

TABLE III.

Sources of Strains

	Urine	Sputum	Nails	Faeces	Vagina	Skin	Throat	Pus	Cerebro-spinal Fluid	Unknown	Total
C. albicans	5	16	8	3	5	6	4	2	-	1	50
Not C. albicans	6	-	11	1	-	8	-	-	1	1	28

did not appear positive until the third day of incubation and five until the fourth day. One strain of Candida of unknown species gave a weakly positive effect indistinguishable with a lens from that given by weakly positive strains of pathogens.

Plating out to give single colonies was superior to "spot-inoculation" to detect the reaction. Only one strain was positive with the second method and negative with the first, whereas six strains gave the reverse result.

DISCUSSION AND CONCLUSIONS

Source of organisms is a very unsatisfactory guide to their pathogenicity, as will be seen from Table III. Of the 50 strains judged to be C. albicans, only 24 were from sources which indicated pathogenicity. These were from cases of paronychia, vulvo-vaginitis, thrush, superficial skin lesions and an infected palm. Of the 28 strains not C. albicans, 19 were from sources which indicated pathogenicity. These were cases of onychia, paronychia and superficial skin lesions.

Of the five tests used on each strain in this investigation, no single test has been found which gives complete correlation with the final decision as to whether an organism was C. albicans or not.

An organism isolated on eosin-methylene blue agar, giving the typical feathery or spidery appearance around the colonies can be accepted as C. albicans. However, if this is the only test, there will be a number of false negative results if the incubation time is limited to two days and still a few with the time of incubation extended to five days. If mere traces of reaction are ignored, there will be no false positive results.

The use of corn-meal extract with Congo red, which takes longer since the organism has first to be isolated, gives reliable results

with most strains, but here again we have the occasional strain which reacts slowly or gives a result which is indefinite and requires the support of a confirmatory test.

The use of animals is not always practical nor economical, and was developed in this series of tests for confirmatory purposes. The results were similar to those found in the above two tests. Most pathogens gave a definite reaction, but some were on the borderline as regards pathogenicity, reacting with one or more, but not all, animals when more than one was inoculated.

Fermentation reactions have limited value. As confirmatory tests, they help to exclude non-pathogens; strains which do not form acid and gas in dextrose and acid in maltose in 48 hours, can be regarded as not C. albicans.

Slide agglutination provides a rapid and simple method of testing. But here again, although the error is smaller than with any of the other tests, it is not completely accurate. One strain, not a Candida (see Table I), gave a positive reaction, but if this strain had been eliminated as not C. albicans because of its microscopic appearance (see above), the use of absorbed anti-serum gave the simplest and most reliable single test for use where a result is required within a few days.

In Table II the results of the various tests on the 50 strains of C. albicans are given, where the results of the tests with corn-meal extract and eosin-methylene blue agar were those obtained after incubation for two days.

SUMMARY

1. A method of using guinea-pigs and mice to detect pathogenicity in strains of C. albicans is described.

2. Agglutination of C. albicans by the slide method has been shown to be reliable.

3. Seventy-eight strains of yeast-like organisms have been submitted to a number of tests to decide upon a method of identifying C. albicans. The tests were fermentation reactions, formation of hyphae and chlamydospores in corn-meal extract, animal pathogenicity tests, slide agglutination with an absorbed anti-serum and colony appearance on eosin-methylene blue agar. No single test was completely accurate.

4. Eosin-methylene blue agar is a valuable medium for isolating and detecting C. albicans.

5. Slide agglutination gives the smallest number of errors where a reasonably rapid decision has to be made.

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ADDENDUM

Since going to press, three further strains, not C. albicans, have been isolated, which were agglutinated by anti-serum Y15. They were similar to Y78 in that they were isolated from urine and were distinguishable from pathogens by the smaller size of the cells.

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THE DISINFECTION OF HOSPITAL BLANKETS

by BRYAN C. STRATFORD, M.B., B.S. (Melb.)

(Deputy Medical Superintendent, St. Vincent's Hospital Melbourne)

R. CHRISTIE, M.Sc.

(Bacteriologist, St. Vincent's Hospital, Melbourne)

- and -

SHIRLEY DIXSON

(Research Officer, Department of Bacteriology,
University of Melbourne).

The control of cross-infection by Staphylococcus aureus in hospital wards requires attention to all possible sources of the organism. One source which has recently received much attention is the woollen hospital blanket.

Although proof is lacking that the sterilization of blankets

will reduce the cross-infection rate (Blowers et alii, 1957), we agree with the suggestion of the Lancet (Editorial, January 24, 1959) that blankets should be regularly disinfected as part of an over-all attack pattern on the reservoirs of Staph. aureus in hospitals. With this aim in mind we considered chemical treatment, this being possibly less laborious and expensive than heat treatment. Quaternary ammonium compounds had been thoroughly investigated, but, whilst sterility of the article at the end of the laundry process seemed assured, any lasting sterility was questionable. Despite this, some manufacturers had claimed that processing with their product effected sterility lasting beyond the laundry process. Chlorhexidine ("Hibitane") was reputed by some trade magazines to endow the blanket with sterility, lasting from "two to six weeks", or to make the blanket "self-sterilizing". In view of these latter claims, it was decided to test chlorhexidine as a product endowing the blanket with some persisting sterility following laundering. When this method failed, investigation of formalin vapour as a disinfecting agent was pursued. This was not in any sense a new process; but by a somewhat different usage a satisfactory result in terms of time and economics was achieved.

METHOD

Chlorhexidine Digluconate Impregnation.

Bed curtains, counterpanes, pillow-slips and blankets were washed by routine laundry methods, save that chlorhexidine digluconate (1:40,000) was added to the final rinse water. These textiles were then dried in the normal way. Two beds in a four-bed ward were made up with these textiles, whilst the corresponding two beds were made up with normal "laundry clean" textiles.

The textiles on both the treated and control beds were sampled by the single, direct contact method of Rubbo and Dixson (1960). The samples were taken 24, 48 and 72 hours after the introduction of the

textile into the ward. Egg-yolk medium was used to antagonize the "Hibitane", and the plates were incubated at 37°C. for 48 hours.

Next, the final rinse of chlorhexidine digluconate was increased to 1:100 concentration and the organism build-up was followed from the moment of drying to 72 hours.

Formalin Treatment.

The object of this investigation was to disinfect blankets by a process which was both inexpensive and rapid; nonetheless, this process had to be effective against the organisms commonly found in hospital cross infection - namely, Staph. aureus, Proteus, Pseudomonas pyocyanea and the enteric bacilli.

Initially, a room of 1000 cu. ft. capacity was sealed off, with the use of sponge-rubber seals and a screw-down door, and blankets were hung in it vertically from ropes near the ceiling in the same way as washing hangs from a line. The temperature of the air in the room was raised to at least 70°F by means of two radiators, and a conventional fan was the source of air circulation. The relative humidity in this room was raised to between 80% and 90% by spraying the walls, floor and blankets with a fine spray of warm water.

Formalin vapour was generated from commercial formalin (containing 10% added ethyl alcohol to reduce polymerization) heated in a metal container on an electric hot-plate. One pint of formalin was used for any one treatment (M.R.C. Report, 1959).

In the early experiments the blankets were exposed to the vapour for two hours, but subsequently the exposure time was reduced progressively to fifteen minutes. As the average colony counts after 15 minutes' exposure were not appreciably higher than those after two hours, we decided to adopt 15 minutes as our exposure time.

Contact sampling suggested that there was some "carry-over" of the formalin on to the agar plates, so the method of sampling adopted in this series was on to 6% blood agar plates by the modified percussion technique of Rubbo and Dixson (1960), initially described by Puck et alii (1946). Colony counts were made after 24 hours' incubation at 37°C.

Blankets used in these tests were taken at random from ward beds, where they had been in use for at least three consecutive weeks.

As the size of the room used in the previous experiments precluded the use of many blankets at any one time, it was decided to seek a larger chamber. The Chemical Division of the Defence Standards Laboratories, Maribyrnong, kindly allowed us to use their gas chamber. This was a sealed room of 100 cu. metre capacity with adjustable heat and humidity, an internal air-circulating system and an exhaust-fan system capable of exchanging the air in the chamber once every four minutes. Intake of near-sterile air was achieved by placing a battery of seven ultra-violet tubes over the source of air to the chamber (an aperture of 1 sq. ft.); the resultant intake of air showed only Bacillus species, and in all our tests no other organism was demonstrated as passing the ultra-violet filter.

Blankets, up to 24 in number, were hung full-length from ceiling ropes and placed approximately one foot apart. Blankets packed in laundry trolleys were also placed in the chamber, but the experimental results on these were found to be unpredictable, so exposure of the total area of the blanket was considered desirable.

The temperature of the chamber was brought to at least 70°F., but not more than 80°F.; humidity was raised to at least 90%, a steam jet being used as the source of water, and the internal air-circulating system was commenced. Next, 3.6 pints of formalin plus 10% ethyl alcohol was vaporized on four conventional hot-plates. Fifteen minutes' exposure

to the gas was allowed, and then the exhaust system was set in use. The blankets were sampled by percussion technique immediately before exposure to the gas, and to minimize any time error, gas masks were donned and the blankets were sampled at the end of the 15 minutes' exposure to the formalin treatment - that is, while the chamber was being cleared.

RESULTS

In our experiments, we found that the use of the final rinse of "Hibitane" in concentrations of either 1:40,000 or 1:100 conferred no lasting sterility. The textiles treated came from the laundry "sterile" but no more so than those subjected to contemporary laundry processes as exhibited by the Central Linen Service of the Royal Melbourne Hospital.

Table I compares the "build-up" of organisms on textiles treated with "Hibitane" (1:40,000) in the final laundry rinse, and those on laundry-clean textiles. The contact method of sampling was used, and the figures shown represent an average of the total on two beds in each case.

It is readily apparent that this treatment initially provides a textile which is just as easily contaminated as the normal laundry article. Further, the treated textile in no way withstands ward contamination.

Table II records a similar experiment using textiles treated with a final rinse of 1:100 "Hibitane" solution; that is, the concentration of the antibacterial agent has been increased 400 times. Again, the results show that even in this strength the antibacterial agent is not capable of exerting its bactericidal effect. There was no doubt of its being present, as the textiles presented a rigid feel to the hand, and small crystals of the "Hibitane" salt could be seen amongst the fibres.

TABLE I.

Comparison of Organism Build-Up on Textiles Treated with "Hibitane" (1:40,000) and Laundry-Clean Textiles, Using Contact Plate Technique

Textile	Average Number of Organisms Isolated from Two Beds					
	First Day		Second Day		Third Day	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
Curtain	9	35	11	31	18	34
Counterpane	12	3	640	350	570	490
Blanket	3	8	27	15	23	48
Pillow-slip	75	8	1000	175	1200	1100

TABLE II.

The Build-Up of Organisms on Textiles Treated with 1:100 "Hibitane" Solution Using Contact Plate Technique

Textile	Average Number of Organisms Isolated from Two Beds				
	Immediately	At 3 Hours	At 24 Hours	At 48 Hours	At 72 Hours
Curtain	8	31	100	40	40
Counterpane	41	>400	300	>400	>400
Blanket	43	40	57	100	100
Pillow-slip	35	142	200	175	150

TABLE III.

The Formalin Treatment of Six Blankets in the Small Room, Exposure Time 15 Minutes, Percussion Sampling Technique

Number of Blankets	Average Count Before Treatment	Average Count After Treatment	Percentage Kill
2	425	9	98
2	325	26	92
2	250	30	88

TABLE IV.

The Formalin Treatment of 86 Blankets in the Gas Chamber, Exposure Time 15 Minutes, Percussion Sampling Technique

Number of Blankets	Average Count Before Treatment	Average Count After Treatment	Percentage Kill	Percentage Kill Less Bacillus Species
20	200	20	90	95
21	197	9	95	98
21	289	34	88	94
24	172	8	95	97

Table III shows the results of the initial formalin treatment in the small sealed room. The results are reported as an average colony count of two blankets in each case. This treatment resulted in a percentage kill of 98%, 92% and 88% on the three occasions. The percussion method was the sampling technique used.

Table IV shows the results of formalin treatment of 86 hospital blankets, all of which had been in use for at least three weeks. Percussion was the method of sampling, and the gas chamber was the locus operandi. These tests were performed in four batches, and the table refers to the average colony counts on each occasion. The total average kill is 91.3% after 15 minutes exposure. When surviving Bacillus species are removed from the total, the average kill is 96%.

DISCUSSION

Reports of infection in which the blanket is regarded as a possible source of pathogenic staphylococci are becoming more common (Frisby, 1957); Schwabacher et alii, 1958; Caplan, 1959). Other investigators have failed to detect a decrease in the cross-infection rate when sterilized blankets were used (Clarke et alii, 1954; Shooter, 1958). Gillespie et alii (1959) suggest that the answer to hospital cross-infection probably lies in attention to more than one object at any one time - for example, blankets, nasal carriers, crockery, etc. They found that this widely-based approach to the problem achieved the desired result.

Nonetheless, unless hospital blankets are visibly soiled, convention has it that they are usually aired and stored until required again, or, even worse, simply left on the bed for the next patient to use. In either event, the blanket, be it wool or cotton, remains to act as a reservoir of organisms in the ward. Because of this, some form of regular disinfection is not only desirable but obligatory.

Small-scale laboratory tests in this hospital showed that

a suspension of Staph. aureus dried on glass had no organisms surviving after one week; on cotton, the organisms were not recoverable after two to three weeks; on the other hand, organisms on woollen blankets were still viable after five weeks in one test and 11 weeks in another. These figures are not included to indicate the limit of survival, but merely reflect the number of samples available for daily testing. Schwabacher and Salsbury (1958) found organisms alive on wool after 30 days, but none on cotton after seven days.

It behoves us now to consider the existing methods of blanket disinfection, which fall into two broad categories -

(a) wet processes, which include chemical disinfection and boiling, and

(b) dry (gaseous) processes, which use ethylene oxide or related compounds and formalin vapour.

All wet processes require shrink-resistant blankets for success on an institutional scale, and although this presents no problem with new purchases, the same does not hold good for existing stocks of blankets. Again, laundering is a relatively expensive process. The charge for washing a blanket in an Australian commercial laundry is approximately 2s. As most hospital blankets are not soiled on the discharge of the patient, because they are protected by both the counterpane and the uppermost sheet, weekly lauhdering appears an unjustified expense. For such unsoiled blankets dry sterilization meets most bacteriological requirements. Soiled blankets must obviously be sent to the laundry, where the washing method used should include a bactericidal treatment.

Wet Processes:

Chemical Additives.

"Fixanol C" and "Cirrasol OD" and other quaternary ammonium compounds are effective so far as immediate sterilization of the blankets

is concerned; but lasting sterility, as claimed by some authors, is questionable (Rountree, 1946; Gillespie et alii, 1958; Marsh, 1958; Frisby, 1957; Blowers and Wallace, 1955). "Hibitane", used in the final rinse in laundering textiles, at a concentration of 1:40,000 has been recommended by some commercial journals. In fact, in our experiments the concentration was increased some 400 times, a 1% solution being used in the final rinse, but without effect. Thus, on a cost basis alone, it is doubtful whether a final "Hibitane" rinse presents any advantage over usual laundry methods.

Boiling.

This is a practical and effective process for disinfecting blankets, provided that a specially selected detergent is used (Cunningham, 1956; Pressley, 1960). But routine boiling of blankets by a central linen service implies both collection and transportation from the hospitals concerned to the central unit; the use of a special boiling technique with detergents; drying and airing; individual packaging of the "sterile" blankets; and finally retransportation to the hospital whence they came. In all, it is undoubtedly an expensive process.

Dry (Gaseous) Processes.

At present, the following three gaseous methods of disinfection are available:

1. Ethylene oxide. This compound is not readily obtainable in Australia, but has been stated to disinfect blankets when used in a concentration of 10% during an overnight treatment (Kaye, 1950).

2. Propylene oxide. This gas behaves in a manner similar to ethylene oxide, and in fact is used to sterilize blood-transfusion and laboratory equipment in this country.

The factors against the general use of these gases are their cost, their explosive nature, the need for an autoclave and the

unnecessarily prolonged time required to achieve sterility.

3. Formalin vapour. It is our contention that, in all respects, it is simpler, cheaper and best for the individual hospital to treat its own blankets, using formalin vapour as the bactericidal agent. This in no way clashes with the boiling process described earlier, which we consider to be complementary to the formalin treatment.

Nordgren (1939) has shown that the bactericidal action of formalin vapour increases with temperature and humidity. Gillespie and Alder (1957) reported satisfactory sterilization of blankets using formalin vapour in an autoclave where a preliminary vacuum was created, facilitating penetration of the vapour. In this instance, after one hour's exposure to the vapour, it was found that 95% of the organisms were killed, the bulk of the survivors being spore-bearing aerobes. Caplan (1959), using the same method, obtained similar results. In addition, he noted that in over 1000 cases there was no evidence of either damage to the blanket or sensitivity in the patient.

The formalin treatment on the limited scale performed in the sealed room (Table III) reduced the average total organism count by 98%, 92% and 88% on three consecutive occasions. No Staph. aureus survived the treatment. As these blankets were taken out of the treatment room for sampling purposes (compare tests in gas chamber, Table IV), some recontamination from the air may have occurred.

However, the tests conducted in the gas chamber gave an average total reduction of organisms of 91.3% after 15 minutes' exposure to the formalin vapour using high temperature and humidity. If one removes surviving Bacillus species from these figures, the average reduction of organisms becomes 96%. Even when blankets artificially contaminated, with both Staph. aureus and Ps. pyocyanea were used, neither of these organisms survived the treatment.

It is not in any way suggested that the formalin treatment confers lasting sterility on blankets - it is doubtful if any known process does (Rubbo, Stratford and Dixson, 1960) - but it has the advantage of being both inexpensive and easily controlled. The design of a suitable unit for any hospital is not difficult to envisage, and ideally we believe that blankets should be treated at least once a week. Moreover, whenever a patient is discharged from hospital, the next patient in the bed should be received into treated blankets, and should not merely inherit those of the man who went before. We recommend that after treatment blankets should be wrapped in sterile plastic envelopes and returned to the general ward or hospital store; but it must be emphasized that blankets soiled with discharge from wounds, faeces, etc., or obviously dirty blankets, should be sent to the laundry for boiling or other comparable sterilization processing.

SUMMARY

1. A review of the literature on disinfection of blankets is presented.

2. Experiments using "Hibitane" in both 1:40,000 and 1:100 concentrations in the final laundry rinse are described. No lasting sterility resulted in either case.

3. Experiments using formalin vapour to treat 92 used hospital blankets in the presence of high humidity and controlled temperature are described. The average reduction of organisms (less Bacillus species) is 96% by this method. The time of exposure to the vapour was 15 minutes.

4. The conclusion is reached that it is practicable to treat all hospital blankets in use at least once a week by this method. However, obviously soiled or dirty blankets should be referred for laundry treatment.

ACKNOWLEDGEMENTS

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HYPERTONIC FRAGILITY OF ERYTHROCYTES

by

R. CHRISTIE, H. J. DOWNING and LYNETTE J. DUMBLE

(From the Red Cross Blood Transfusion Service, Melbourne)

(Submitted for publication, 3rd March, 1969)

The osmotic fragility test with erythrocytes selects cells which are more sensitive to a lower saline concentration than are normal cells, and is much used in clinical work. It is carried out by adding erythrocytes to a series of concentrations of saline (0.9 per cent to 0.1 per cent) and noting the concentration of saline at which haemolysis occurs. Normally it begins at 0.5 per cent and complete haemolysis is obtained with solutions of 0.25 per cent.

Another haemolytic phenomenon, involving hypertonic solutions is reported here. When human erythrocytes are suspended in saline concentrations ranging from 1 per cent to 10 per cent, no visible change occurs, but when the cells are then resuspended in 1 per cent saline haemolysis commences with those cells which were previously suspended in 5 per cent saline. This haemolysis is complete or nearly so for the cells which were originally suspended in 10 per cent saline.

The method used was to add a drop (0.05 ml.) of heparinized blood to 1 ml. of each of 10 concentrations of saline ranging from 1.0 per cent to 10 per cent. Distilled water was then added to each tube, slowly and with constant shaking, to adjust the final concentration in all tubes to 1 per cent. The volume in each tube was then adjusted to 10 ml. by the addition of more 1 per cent saline. The suspensions were then centrifuged to remove stroma and unlysed cells and the haemolysis in each tube was measured with a colorimeter. A control tube containing one drop of erythrocytes in 10 ml. distilled water was used to give the reading for complete haemolysis. The other readings were then expressed as percentages of this reading.

The tests were carried out on 100 individuals with the possibility of finding definite variations but no appreciable variations from the average figures were found. To obtain definite differences it was necessary to use other species.

Cells from horses, sheep, cattle, guinea pigs and rabbits showed increased resistance to the action of hypertonic saline. For these species haemolysis commenced in the cells suspended in 6 to 7 per cent saline and was only 20 to 40 per cent complete in 10 per cent saline.

Conversely cells from species with nucleated red cells - pigeons, hens and toads - showed a greater sensitivity than those of humans, haemolysis commencing at 3 to 4 per cent and being complete, or nearly so at 6 to 7 per cent saline.

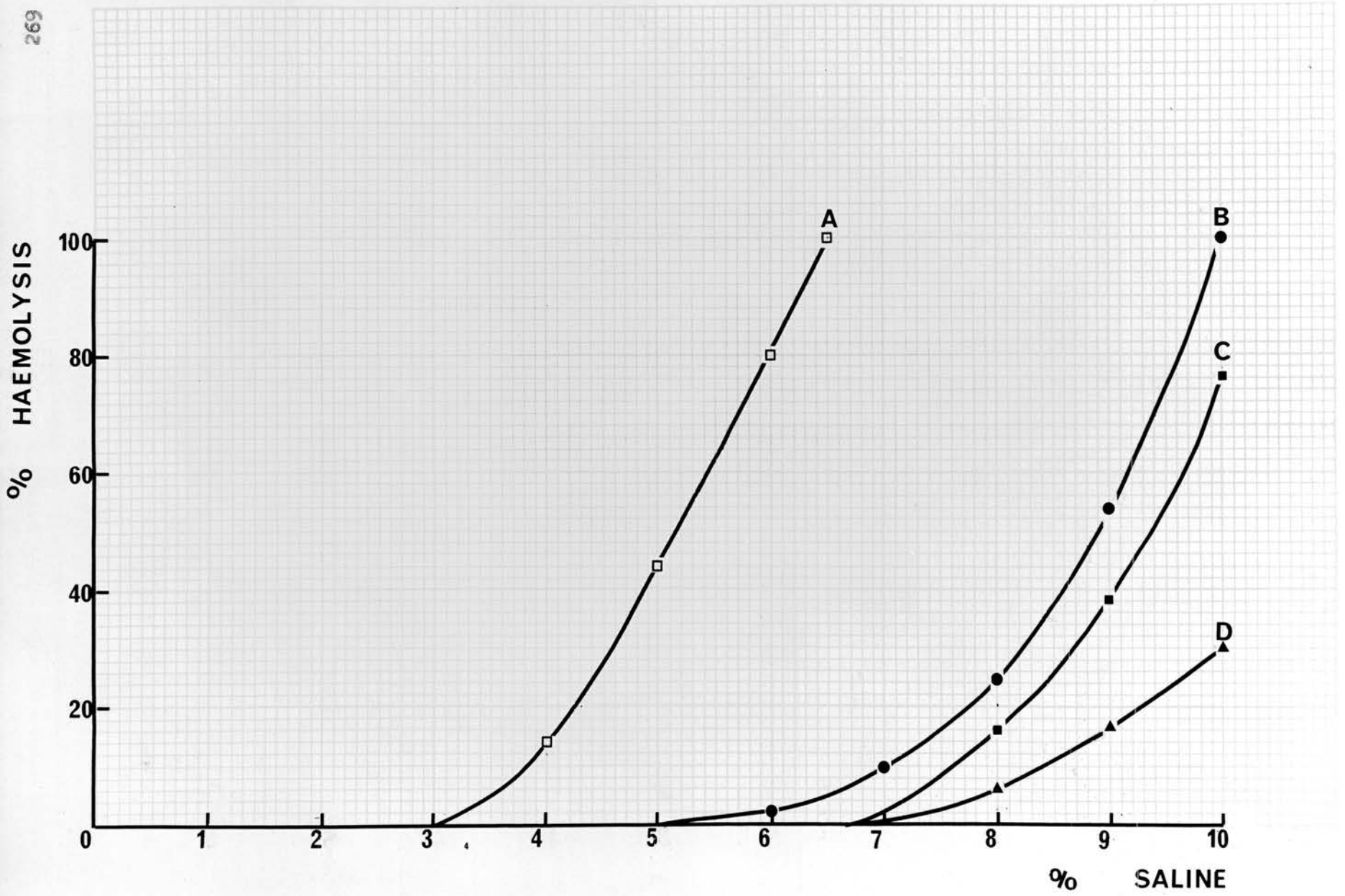
Cells from dogs, cats and monkeys gave figures intermediate between those for humans and such herbivors as were tested. The average results are shown in Figure 1.

The possibility that the haemolysis was caused by the distilled water affecting the cells before adequate mixing with saline

was eliminated by repeating the tests with a procedure that did not employ distilled water, the cells being suspended in the 10 concentrations of saline, centrifuged and resuspended in 1 per cent saline. The results did not differ significantly from those obtained by the original procedure.

The possibility that loss of potassium by diffusion might have been a factor concerned with the haemolysis was discounted when the tests were repeated in saline concentrations made from a stock solution of 9 per cent NaCl and 1.3 per cent KCl instead of the usual 10 per cent NaCl. No changes in the curves were obtained.

It is possible that differences in curves may be detectable for people with various dyscrazias if the tests are repeated with more rigid conditions of temperature, pH and time intervals. Work is proceeding along these lines.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THE HL-A ANTIGEN SYSTEM - THE INCLUSION OF THE 7c ANTIGEN IN THE 4b ANTIGEN

by

R. CHRISTIE, LYNETTE J. DUMBLE, H. DOWNING
and M. BERAH

(Red Cross Blood Transfusion Service, Melbourne)

(Submitted for publication March, 1969).

In studying transplantation antigens of the HL-A system, Kissmeyer-Nielsen et al (1) have shown that in a Scandinavian population the 7c antigen on lymphocytes, detected by cytotoxicity tests and on platelets, detected by complement-fixation tests was included in the 4b antigen. However this was not found by Dausset et al (2).

In the present communication we present the results of a series of tests, using leuco-agglutination as well as lymphocytotoxicity supporting the view that the 7c antigen is included in the 4b antigen.

The antigens on the leucocytes of 120 unrelated Australians (Caucasians) were studied by means of the leuco-agglutination technique of van Rood (3) and by the macrocytotoxicity test of Walford (4). The agglutinating anti-4b and anti-7c sera were numbers 1057 and 594

respectively from Dr. van Rood's panel. For the cytotoxicity tests the anti-4b sera were two of our own (Hinchcliffe and Walls) while the anti-7c sera included one of our own (Pellegrinelli) and a serum (Vujevic) from Mr. Alex Sharp, blood Transfusion Service, Sydney.

The results obtained by both methods are presented in Table 1.

TABLE 1

Comparison of the Incidence of Antigens 4b and 7c in 120 Unrelated Australians

Antisera		+/+	+/-	-/+	-/-	Total
4b	7c	32	69	0	19	120

It will be seen that of the 120 tested the 7c antigen was never found in the absence of the 4b antigen although the reverse occurred in 69 cases. This supports the contention of Kissmeyer-Nielsen that the 7c antigen is included in the 4b antigen using the same technique he used and a further technique of leuco-agglutination.

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INCIDENCE OF LEUCOCYTE ANTIBODIES INDUCED BY PREGNANCY AND THEIR CONNECTION WITH ABORTION

by LYNETTE J. DUMBLE, R. CHRISTIE
and H. J. DOWNING

(Red Cross Blood Transfusion Service, Melbourne).

It is now well recognised that pregnancy can be responsible for the formation of leucocyte antibodies in the serum of pregnant women when the foetus has an antigen possessed by the father and not by the mother (1 and 2).

During a survey carried out here on the sera of women who had had three or more pregnancies it was noticed that the percentage of cases with cytotoxic leucocyte antibodies in the serum was significantly higher in the series of women with a history of abortion than in the series without such a history.

307 sera were examined using the lympho-cytotoxicity test of Walford, (1965), (3). Fifty-three (17.3%) of these sera contained cytotoxic leucocyte antibodies. Of the 118 sera from women with a history of one or more abortions, 28 (23.7%) contained cytotoxic leucocyte antibodies, whereas in the 189 sera from women with no history of abortions, only 25 (13.2%) showed the presence of antibodies. These

results are shown in Tables 1a and 1b.

TABLE 1a

Presence of Cytotoxic Leucocyte Antibodies in Multigravidae With and Without History of Abortion

	One or more abortions	No abortions	Total
With cytotoxic leucocyte antibodies	28	25	53
Without cytotoxic leucocyte antiobodies	90	164	254
Total	118	189	307

$\chi^2 = 5.6$; $p < 0.05$ (one degree of freedom).

TABLE 1b

The Data in Table 1a, subdivided Into Groups, According to Number of Pregnancies

No. of pregnancies		One or More Abortions	No Abortions
3	antibodies +	9 (20%)	14 (14%)
	" -	36	85
4	" +	5 (15%)	4 (10%)
	" -	29	41
5	" +	6 (35%)	2 (12%)
	" -	11	15
6	" +	3 (30%)	1 (6%)
	" -	7	16
7 or more	" +	5 (42%)	4 (36%)
	" -	7	7

In seeking a causal connection between the cytotoxic antibodies and abortions, one hypothesis could be that the antibodies cause the abortions. This would be a reasonable suggestion as the cytotoxic antibodies belong to the IgG class of immunoglobulins, which are capable of crossing the placenta (4). Furthermore it has been shown in vitro (5) that cytotoxic leucocyte antibodies can destroy tissues possessing the appropriate antigen.

However our records do not support this hypothesis but rather the second possibility that the abortions precede antibody formation. Foeto-maternal bleeding, at the time of the abortion, may be responsible, resembling the formation of Rh antibodies following delivery. This possibility was supported by examination of the obstetric history of each of the 28 cases with a record of one or more abortions where antibodies were present. In these 28 cases, 11 were found to have had one or more abortions <u>before</u> the first normal birth, as shown in Table 2. (This high proportion supports the hypothesis that abortion precedes antibody formation).

<u>TABLE 2.</u>

<u>Obstetrical Details of 28 Multigravidae with Cytotoxic Antibodies in Their Sera and a History of Abortion</u>

One or more abortions preceding a normal delivery	No.	Abortions following one or more normal deliveries	No.
$A_1\ T_{2,3\ or\ 4}$	6	$T_1\ A_1\ T_{1,2\ or\ 3}$	7
$A_2\ T_{1,2\ or\ 5}$	4	$T_1\ A_2\ T_{1,2\ or\ 3}$	2
$A_3\ T_3$	1	$T_2\ A_1\ T_{1,2\ or\ 3}$	2
		$T_3\ A_{1\ or\ 2}\ T_{1,2\ or\ 3}$	5
		$T_5\ A_2\ T_1$	1
	11		17

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Table 2 cont'd...

A = abortion; T = terminal.
Subscripts represent numbers of abortions or terminal deliveries.

SUMMARY

The results of testing 307 sera from pregnant women for the presence of leucocyte antibodies suggest that abortion is a stronger stimulus in the production of these antibodies than a full term pregnancy.

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THE PLATE TEST FOR COAGULASE PRODUCTION BY STAPHYLOCOCCI

by E. F. WOODS and BARBARA J. PARKIN

(From the Commonwealth Serum Laboratories, Melbourne)

(Accepted for publication 16th November, 1945)

The ability of staphylococci to coagulate plasma is regarded as the best single test of their pathogenicity. In the usual method diluted plasma in test tubes is inoculated with staphylococci, the presence or absence of a clot being noted after incubation at 37°C.

Penfold (1944) used a solid medium for the test. Colonies of coagulase-producing staphylococci grown on agar containing 20 p.c. human plasma were surrounded by zones of opacity in the medium. Reid and Jackson (1945) described a similar test and recommended the use of plasma agar plates in the primary isolation of pathogenic staphylococci. The medium could be stored in the cold for at least a month.

The purpose of this paper is to report the results obtained here when this medium was used to test a number of strains of staphylococci of known characteristics.

MATERIALS AND METHODS

Plasma Agar.

Nutrient agar was melted, cooled to 45°C. and 20 p.c. oxalated human plasma added. The mixture was then poured into Petri dishes, allowed to solidify and stored at 4°C. until required.

Nine strains were "spot inoculated" on to each plate with a heavy inoculum from a 24 hours' agar slope culture and readings made after 4 hours' and 24 hours' incubation at 37°C. Inocula from broth cultures were found to be less satisfactory since results were less definite after 4 hours.

Citrated plasma gave the same results as oxalated plasma.

Tube Test for Coagulase.

Oxalated human plasma, diluted ten times with normal saline, was dispensed in small tubes, 2 ml. per tube. These were inoculated from 24 hours' agar slope cultures and the presence or absence of a clot noted after 4 hours' and 24 hours' incubation at 37°C.

Fibrinolysin Production.

This was determined by inoculation of agar plates rendered turbid with heat-precipitated fibrinogen (Christie and Wilson, 1941).

Fibrinogen Solution.

A saturated solution of sodium chloride was added to an equal volume of oxalated human plasma and the mixture stored overnight at 4°C. The precipitated fibrinogen was separated by centrifugation and re-dissolved in an amount of distilled water equal to that of the original volume of plasma.

RESULTS

Table 1 shows the results obtained when 151 strains were tested for coagulase production by the tube and the plasma agar plate methods. Of 86 strains positive by the tube test, 76 gave a positive reading on the solid medium after 4 hours and 82 after 24 hours. Two strains were negative after 4 and 24 hours, 8 were negative after 4 hours and positive after 24 hours, while 2 were positive after 4 hours and negative after 24 hours. Of the 65 strains which were negative by the tube test, 5 showed narrow faint bands on the plates after 4 hours and 22 after 24 hours.

The zones of opacity varied in intensity and width with different strains and with different samples of plasma. Some strains gave a narrow intense zone around the colony, others a broad faint zone, while some showed both types of opaque zones.

The broad faint zone appearing in 4 hours was in no case given by a coagulase-negative strain. All these zones at the end of 24 hours' incubation were more extensive and some gave an annular effect with a clear zone between the colony and the opaque zone (as reported by Reid and Jackson).

Table 1.

Comparison of the Tube Test for Coagulase Production by Staphylococci and the Formation of Opaque Zones on Plasma, Serum and Fibrinogen Agar Plates.

	Tube test for coagulase.	No. of strains		Plasma agar No. pos.	Serum agar No. pos.	Fibrinogen agar No. pos.
Human strains	Positive	62	4 hrs	58	22	60
			24 hrs	58	60	59
	Negative	65	4 hrs	5	5	0
			24 hrs	22	24	18
Animal strains	Positive	24	4 hrs	18	18	22
			24 hrs	24	24	24

Difficulty was experienced in detecting the opacity given by positive strains with some samples of plasma.

The false positive zones given by coagulase-negative strains at the end of 4 hours were all much narrower, but this cannot be used as a distinguishing feature, since a few of the "true positives" were similar. Of the 22 "false positives" after 24 hours' incubation, 7 were distinguishable from the true reaction in that they gave only narrow intense zones close to the colony, but one coagulase-positive strain gave a similar reaction. Six of the false positive reactions were indistinguishable from those given by coagulase-positive strains. The remaining 9 gave zones, similar to the "true positives" in appearance, but fainter and less extensive.

The tests were then repeated using serum agar in place of plasma agar. The tests were done in triplicate using serum obtained by clotting oxalated plasma and two different batches of serum obtained from naturally clotted blood samples. Very faint zones of opacity were seen with some strains at the end of 4 hours. After 24 hours, 84 of the coagulase-positive strains and 24 of the coagulase-negative strains showed zones of opacity (see Table 1). Of these, 51 were similar to those shown in plasma agar by coagulase-positive strains and 57 were of a narrow intense kind. Six of the coagulase-negative strains gave zones similar to those of the coagulase-positive strains. The intensity of all these reactions varied with the batch of serum used.

The appearances suggested that there were 3 agents capable of causing opacity, one given by coagulase-positive strains, one with a similar effect given by coagulase-negative strains, and a third causing the intensely opaque zones. That the first two of these were different although they gave a similar effect, could be shown by growing a colony of each close to each other on a plasma agar plate. Where the faintly opaque zones overlapped, an area of intense opacity appeared. This overlap phenomenon did not show when two coagulase-positive strains were grown together.

It seemed possible that the false positive reactions obtained on plasma agar were due to one or two of these agents and that the true reactions given by coagulase would be the only one which would show if fibrinogen agar were used. Plates of this medium were prepared from agar and fibrinogen solution mixed at 45°C. The results are shown in Table 1. Eighty-two of the 86 coagulase-positive strains gave faint broad zones of opacity after 4 hours. There were no false positive reactions after 4 hours but 18 of the 65 coagulase-negative strains gave zones of opacity after 24 hours, 9 of these being similar in appearance to those of the coagulase-positive strains, the reaction given by the other 9 being much weaker. In the case of the fibrinolytic strains, the zones of opacity, after 24 hours' incubation, were indefinite and much fainter than after 4 hours, while in the case of the non-fibrinolytic strains, the zones of opacity were of the same intensity but broader. None of the zones of intense opacity which showed with plasma or serum appeared on fibrinogen agar, so that this effect is probably due to the action of an agent other than coagulase on serum albumin or globulin.

Since plasma agar is more easily obtained than purified fibrinogen agar, an attempt was made to modify the test so that only the effect on the fibrinogen would be shown. Plates were prepared from nutrient agar containing 2 p.c. human plasma and, after the staphylococci had been incubated on them for 24 hours, the fibrinogen was precipitated by flooding the plates with 1 p.c. acetic acid. Clear zones showed around some of the colonies, the medium unaffected by exudate becoming turbid. The staphylococci which gave clear zones were nearly all coagulase-positive, but some of the results were indefinite and control plates with serum instead of plasma showed that an effect, though much weaker, was obtainable in the absence of fibrinogen. The tests were therefore repeated on nutrient agar containing isolated fibrinogen in place of plasma. Definite clear zones were again obtained, indicating that some of the staphylococci had so altered the fibrinogen that it was no longer capable of being precipitated with acetic acid as it normally is. A change of

the nature of fibrinolysis must have occurred. When the results with a series of strains were examined, there was good but not complete agreement with the results of the coagulase tests, and with the results of lysis of heat-precipitated fibrinogen. Three coagulase-positive strains which did not lyze heat-precipitated fibrinogen, as most pathogenic strains do, gave positive results with the above test while two others were negative by both tests. Of 27 coagulase-negative strains, 23 were negative by both tests, the remaining four being positive with the acetic acid test only.

Liquefaction of gelatine by the various strains did not correlate with any of the tests with fibrinogen.

DISCUSSION

The use of solid medium for testing for coagulase production has not been as successful in our hands as in those of Penfold, Reid and Jackson. This is possibly due to the larger number of strains we used. The most reliable results were obtained when fibrinogen solution was added to the agar instead of plasma. This medium gave four false-negative but no false-positive results out of 86 coagulase-positive strains tested, provided readings were made after four hours' incubation. After 24 hours' incubation, one false negative and 18 false positive results were obtained.

There are at least four agents elaborated by staphylococci which give opacity on human plasma agar. One of these is coagulase. Staphylococcal β toxin which causes opacity in human serum (Christie and North, 1941; Fulton, 1943) is another, and two others are elaborated by coagulase-negative strains, none of which produces β toxin. At least one of the agents produces the opacity from serum in the absence of fibrinogen.

From the results with fibrinogen agar, it is evident that

staphylococci can affect fibrinogen in three ways. They can induce coagulation, cause lysis of fibrinogen in the heat-precipitated form or alter it so that it is no longer precipitable with acetic acid.

There are at least two agents responsible for these three effects since coagulase-negative strains can produce the lytic reactions. Whether the lytic phenomena are due to two agents or are both caused by the same agent in differing degree or with interfering substances preventing complete agreement, is not certain. Until these reactions have been more completely investigated, the results of the plate test for coagulase should be treated with reserve.

SUMMARY

Eighty-six coagulase-positive strains and 65 coagulase-negative strains were tested for coagulase production on plasma agar plates. Four false negative and 22 false positive reactions were obtained. A further lack of specificity of the test for coagulase was shown by the fact that zones of opacity also formed on serum agar.

More accurate results were obtained if fibrinogen agar plates were used and readings were made after four hours. There was evidence that at least four agents can be elaborated by staphylococci capable of producing opacity on human plasma agar, only two of which have been shown to be indicative of pathogenicity.

Two different effects on fibrinogen, apart from coagulation, induced by staphylococci, are described.

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PENICILLIN RESISTANCE OF STAPHYLOCOCCI

by DOROTHY H. CARD

(Pathology Department, Saint Vincent's Hospital
Melbourne)

When penicillin therapy was introduced, it was soon realized that resistant strains of organisms normally sensitive to the drug were likely to be encountered. The danger that this acquisition of resistance to penicillin might become a property of infecting staphylococci has been stressed frequently.

In an examination of more than 100 strains of staphylococci isolated where penicillin was not in use, Spink, Hall and Ferris (1945) found that all were sensitive to penicillin. This was also found by North and Christie (1945) using a stock collection of 88 strains of coagulase-producing staphylococci collected before penicillin was used for clinical purposes. North, Christie and Rank (1946), in a study of 59 strains isolated from wounds during intensive penicillin treatment, found a considerable number of highly resistant strains. Barber (1947), in an examination of strains from 100 patients, found that 38 were penicillin-resistant; eight of these 38 were isolated from patients who had had no penicillin treatment.

In routine testing of staphylococci for penicillin resistance in this laboratory, it was noticed that a number of strains showed

a resistance which was appreciably greater than that of normal strains. Many were from patients who had received no penicillin treatment, and in view of this it seemed likely that the penicillin sensitivity of the average infecting staphylococcus was already being affected by the general use of the drug. To examine this possibility strains were collected from patients who had no past history of penicillin treatment; staphylococci from all "doubtful" patients were excluded. As the use of penicillin both parenterally and in the form of lotions et cetera is so general, some difficulty was experienced in making a collection, which accounts for the small number (28) of strains used in this survey.

Of the 28 strains, seventeen were isolated from skin eruptions, three from urine, three from sputum, four from eye and ear infections and one from the throat. All were pigmented, coagulase-producing, haemolytic and isolated at an early stage of the disease.

Sensitivity to penicillin was tested by the tube method. Ten millilitre quantities of nutrient broth containing increasing concentrations of penicillin were inoculated with a four milli-metre loop from a twenty-four hour broth culture of the organism and incubated overnight at 37°C. The concentration of penicillin which just inhibited growth, judged by lack of turbidity, was taken as the end point. A known sensitive strain (F.D.A. 209) and a known resistant strain (Crotty) were used as controls.

The standard sensitive strain was inhibited in all tests by one-twentieth of a unit of penicillin per milli-metre and the standard resistant strain by twenty units of penicillin per millilitre.

Of the test strains, sixteen were inhibited by one-twentieth of a unit per millilitre, one by one-tenth of a unit, two by one-fifth of a unit, three by one unit, three by ten units, one by eighty units and two by 160 units.

If one-tenth of a unit per milli-litre is taken as the upper

limit of sensitivity of normal strains, 11 of the 28 strains showed a resistance to penicillin which is definitely above this figure; six strains were able to grow in the presence of five units per millilitre - a concentration above that which can be achieved and maintained in the blood.

The number of strains used in this survey is relatively small, but the proportion of resistant strains is sufficiently high to be significant, especially when compared with the complete lack of resistant strains found in the early surveys.

Not all patients from whom resistant strains were isolated were treated with penicillin, and several failed to report after treatment, so that the records give no adequate picture of the success or failure of penicillin with resistant strains as compared with the sensitive strains.

In view of the results of Warmer and Amluxen (1945) and of North and Christie (1946), which showed that resistance in vivo paralleled resistance in vitro, the present tendency towards indiscriminate use of penicillin should be discouraged.

SUMMARY

An examination of 28 strains of Staphylococcus aureus isolated from patients with no history of penicillin treatment showed that eleven had a resistance to penicillin higher than that shown by normal strains.

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A NOTE ON THE USE OF ANTIBIOTICS IN THE TREATMENT OF INFECTIONS WITH SOME GRAM-NEGATIVE BACILLI

by SHIRLEY V. KEATING, B.Sc.

(Pathology Department, Saint Vincent's Hospital, Melbourne)

With the increasing supply and range of action of antibiotics it is becoming common practice, where the assistance of modern laboratory technique is not readily available, to presume the nature of the infecting organism or organisms on clinical grounds or from simple microscopic examination; the selection of antibiotic is then guided by previous experience or by consultation of a chart showing the action of antibiotics against the various organisms. A number of such charts is available. They are very useful as a preliminary guide, but they fail where there is variability in sensitivity amongst members of the same genus due to inherent differences and/or resistance acquired during inadequate treatment. Even within the same species differences may be found (Enticknap and Stephens, 1951; Garrod, 1951).

When the selected antibiotic fails to suppress the organism, the other antibiotics are usually tried. This empirical procedure runs counter to the essentially scientific character of modern clinical medicine. Not only is this type of empiricism expensive and time-consuming, but possibly it allows unnecessary harm to be caused by the unchecked organism and encourages the development of resistance in other pathogens harboured

by the host (for example, staphylococci).

Where the facilities are available, the infecting organism should be isolated and tested for sensitivity to antibiotics as a guide to selection of the antibiotic and appropriate dosage. The results obtained here with a number of organisms isolated during routine work, particularly strains of Proteus, illustrate the point just raised.

In the testing of sensitivities nutrient agar plates incorporated with the appropriate amount of antibiotics were prepared. One loopful of a six-hour or seven-hour nutrient broth culture of the organism to be tested was diluted in five millilitres of saline and a loopful of this diluted preparation was streaked on plates containing various concentrations of the antibiotic. As many as nine different organisms, including a control Staphylococcus aureus (strain F.D.A. 209), could be inoculated as a single streak on each plate. Readings were made after overnight incubation at 37°C. The results with 51 strains of Proteus, 46 strains of Pseudomonas pyogyanea and 12 strains of lactose-fermenting "coliform" bacilli are shown in Table I. These organisms were collected from specimens of sputum, pus, faeces and infected urine.

It is apparent from the results given in the table that there is wide variation in sensitivity to the antibiotics in the Proteus group, some organisms being sensitive to all three agents and some resistant to all three. In the largest subgroup (37%) aureomycin is definitely not indicated for treatment. In the Pseudomonas group of organisms there is some but lesser variation in reaction, 58% being resistant to all concentrations of each antibiotic used, while only two strains of the 46 tested showed reasonable sensitivity, and this was to aureomycin only. The number of strains in the "coliform" group was small, but it was still sufficiently large to show that random use of antibiotics is not to be recommended.

TABLE I.

Results of Tests with Antibiotics.

Organism and Number of Strains Reacting as Shown	Concentration of Antibiotic (γ per Millilitre)											
	Streptomycin				Aureomycin				"Chloromycetin"			
	1	10	20	500	0.1	1	5	10	1	5	10	20
Proteus (51 strains):												
1	-	-	-	-	+	-	-	-	-	-	-	-
1	-	-	-	-	+	+	-	-	+	-	-	-
1	-	-	-	-	+	+	+	+	+	+	-	-
6	+	-	-	-	+	+	-	-	+	-	-	-
3	+	-	-	-	+	+	+	-	+	-	-	-
19	+	-	-	-	+	+	+	+	+	-	-	-
4	+	-	-	-	+	+	+	+	+	+	-	-
1	+	+	-	-	+	-	-	-	+	-	-	-
1	+	+	-	-	+	+	-	-	+	-	-	-
1	+	+	+	+	+	-	-	-	+	-	-	-
1	+	+	+	+	+	+	-	-	+	-	-	-
6	+	+	+	+	+	+	-	-	+	+	-	-
3	+	+	+	+	+	+	+	+	+	-	-	-
2	+	+	+	+	+	+	+	+	+	+	-	-
1	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas pyocyanea (46 strains):												
1	+	+	-	-	+	+	+	-	+	+	+	+
4	+	+	-	-	+	+	+	+	+	+	+	+
1	+	+	+	-	+	+	-	-	+	+	-	-
3	+	+	+	-	+	+	+	-	+	+	+	+
9	+	+	+	-	+	+	+	+	+	+	+	+

cont'd...

Table I. cont'd...

1	+	+	+	+	+	+	+	-	+	+	+	+
27	+	+	+	+	+	+	+	+	+	+	+	+
"Coliform" bacillus (12 strains):												
7	+	-	-	-	+	+	-	-	+	-	-	-
1	+	+	+	-	+	+	-	-	+	-	-	-
1	+	+	+	-	+	+	-	-	+	+	+	+
2	+	+	+	+	+	+	-	-	+	-	-	-
1	+	+	+	+	+	+	-	-	+	+	-	-

"+" indicates growth on nutrient agar containing the stated amount of antibiotic.

SUMMARY

The results of sensitivity tests against streptomycin, aureomycin and "Chloromycetin", with 51 strains of Proteus, 46 strains of Pseudomonas pyocyanea and 12 strains of "coliform" bacilli, are given.

The results indicate that rational therapy in cases of infection with these Gram-negative bacilli requires preliminary testing of each organism.

ACKNOWLEDGEMENTS

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THE STAINING OF FUNGI IN SKIN AND HAIR PREPARATIONS

by J. F. HORE

(Senior Technician, Pathology Department, St. Vincent's Hospital, Melbourne).

The detection of dermatophytes in skin scrapings or hairs, is usually attempted by direct microscopic examination of a specimen which has been treated with potassium hydroxide solution. With grossly infected material, the diagnosis is easily made, but where the number of organisms is small, when they are present as spores with little or no mycelium and where there is much debris, it may be difficult to form an opinion.

The differential staining technique for the detection of ringworm in hairs, recommended by Lewis and abstracted in this Journal* (1951, Vol. 1) has been applied here, using specimens of hair and skin and found of great help.

Two difficulties were encountered, however, The first was to prevent the material from leaving the microscopic slide during staining, particularly with hairs and very fine particles of skin obtained by scraping. The second was that most finished preparations appeared over-stained and prolonged decolorisation made little improvement.

Slight alteration of the technique results in overcoming both these difficulties.

The method now in routine is as follows: The specimen is placed on a slide previously coated with a fine film of glycerine-egg albumen as used in histological preparations. After 30 minutes drying at 37°C. the slide is then dipped into a 1% solution of celloidin in equal parts of absolute alcohol and ether. It is then allowed to drain in an upright position. When dry it is covered with chloroform for five minutes. This serves the double purpose of hardening the film of celloidin and removing grease and fat from the specimen. After drying it is stained with a mixture of 5% alcoholic gentian violet (1 part) and aniline water (3 parts) for 1 minute, followed by Gram's Iodine solution for 1 minute. It is then decolourised with aniline for 20 minutes. The aniline is then removed with Xylol and the preparation mounted in Canada balsam.

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Numbers 1-4 p. 13.

A NOTE ON THE DETECTION OF CANDIDA ALBICANS

by MARY M. MORTON

(Pathology Department, St. Vincent's Hospital, Melbourne)

Weld (1952, 1953) reported that colonies of C. albicans grown at 37°C. on Levine's eosin-methylene blue agar in an atmosphere containing about 10% CO_2, are identifiable by their morphology in that they form spidery or feathery out-runners. This appearance is visible to the naked eye or with a hand lens.

In an effort made here to link up the phenomenon with one or more ingredients of the medium it was found, by eliminating constituents one at a time, that a much simpler medium was just as effective. The medium now in use consists of peptone (1%) and agar (2%) in tap water. Increasing the concentration of CO_2 in the atmosphere gave better results, although it was not always essential. The hydrogen ion concentration of the medium was not critical; three batches of media of pH 5, 7 and 9 all gave the same results. Incubation at 30°C. was found suitable, although most of the routine tests were carried out at 37°C.

Where there is gross contamination of the inoculum, it is

advisable to add aureomycin (10 mgm. per 100 c.c. of medium) as recommended by Weld. However, even without aureomycin and where numerous other organisms have grown, colonies of C. albicans may be detected, usually within twenty-four hours, particularly if a plate microscope is available.

The typical appearance of colonies has been less marked with stock cultures than with freshly isolated strains. With plates of medium on which a stock culture has been plated out, many of the colonies may fail to show the reaction.

So far only one Candida has been found which gave a suspicious reaction on peptone agar, although subsequent tests showed it was not C. albicans. The outrunners from this organism were finer and much more numerous than those given by a pathogenic Candida (see Figures I and II). With the exception of a Candida of this nature, a Candida giving the typical appearance as described by Weld may be accepted as Candida albicans. Colonies not showing the reaction should be further tested. The routine system used here is to inoculate such a colony on to an agar slope and incubate overnight at 30°C. for slide agglutination test (Christie and Morton, 1953) and into corn-meal extract for detection of hyphae and chlamydospores (Anderson, 1946). If the agglutination test is negative, the organism is discarded as not C. albicans; if positive, the corn-meal extract is then examined after two, three and five days' incubation.

Drouhet and Couteau (1954) have also found a simplified medium suitable, this medium consisting of 1% peptone and 0.2% KH_2PO_4 in 1.5% agar. Rosenthal (1954) found that on a medium consisting of 0.5% neopeptone, 0.5% glucose and 2% agar, filament formation by C. albicans could be observed in areas under a coverslip, no filaments being seen in uncovered areas.

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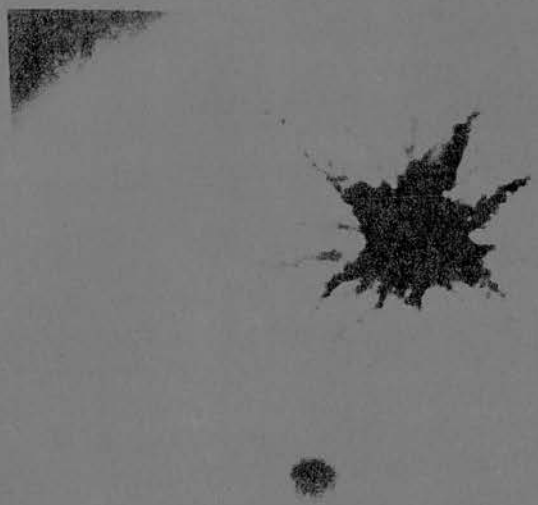

FIGURE I.
Colony of *C. albicans* on peptone agar. ×15.

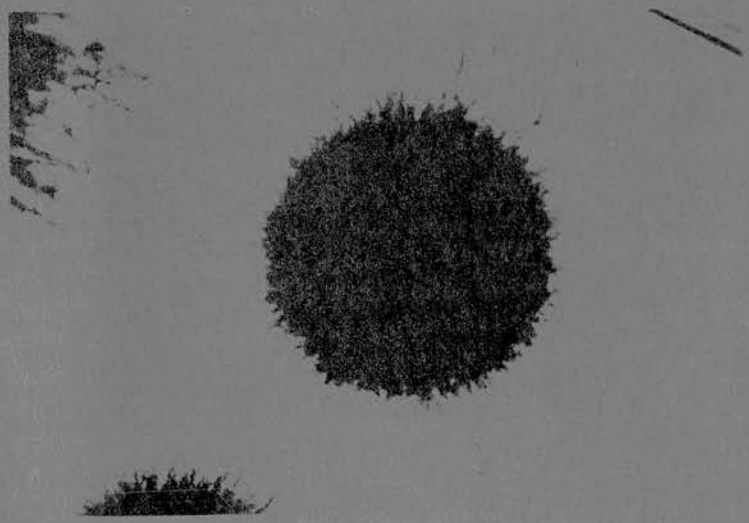

FIGURE II.
Colony of *Candida*, not *C. albicans*. ×10.

(Photographs by W. Sullivan.)

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A BIOCHEMICAL AND SEROLOGICAL STUDY OF THE GENUS PROTEUS

by SHIRLEY V. KEATING, B.Sc.

(Pathology Department, Saint Vincent's Hospital, Melbourne)

Rustigian and Stuart (1945) studied 454 cultures of Proteus isolated from various sources, and found that this genus could be divided biochemically into four species - Proteus vulgaris, P. mirabilis, P. morgani and P. rettgeri. Cook (1948) working with 120 Proteus strains isolated from human faeces, was also able to place his strains in four well defined groups as a result of biochemical tests and suggested a tentative classification based on these (see Table I). Mushin (1950) applied biochemical criteria to 217 strains of Proteus isolated from faeces, and was able to place all organisms in three well-defined groups, no P. rettgeri being encountered. Cook's suggested classification is set out in Figure I.

In this laboratory 224 strains of Proteus isolated from various sources were examined biochemically as indicated in Figure I for species differentiation. Approximately 23% of these organisms, while similar to each other, were unable to be allotted to any one of the four species in Figure I. This prompted a further study of these strains and their relationship to the remainder of the series.

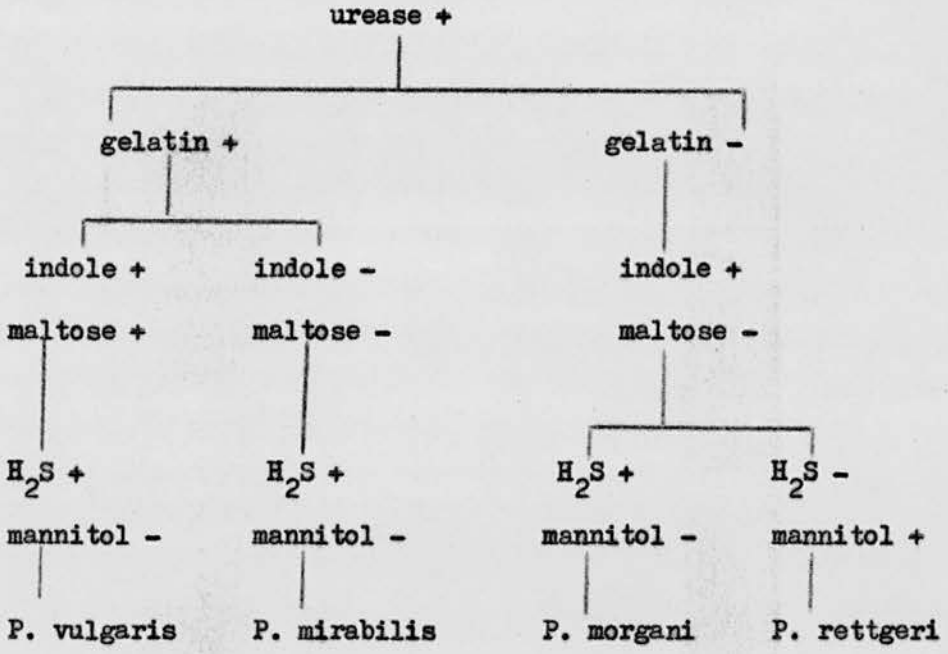

Figure I.

Biochemical classification of Proteus suggested by Cook.

MATERIALS AND METHODS

Source of Strains.

One hundred and twelve strains of Proteus, numbered serially as obtained, were maintained on nutrient agar slopes at 0°C. to 4°C. The strains were isolated from urine, sputum, faeces and pus. The term "pus" was used collectively for frank pus and for specimens such as eye fluids, nasal swabs, prostatic fluids et cetera. One strain was isolated from dust, and the sources of six strains were unrecorded. A strain of P. rettgeri, forwarded by Dr. C. A. Stuart, was also included.

Bacilli were accepted as Proteus if they were Gram-negative, hydrolysed urea rapidly, fermented dextrose with or without gas and did not ferment lactose.

Urease Test.

Urea agar slopes (medium B, Christensen, 1946) were used. The formation of a definite red colour within six hours was regarded as a positive reaction.

Carbohydrate Fermentation Tests.

The carbohydrates used were dextrose, lactose, mannitol, maltose, sucrose and salicin, made up in a concentration of 1% in peptone water with brom-cresol-purple as indicator. Twenty-one days' incubation was allowed before a result was regarded as negative.

Sulphuretted Hydrogen Production.

The medium used was 2% peptone water. A dried strip of filter paper previously soaked in a saturated solution of lead acetate and sterilized by autoclaving was placed under the cotton stopper of each tube, and the appearance of a black stain after twenty-four hours' incubation was recorded as a positive reaction.

Gelatin Liquefaction.

Stabs of medium (10% gelatin in nutrient broth) were used. After incubation the tubes were placed in the refrigerator for one hour before being examined for liquefaction. The tests were carried out in duplicate, one series being incubated at 37°C., the other at 22°C. The first series was examined for three weeks and the second for eight weeks.

Glucose MacConkey Broth.

Single-strength glucose MacConkey broth was used, tubes being incubated in a 44°C. water bath for forty-eight hours before readings for acid and gas production were made. When no signs of fermentation occurred, twenty-four hours' further incubation at 37°C. was given as a test for viability.

Serum Liquefaction.

Löffler's inspissated serum was used and the results were read after fourteen days.

Koser's Citrate Test.

Tubes of Koser's citrate medium were inoculated with a large loopful of each culture tested. If growth occurred in twenty-four hours, sub-cultures were made into fresh tubes of the medium, which were then incubated for five days. Growth in these tubes was regarded as a positive result.

Miscellaneous Tests.

The indole, methyl-red, Voges-Proskauer and litmus milk tests were carried out in the usual way.

Inoculations for all tests were made from twenty-four hour agar slope cultures, and unless otherwise stated the temperature of incubation was 37°C.

Antibiotic Sensitivity Tests.

Antibiotic sensitivity tests were carried out by the use of nutrient agar plates incorporated with the appropriate amount of antibiotic. One loopful of a six-hour to seven-hour nutrient broth culture of the organism to be tested was diluted in five millilitres of saline, and a loopful of this diluted preparation was streaked on plates containing various concentrations of the antibiotic. As many as nine different organisms, including a control Staphylococcus aureus (strain F.D.A. 209), were inoculated as single streaks on each plate. The concentrations of streptomycin used were 1, 10, 20 and 500 per millilitre, of chloramphenicol 1, 5, 10 and 20 per millilitre and of "Aureomycin" and "Terramycin" 0.1, 1, 5 and 10 per millilitre. Readings were made after overnight incubation at 37°C., and results were recorded as the lowest concentration of antibiotic at which growth was inhibited.

Preparation of Antisera.

The growth from twenty-four-hour agar slope cultures was emulsified in normal (0.85%) saline, to give a turbidity of approximately 2000 x 10^6 organisms per millilitre, and the resulting suspensions were killed by the addition of 0.3% formalin solution. Rabbits were then inoculated intravenously twice weekly with the suspensions in doses of 0.2, 0.4, 0.6, 1.0, 1.0 millilitre, and finally with 1.0 millilitre of a living twenty-four-hour broth culture. The rabbits were bled from the heart one week after the final injection, and the serum, preserved with chloroform, was stored at 0°C. to 4°C.

Absorptions were carried out by adding varying quantities of the absorbing organisms to one millilitre quantities of 1:10 dilution of the serum, the mixtures being allowed to stand at 37°C. for one hour and then clarified by centrifugalization. Absorbed sera were also stored at 0°C. to 4°C. with chloroform as preservative.

Technique of Agglutination Tests.

The suspensions used in agglutination tests were prepared by washing the organisms from a twenty-four-hour nutrient agar slope with approximately two millilitres of normal saline. A drop of this suspension was added to a drop of the serum, suitably diluted, on a slide; the mixture was rocked for two minutes and readings were made with the aid of a hand lens. A control test was carried out at the same time, a drop of saline being used in place of the serum.

RESULTS

The series of 113 strains examined comprised 65 strains of P. mirabilis, seven strains of P. vulgaris, 11 strains of P. morgani, two strains of P. rettgeri and 28 unclassifiable strains. The last-mentioned will be referred to as odd strains.

Motility and Swarming.

The motility of the bacilli tended to be sluggish, some strains being non-motile when grown in peptone water or broth at 37°C. However, when cultivated in nutrient broth at room temperature overnight, all strains were motile.

Ability to swarm was determined on moist 1.75% agar plates at 22°C. Those strains failing to swarm were retested on 1% agar. Only one strain failed to swarm; this was a strain of P. morgani.

Biochemical Results.

The majority of the biochemical results are summarized in Table I.

The unusually high percentage of strains, not P. vulgaris, fermenting maltose suggested that the maltose may have been impure. Inoculations into maltose were therefore repeated, three different brands of this carbohydrate being used, and also medium sterilized by Seitz filtration as well as by the usual three days' steaming. The results were identical with all batches of medium. The strains were also plated out on maltose MacConkey agar, but no non-maltose fermenting variants were detected.

Acid produced in mannitol by the P. rettgeri strains reverted to a trace only in three days, but by six days the medium was fully acidified again.

All strains produced sulphuretted hydrogen in twenty-four hours. Production of sulphuretted hydrogen by the two strains of P. rettgeri could be explained only by the variability of results found for this test according to the medium used.

Gelatin was found to be more rapidly liquefied at 37°C. than at 22°C. The majority of P. mirabilis strains liquefied the gelatin in two days. P. vulgaris strains mostly took seven days, and the odd strains were approximately equally divided into slow and rapid liquefiers.

Liquefaction of inspissated serum was likewise more easily demonstrated at 37°C.

Litmus milk was either not attacked or else was turned strongly alkaline by P. morgani strains, while the majority of the P. vulgaris, P. mirabilis and the odd strains decolorized the milk and clotted it; this was followed by peptonization. The P. rettgeri strains turned the milk strongly alkaline.

Twenty-nine strains representative of all groups, were tested for growth in citrate. All failed to grow.

Neither strain of P. rettgeri produced gas in carbohydrate media. The strain isolated here differed from the imported strain by producing acid in glucose MacConkey broth at 44°C., and in giving a negative response to the methyl-red test.

Another series of 112 strains of Proteus bacilli, of which each strain was tested directly on isolation, was classified according to Figure I. Twenty-four strains (22%) were unclassifiable, and no strain of P. rettgeri was encountered.

Serological Results.

Antisera were prepared for three strains each of P. mirabilis and P. vulgaris, two strains of P. morgani, two odd strains and the standard strain of P. rettgeri (R).

By the use of doubling dilutions of antiserum, the titre of agglutination of each organism for which an antiserum was prepared was determined by slide agglutination. Also each antiserum, in a doubling dilution range of from 1:10 to 1:2560, was tested against organisms from which the other antisera had been prepared, to detect evidence of cross-agglutination. The results are recorded in Table II.

TABLE I.

Results of Biochemical Tests[1]

Sub-Group	Number Tested	Indole Production	Sulphuretted Hydrogen Production	Gelatin Liquefied	Serum Liquefied	Methyl Red Test: Positive Result.	Voges-Proskauer Test: Positive Result.	Glucose MacConkey Broth Incubation at 44°C.		Days	Dextrose Fermentation.	Lactose Fermentation	Mannitol Fermentation	Maltose Fermentation	Sucrose Fermentation	Salicin Fermentation
								Acid Production	Acid and Gas Production							
P. mirabilis	65	0	65	65	62	65	0	19	45	1-2	65	0	0	0	1	2
										3-21	-	0	0	0	51	-
P. vulgaris	7	7	7	7	1	7	0	6	0	1-2	7	0	0	7	7	4
										3-21	-	0	0	-	-	-
P. morgani	11	11	11	0	0	3	0	6	1	1-2	11	0	0	0	0	0
										3-21	-	0	0	0	2	0
Odd strains	28	0	28	28	23	28	0	27	0	1-2	28	0	0	28	28	0
										3-21	-	0	0	-	-	0
P. rettgeri	2	2	2	0	0	1	0	1	0	1-2	2	0	2	0	0	2
										3-21	-	0	-	0	2	-

1 Results for the carbonydrate fermentations were recorded as positive if acid, with or without gas, was formed.

TABLE II.

The Highest Titre of Agglutination, with Unabsorbed Antisera, of the Strains Used in Their Preparation

Organisms		Antisera										
Sub-Group	Serial Number	78	58	112	81	27	70	79	19	16	94	R
P. mirabilis	78	1:160	1:10	1:10	-	-	-	-	-	-	-	-
	58	-	1:160	-	-	1:80	-	-	-	-	-	-
	112	-	-	1:160	-	-	-	-	-	-	-	-
P. vulgaris	81	-	-	-	1:80	1:10	-	-	1:10	-	-	-
	27	-	1:80	-	-	1:320	-	-	-	-	-	-
	70	-	1:10	1:10	-	-	1:80	-	-	-	-	-
P. morgani	79	-	-	-	-	-	-	1:160	-	-	-	-
	19	-	-	-	-	-	-	1:20	1:80	-	-	-
Odd	16	1:80	1:40	1:80	1:40	1:160	-	1:40	-	1:1280	1:640	-
	94	-	1:20	-	1:20	-	-	-	-	1:320	1:640	-
P. rettgeri	R	-	-	-	-	-	-	-	-	-	-	1:80

When necessary, absorption experiments were carried out to prepare the antisera as free as possible from those cross-reactions demonstrated in Table II.

One hundred and thirteen strains were tested for agglutination in these absorbed sera diluted 1:10. The results are summarized in Table III.

One strain of the series P18, biochemically regarded as a strain of P. rettgeri, was agglutinated by the anti-serum R prepared from the American strain of P. rettgeri, and by none of the other antisera. This antiserum was made up in doubling dilutions from a titre of 1:5. The American strain was agglutinated strongly in all dilutions up to 1:40, while strain P18, although agglutinating to this titre, did so weakly, the result in 1:5 dilution being no stronger than that in 1:40. This indicated a sharing of antigens between these two strains, although they were not identical. This, together with its biochemical behaviour, strongly favoured P18 being a strain of P. rettgeri.

None of the odd strains agglutinated in any of the absorbed antisera prepared for the other species.

Antibiotic Sensitivity Tests.

While allowance is made for some variation in sensitivity from strain to strain with all species, strains of P. mirabilis, generally speaking, were almost all completely resistant to "Aureomycin" and "Terramycin" and sensitive to chloramphenicol. Two-thirds of the strains were sensitive to streptomycin.

P. vulgaris strains were more resistant to "Terramycin" than to "Aureomycin", most strains being quite susceptible to the latter. They were uniformly sensitive to chloramphenicol, but showed wide variation in sensitivity to streptomycin, some strains being completely sensitive while others were completely resistant.

TABLE III.

Results of Agglutination Tests with Absorbed Antisera

Organisms		Absorbed Antisera										
Species	Number of strains	P. mirabilis			P. vulgaris			P. morgani		Odd		P rettgeri
		P58	P78	P112	P27	P81	P70	P19	P79	P16	P94	
P. mirabilis (65 strains)	42	-	+	-	-	-	-	-	-	-	-	-
	8	+	-	-	-	-	-	-	-	-	-	-
	2	+	+	-	-	-	-	-	-	-	-	-
	2	-	+	-	-	-	-	-	-	-	+	-
	1	+	+	-	-	-	-	-	-	-	+	-
	1	-	-	+	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	+	-
	5	-	-	-	-	-	-	-	-	-	-	-
P. vulgaris (7 strains)	3	-	-	-	+	-	-	-	-	-	-	-
	1	-	-	-	-	-	+	-	-	-	-	-
	1	-	-	-	-	+	-	-	-	-	-	-
	1	-	-	-	-	+	-	-	-	-	+	-
	1	-	+	-	-	-	-	-	-	-	-	-
P. morgani (11 strains)	7	-	-	-	-	-	-	-	+	-	-	-
	1	-	-	-	-	-	-	+	-	-	-	-
	1	-	-	-	-	-	-	+	+	-	-	-
	1	-	-	-	-	-	-	-	-	-	+	-
	1	-	+	-	-	-	-	-	-	-	-	-
Odd (28 strains)	17	-	-	-	-	-	-	-	-	-	+	-
	5	-	-	-	-	-	-	-	-	+	+	-
	6	-	-	-	-	-	-	-	-	+	+	-
P. rettgeri (2 strains)	2	-	-	-	-	-	-	-	-	-	-	+

TABLE IV.

P. mirabilis				P. vulgaris				P. morgani				Odd				P. rettgeri			
Number of Strains	Streptomycin	Chloramphenicol	"Aureomycin	"Terramycin"	Number of strains	Streptomycin	Chloramphenicol	"Aureomycin"	"Terramycin"	Number of Strains	Streptomycin	Chloramphenicol	"Aureomycin"	"Terramycin"	Number of Strains	Streptomycin	Chloramphenicol	"Aureomycin"	"Terramycin"	Number of Strains	Streptomycin	Chloramphenicol	"Aureomycin"	"Terramycin
1	10	5	5	1	1	1	5	5	10	1	1	5	10	1	1	20	20	10	>10	1	1	5	5	10
19	10	5	>10	>10	1	1	5	5	>10	2	10	5	5	10	1	>500	5	1	10	1	10	10	>10	>10
15	10	10	>10	>10	1	1	5	>10	>10	4	10	5	10	10	2	>500	20	1	5					
4	10	20	>10	>10	1	10	5	5	>10	1	10	10	>10	>10	1	>500	5	5	10					
8	>500	10	>10	>10	1	10	5	10	>10	1	10	20	>10	>10	4	>500	10	5	5					
7	>500	5	>10	>10	1	500	5	5	10	1	20	20	>10	>10	5	>500	10	5	10					
4	>500	20	>10	>10	1	500	5	10	>10	1	20	>20	>10	>10	1	>500	20	5	1					
2	500	10	>10	>10											1	>500	20	5	5					
2	20	20	>10	>10											2	>500	20	5	10					
2	1	5	>10	>10											3	>500	20	10	10					
1	1	5	>10	>10											1	>500	>20	5	5					
															6	>500	>20	5	10					
65					7					11					28					2				

Results of Sensitivity Tests with Four Antibiotics

P. morgani strains gave variable results with "Aureomycin" and "Terramycin", but the majority were resistant to these antibiotics. Results were also very variable for chloramphenicol and streptomycin, but with these the majority were sensitive.

In view of the small number of strains of P. vulgaris and P. morgani studied, it would be unwise to draw inferences from these results. Likewise, with the two strains of P. rettgeri studied, all that can be said is that these two strains were sensitive to streptomycin and chloramphenicol; one strain was sensitive to "Aureomycin" and more sensitive to "Terramycin" than the other, which was resistant to both these antibiotics.

The odd strains were almost uniformly resistant to streptomycin, mostly sensitive to "Aureomycin" and "Terramycin", and variable in response to chloramphenicol, about 60% of strains being resistant.

The results are summarized in Table IV, where results are recorded as the lowest concentration of antibiotic in γ per millilitre inhibiting growth.

DISCUSSION

From the work done here, it will be seen that if Cook's table is used to subdivide the genus into four groups, there is still a fifth group which cannot be classified and which includes a fairly high percentage of strains.

Examination of the source of this group (see Table V) showed that a higher percentage occurred in urine than in any other material. The absence of strains of this nature in the work of Cook and of Mushin may have been due to the isolation of their strains from faeces only.

Biochemically, this group of organisms resembled P. vulgaris in fermentation of maltose, P. mirabilis in failure to produce indole and both these species in liquefaction of gelatin. It showed greater simi-

TABLE V.

Source of Strains.

Sub-Group	Number of Strains	Source					
		Urine	Sputum	Faeces	Pus	Dust	Unknown
P. mirabilis	141	70	9	5	48	1	8
P. vulgaris	13	7	1	2	2	0	1
P. morgani	17	8	1	6	3	0	0
P. rettgeri	1	0	0	0	1	0	0
Odd	52	37	0	0	9	0	6

larity to these species than to either P. morgani or P. rettgeri, as these latter produce indole and neither liquefy gelatin nor ferment maltose. The results of other biochemical tests proved on the whole too variable to be of value in comparing species.

Results of the agglutination tests, though on a small number only of some species, were sufficient to verify what has long been known - that serology offers no simple means of classifying strains in this genus. However, the odd strains were agglutinated only in the absorbed antisera prepared for their own group, and they showed no cross-reaction with the other species. Hence, in the series studied an organism biochemically classified as an odd strain could be verified as such serologically, although the reverse of this did not always apply.

The results of the antibiotic tests gave further support to the view that the odd strains formed a distinct group. The findings for the four recognized species agreed in broad outline with the results of Potee and his co-workers (Potee, Wright and Finland, 1954). However, the odd strains differed from these species in being uniformly resistant to streptomycin and sensitive to "Aureomycin" and "Terramycin", and in showing greater resistance to chloramphenicol.

As the pattern of sensitivity to antibiotics of Proteus strains seems to be correlated to some extent with their classification into four species (Poole, 1954), so the pattern for these odd strains is sufficient to delineate them from the other species and correlates with their different biochemical and serological behaviour.

The results of biochemical, serological and antibiotic sensitivity tests would indicate that the odd strains were a group distinct from the other species rather than variants of any one particular species.

The classification put forward by Cook to differentiate species by biochemical tests would have to be modified to accommodate these strains,

and it is proposed that, in view of the variability of the results for gelatin liquefaction and sulphuretted hydrogen production, these tests be erased, as they are not essential for any specific differentiation. Once an organism has been recognized as a Proteus by rapid hydrolysis of urea, P. vulgaris, P. mirabilis and the odd strains can be differentiated on maltose fermentation and indole production alone, while fermentation of mannitol is a further requisite to distinguish between P. morgani and P. rettgeri. The proposed modification is given in Figure II.

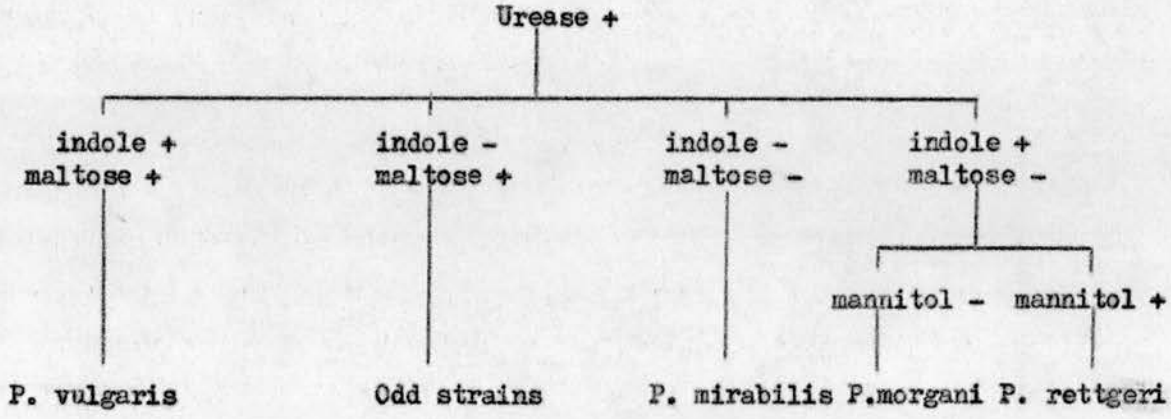

FIGURE II.

Proposed simplified table for species differentiation of Proteus.

SUMMARY

1. Evidence is produced for the existence of another sub-group in the genus Proteus, which is distinct from the four already recognized - P. mirabilis, P. vulgaris, P. morgani and P. rettgeri.

2. The members of this sub-group both ferment maltose and fail to produce indole. They give serological reactions which differentiate them from the other sub-groups, and they show a different pattern of sensitivity to antibiotics, in particular a greater resistance to streptomycin.

3. A classification is proposed for the differentiation of the

five sub-groups of the genus, based on simple biochemical tests.

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THE USE OF DISPERSOL ‡ IN CULTURE-MEDIA FOR THE ISOLATION OF STREPTOCOCCI

by LYNETTE J. DUMBLE

(Bacteriology Department, St. Vincent's Hospital, Melbourne).

There are several methods of adjusting media to prevent the spreading of PROTEUS, where this organism would mask the presence of others. The addition of Dispersol to blood agar prevents this spreading, and was used here with apparent success where urine samples were being examined. Where specimens, not obviously contaminated, were being examined, as many as four could be inoculated on one plate. When used for specimens other than urine, however, it was found that growth of some streptococci was partly or completely inhibited and their presence could only be detected from direct smears of the sample, or culture on medium without Dispersol.

It was also noticed that some colonies of streptococci on Dispersol blood agar had dark centres; where these were very numerous a brown discoloration of the medium surrounding the colonies appeared, resembling the discoloration produced on maltose blood agar by streptococci of Group B (Lancefield). (See prints).

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‡ FOOTNOTE: DISPERSOL L.N. - a wetting agent obtained from I.C.I. and used in concentration 0.3%.

A number of streptococci were isolated and their appearance on Dispersol blood agar noted after 24 hours' incubation at 37°C. Further tests were carried out to determine their species, using the methods given in Bergey's Manual of Determinative Bacteriology.

RESULTS

When testing for formation of colonies with dark centres and/or surrounding brownish discoloration, horse, sheep or human blood agar gave the same results. Nutrient agar without blood gave no discoloration.

Table I shows the results obtained with 103 strains of streptococci.

Table II shows the numbers of strains which grew without inhibition on Dispersol blood agar with or without darkening or discoloration.

The four species which produce discoloration of medium are all included in Bergey's group D. (Enterococcus group). Of the strains not in this group none gave discoloration.

The discoloration phenomenon could be shown in liquid cultures as well as solid. Tubes of broth containing 5% horse blood, some with and some without Dispersol were inoculated with Enterococci. After 24 hours' incubation lysis of red cells could be seen in all tubes. After 72 hours there was little change in the tubes without Dispersol. Where Dispersol was present, however, dark brown discoloration appeared which became more pronounced with further incubation. Samples of liquids were clarified by centrifugalisation and examined spectroscopically. Absorption bands were found in the specimens which contained Dispersol. The position of these bands was similar to those given by methaemoglobin. This was further confirmed by the disappearance of the absorption bands on the addition of sodium hydrosulphite.

TABLE I.

Numbers of Strains of Streptococci Showing Source and Ability to Grow on Dispersol Blood Agar.

Source	Number	Name of Organism	No.	Dispersol Agar	
				No. Grown	No. Inhibited
Urine	50	Strep. faecalis	11	11	0
		Strep. agalactiae	2	0	2
		Strep. zymogenes	20	20	0
		Strep. liquefaciens	16	16	0
		Viridans grp.	1	1	0
Pus	40	Strep. agalactiae	9	0	9
		Strep. pyogenes	9	0	9
		Strep. liquefaciens	4	4	0
		Strep. zymogenes	5	5	0
		Strep. durans	1	1	0
		Viridans group	2	2	0
		Lactic group	2	2	0
		Strep. equinas	1	1	0
		Strep. faecalis	6	6	0
		Strep. intermedius (anaerobic)	1	1	0
Vaginal Swabs	2	Strep. zooepidemicus	1	0	1
		Strep. zymogenes	1	1	0
Blood	4	Strep. liquefaciens	3	3	0
		Viridans group	1	1	0
Sputum	4	Viridans group	3	3	0
		Strep. pyogenes	1	0	1
C.S.F.	1	Strep. zymogenes	1	1	0
Throat Swabs	1	Strep. pyogenes	1	0	1
Mouth Swabs	1	Lactic Group	1	1	0
TOTAL	103		103	80	23

TABLE II.

Numbers of Strains Which Grew Without Inhibition on Dispersol Blood Agar

Name of Organism	Number	Discoloration Produced	No Discoloration
Strep. faecalis	17	17	0
Strep. zymogenes	27	27	0
Strep. liquefaciens	23	23	0
Strep. durans	1	1	0
Viridans group	7	0	7
Lactic group	3	0	3
Strep. equinas	1	0	1
Strep. intermedius (anaerobic)	1	0	1

SUMMARY

Using Dispersol blood agar to isolate streptococci from urine samples, all grew freely except two in fifty attempted isolations.

With other specimens, however, pus, sputum. etc., twenty-one of fifty-three either failed to grow or grew poorly.

Some streptococci grew with dark-centred colonies with or without surrounding discoloration of the medium. These were all of the Enterococcus group. (Bergey group D).

Spectroscopic examination suggests that the brown discoloration was due to the presence of methaemoglobin.

Dispersol blood agar provides a simple confirmatory test for streptococci of the Enterococcus group.

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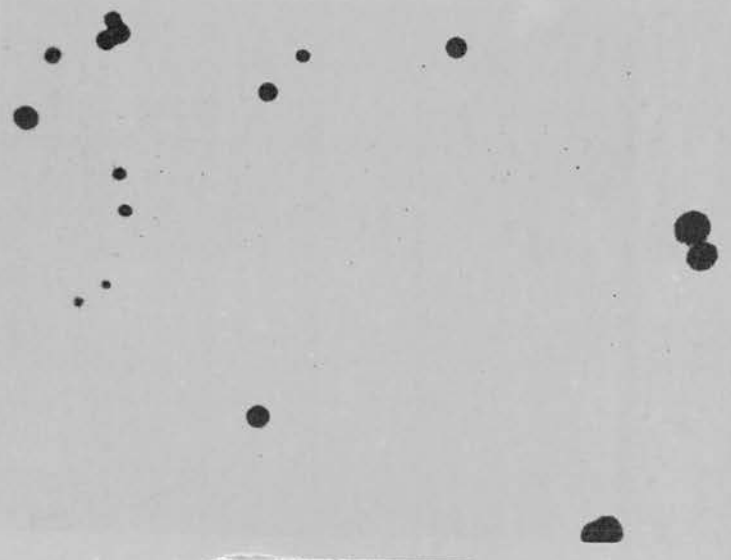

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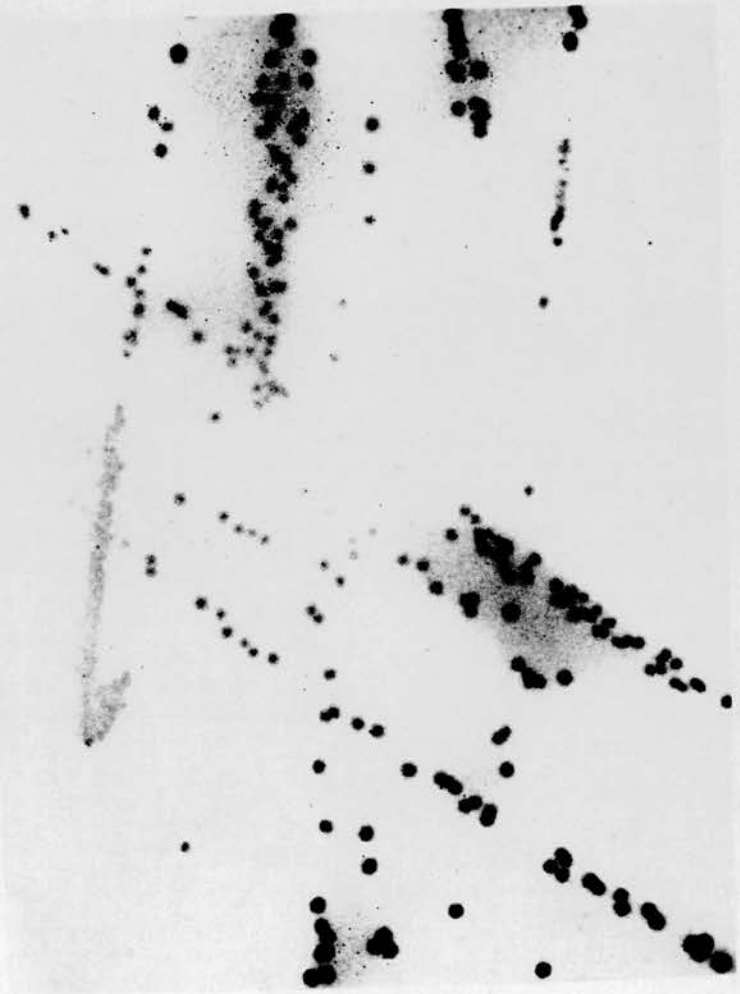

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