A COMPARISON OF PATHOGENICITY TESTS FOR STAPHYLOCOCCI

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A SERIES of unselected staphylococcus strains was subjected to a number of tests, and the results obtained were examined as to their value for the assessment of pathogenicity.

In all, 325 different strains were examined, 200 aureus and 125 albus strains. All staphylococci showing pigmentation, however slightly, were ranged with the aureus strains, with the exception of definitely lemon-coloured *Staphylococcus citreus*, of which only a small number were examined. All strains labelled albus showed no pigmentation at all.

The sources of the isolated staphylococci varied considerably. It should be pointed out, however, that though some of the strains were obtained from pus of abscesses, cellulitis and osteomyelitis, the rarer places of origin as blood stream, joints, spinal canal, peritoneum, lungs, pleural sacs and the female genital tract were represented as well.

Before describing the methods adopted it will be advantageous to discuss briefly the work done in this field of research and, where necessary, to contrast or support this with our own findings.

Biochemical Reactions.—The fermentation of mannite as an aid in differentiating pathogenic strains was introduced by Gordon in 1903. But, as Blair[1] pointed out in his review, " an appreciable proportion of non-pathogenic staphylococci, varying from 11 to 55 per cent., has been reputed also to ferment mannitol, rendering this test less specific."

Christie and Keogh[2] recommended the use of trehalose and mannose as a further means in separating the saprophytic from pathogenic strains. But in their own hands mannose proved of little help in this respect ; our own results were even less favourable. We therefore substituted sorbite for mannose and thus tested our strains for their ability to ferment—apart from glucose—lactose, mannite, trehalose and sorbite. It will be shown that there exists some difference between coagulase positive and coagulase negative strains in their ability to use these sugars.

Coagulase Production.—The power which certain staphylococci possess of coagulating plasma was first described in 1903.[3] It had, however, to be rediscovered twice[4,5] before its use for routine purposes was adopted. There are certain aspects of this simple test which require detailed consideration.

1. *Source of Plasma.*—The plasma of various animal species is used for the coagulase test. While rabbit plasma is commonly regarded as being clotted within the shortest time, the clot produced has sometimes the disadvantage of being redissolved when incubated overnight.[6, 7] It is also recorded that coagulation occurs with *B. subtilis* and *B. pyocyaneus*.[7] Most workers, therefore, prefer human plasma, which yields a firm clot after comparatively short incubation and is not clotted by other organisms. It might be noted here that in a recent paper, Smith and Hale [8] described their success in largely eliminating the difference in coagulability of various animal plasmas by staphylococcal coagulase by means of the addition of an activating substance, to which reference is made below.

2. *Whole Blood.*—The use of whole blood has been advocated for the coagulase test. We have been unable to confirm the favourable results obtained. In repeated trials oxalated human blood was found to react very slowly and to yield only a slight and loose clot. In a few instances no clot formation was observed after inoculation with definitely pathogenic staphylococci.

3. *Prothrombin Solution.*—A prothrombin solution has been employed by different authors,[7, 9, 10, 11] mainly for the slide agglutination test, but also for the orthodox coagulase test. For the latter purpose the prothrombin solutions were found to be a poor substitute and for the former also no obvious advantage was obtained with its employment. Moreover, after three weeks, and with one batch after one month, the solution had suddenly lost its potency. As ordinary plasma can be stored at room temperature or, better, at 4° C. for several weeks,[12, 13, 14, 15, 16] the need to embark on the preparation of prothrombin solutions is not apparent.

4. *Dried Plasma.*—Colbeck and Proom [17] have used dried rabbit plasma with good results. In this laboratory unfiltered, pooled, human plasma, dried *in vacuo*, has been found to be equally suitable for the coagulase test as well as for the slide agglutination. By employing pooled plasma the risk of obtaining unsuitable material from one particular donor was eliminated. Stored at 4° C. this dried plasma kept its properties for over one year. When required, 5 per cent. solutions in sterile distilled water were prepared and stored in the ice chest. Hyperconcentration of plasma (10 and 20 per cent. solutions) gave late and slight clotting only. Dried *filtered* plasma proved to have lost most of its coagulating properties.

5. *Dilution of Plasma.*—Some workers have advocated comparatively high saline dilutions for the coagulase test. We would like to support Chapman,[6] who warns against the use of higher dilutions than 1 : 4. Sometimes only minute coagula are produced when greater plasma dilutions are employed, and these can easily be overlooked.[6, 14] An additional advantage of neat plasma or slightly diluted plasma is that, besides yielding a firmer clot, this clot is produced after a shorter time of incubation.

6. *Shortening of Clotting Time.*—Smith and Hale,[8] working with cocci-free broth filtrates which contained the enzyme, have shown that by the addition of up to 10 per cent. of an activating substance— produced by preparing a 10 per cent. extract of ground human or rabbit testicle in distilled water—they could appreciably shorten the clotting time of " atypical " human plasma or slowly working plasma from various animal species. We have tried this testicle extract in a small series of tests, adding 0·05 or 0·01 c.c. to 0·5 c.c. of plasma and have found it to shorten the clotting time in some cases. In pre-liminary tests no appreciable advantage was obtained by this enriched plasma for the slide agglutination. As we observed spontaneous clotting in a case of deteriorated plasma after addition of this extract, the necessity of always putting up controls is emphasised.

7. *Sterility of Glassware, and Contaminating Organisms*—The use of sterile test-tubes for the coagulase test in order to avoid false positives has been advocated.[19] This, in our experience, is not strictly necessary. False negative results, however, can be obtained when the culture used for inoculation includes also *B. proteus* or fibrinolytic streptococci (of Group A or C). These organisms will readily destroy any clot present (if added afterwards), or prevent clot formation, if present from the start of the test. Of a great variety of organisms tested no other appeared to have coagulative powers.

8. *Comparison of Cultures on Solid and in Fluid Media.*—In our experience either of these methods may be used, with similar results. We prefer culture on solid medium, but trypsin broth cultures are also very good, probably due to a more luxuriant growth if compared with other fluid cultures. Plasma diluted with broth is in general not superior to saline dilutions.

9. *Length of Incubation.*—On the whole, overnight incubation at 37° C. seems preferable to 3 hours' water-bath incubation and subse-quent standing at room temperature, though the difference is slight. One point in favour of the latter method is the fact that in a very few instances, using overnight incubation in the water-bath, a partial dissolution of the clot was observed. This process should be distinguished from a retractible clot which is mainly seen when using higher dilutions of plasma.

Slide Agglutination.—The clumping of staphylococci in the presence of plasma was noted in 1908,[4] and its use for routine purposes was recommended by Birch-Hirschfeld in 1924, and recently by Cadness-Graves *et al.* in this country. We have not been as fortunate as some workers, who recorded a correlation of 100 per cent. between this and the coagulase test, having obtained, as will be shown below, false negative as well as positive results. Another and more disturbing feature was the phenomenon that in a few instances an obvious aureus strain failed to be clumped at first attempt, while a second test gave a positive result, using the same (fresh) human plasma and the same culture in approximately the same proportions. Only the latter result

has been recorded, thus reducing—perhaps unjustifiably—the numbers of false negatives. It should be remembered that according to some authors [6, 20] pathogenic strains split off coagulase negative variants. If the slide test is carried out with only one colony, it should be repeated in cases where the outcome is questionable.

TECHNIQUE OF TESTS USED

Biochemical Examination

The carbohydrates were used in 1 per cent. peptone water solutions, inoculated with a loopful of a 24 hours' growth on agar or Löffler slopes, and incubated at 37° C. for 48 hours. This time limit was taken as it has been observed that some pathogenic strains were somewhat slow in fermenting mannite and other sugars, whereas some saprophytic strains will ferment mannite after further incubation [12, 21]. Gelatin stab cultures were also made and left at room temperature for eight weeks.

Coagulase Test

One half c.c. of neat human plasma (fresh, or dried and diluted with distilled water), or occasionally a 1 : 2 saline dilution of plasma was inoculated with a loopful of culture on solid medium. The tubes were incubated at 37° C., readings being made at frequent intervals, the last after 18 to 24 hours.

Slide Agglutination

A drop of saline and a drop of plasma were placed on a glass slide. A loopful of culture or one colony was then taken up with the platinum loop and approximately the same quantity of growth was deposited next to each drop. The fluids were then brought into contact with the cocci while stirring for about a dozen times. Unless the growth was of an R type, the saline suspension remained smooth and even, whereas in the case of virulent staphylococci the plasma prevented the formation of a smooth suspension, or caused clumping after an interval of up to 15 seconds. Avirulent strains yielded as even a suspension with plasma as with saline.

RESULTS

As indicated above, 200 aureus and 125 albus strains were examined. Fourteen aureus strains proved to be coagulase negative, which fact tallied in most cases with the outcome of slide agglutination and fermentation tests. Twenty-five albus strains were found to be coagulase positive.

As Table I shows, the aureus and coagulase positive albus strains fermented in most instances lactose, mannite, trehalose and sorbite, whereas coagulase negative albus strains did so to a very much lesser degree. The difference is particularly great with mannite, but sorbite also proved of value, as only a quarter of the albus strains were able to ferment this sugar. Gelatin was liquefied by the latter in about a third of the cases. It is a point of interest that the coagulase negative

aureus strains showed a less marked difference, as they were able to use the various sugars in 40 to 60 per cent. The citreus strains— not tabulated—were found to have varying fermentation properties, but

TABLE I

Positive Results obtained with the Following Media

Groups.	Number examined.	Glucose.	Lactose.	Mannite.	Gelatin.	Trehalose.	Sorbite.
Coagulase positive aureus	186	186 100%	184 98·9%	184 98·9%	184 98·9%	99 of 107 92·5%	74 of 82 90·2%
Coagulase positive albus	25	25 100%	25 100%	24 96%	24 96%	15 of 15 100%	15 of 15 100%
Coagulase negative albus	100	87 87%	64 64%	18 18%	35 35%	25 of 56 44·6%	13 of 50 26%
Coagulase negative aureus	14	14 100%	9 64·3%	6 42·9%	6 42·9%	6 of 10 60%	5 of 8 62·5%

The positive results indicate fermentation of the sugars and liquefaction of gelatin.

none fermented mannite, most did not ferment any of the carbohydrates used ; all liquefied gelatin.

The same relation between coagulase positive and coagulase negative staphylococci becomes evident if one notes the number of sugars fermented. In Table II only lactose, mannite, trehalose and sorbite are considered. Thus 96 per cent. of the aureus strains and 93·5 per cent. of the coagulase positive albus strains fermented either 4 or 3 sugars.

TABLE II

Groups.	Number examined	Number of Carbohydrates fermented.				
		4	3	2	1	0
Coagulase positive aureus .	77	63 82%	11 14%	3 4%	... ...	... ...
Coagulase positive albus .	15	13 87%	1 6·5%	1 6·5%	... ...	... ...
Coagulase negative albus .	58	4 6%	4 6%	11 20%	34 60	5 8
Coagulase negative aureus .	7	1 13·5%	2 28·8%	2 28·8%	2 28·8%	... ...

In contrast to this, only 12 per cent. of the coagulase negative albus strains showed fermentation of 4 or 3 sugars ; probably this figure is really still smaller, the cause for the apparent increase being that several of the albus strains were isolated from urine. There exists some ground for supposing that the aureus as well as virulent

albus strains may lose their power of coagulase production in the urinary tract, though still being pathogenic to man. Nevertheless, the bulk of the albus strains, 80 per cent., fermented only one or two of the sugars, while 8 per cent. were unable to use even one. Though these are only group characteristics, they may be of some help in some cases in determining the virulence of an unknown staphylococcus strain.

On the whole, the slide agglutination tallied well with the coagulase test ; but, as Table III shows, some discrepancies were observed. In the case of coagulase positive albus strains, however, false negatives

TABLE III

Discrepancies between the Slide Agglutination and Coagulase Test

Groups.	False Positive Slide Agglutination.	False Negative Slide Agglutination.
Coagulase positive aureus .	...	5 (2·6%)
Coagulase positive albus .	...	7 (28%)
Coagulase negative albus .	3 (3%)	...
Coagulase negative aureus .	1 (7%)	...

amounted to as much as 28 per cent. The numbers tested were, of course, small. Whether this result is only a chance outcome, or whether it holds good as group characteristic for all albus variants, thereby indicating a loss in pathogenicity coupled with loss of pigment, only further investigation can prove. None of the *citreus* strains was clumped by plasma.

Submitting the outcome of the coagulase tests to further examination, some disputable results become apparent. It is a mistake, as some workers have done, to test only definite aureus strains from abscesses or, on the other hand, definite albus strains, for example, from the air. There are some strains which, in respect of their pathogenicity, stand somewhere in between. Though the coagulase test is the best guide which we possess as to the virulence of staphylococci, it is bound to fail sometimes. What interpretation has to be made, for example, in the case of a strain which readily ferments glucose, lactose, mannite, trehalose and sorbite, liquefies gelatin in a few days, gives a positive slide agglutination, but fails to clot ·fresh plasma ? Two strains of this kind were obtained from sputum and one from blood culture. In cases of R strains, too, some questionable negative results were obtained.

Staphylococcal infections of the urinary tract merit some detailed consideration. Repeatedly strains were isolated which, judging by the signs and symptoms of the patient, the number of polymorphs and cocci in the centrifuged deposit of the urine, and the outcome of the

fermentation tests should be regarded as pathogenic, while both the coagulase test and the slide agglutination failed to give a positive result. Frequently those strains were also unpigmented. This should prove a fruitful field for further investigation.

In determining the relative importance of staphylococci isolated from the blood stream the coagulase test is obviously of great value. In this series among 48 strains there were 6 which showed golden pigmentation, but which by the coagulase test were shown to be presumably non-pathogenic. It should be remembered, however, that with these methods only an opinion can be given on the relative importance of an organism, as coagulase negative staphylococci have been shown to be the causative organism in cases of acute and sub-acute endocarditis.[22, 23, 24]

None of the *citreus* strains proved to be coagulase positive.

SUMMARY AND CONCLUSIONS

Three hundred and twenty-five strains of staphylococci, isolated from various sources, were examined. They were divided into 186 coagulase positive aureus, 25 coagulase positive albus, 100 coagulase negative albus and 14 coagulase negative aureus strains. The biochemical properties were investigated. If a strain ferments four or at least three of the carbohydrates used, the probability of its proving to be pathogenic is increased. The coagulase test was found to give reliable results in probably over 99 per cent.

The slide agglutination, while not without value for presumptive testing, is liable to yield false positive as well as false negative results.

Different methods of carrying out the coagulase test are discussed. Unfiltered human plasma, dried *in vacuo*, was found to be a reliable substitute for fresh plasma and could be stored at least for 14 months at 4° C.

The addition of testicle extract to plasma which proved to work slowly was found to be advantageous.

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