Technical method

A method for the electrophoresis of colicines in agar gel

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The design, construction, and use of an apparatus for the electrophoresis of colicines in agar gel is described, as are some preliminary experiments with it. The possibilities of this method in typing strains of colicinogenic organisms and for the study of colicines are also discussed.

In 1953, Ludford and Lederer, using a simple apparatus, demonstrated that substances known as colicines consisted of complexes whose components could be separated by means of electrophoresis inside agar gel. The technique consisted of growing a colicine-producing organism as a spot inoculum in the centre of a strip of agar. The presence of colicine was demonstrated by areas of inhibition in a carpet of a sensitive organism subsequently grown on the surface of the agar.

APPARATUS AND MATERIALS

The apparatus had a direct current power supply with a built-in time switch and the electrophoresis stand was made of Perspex. Platinum electrodes made contact with the buffer in the electrolyte trays and blocks of agar containing buffer linked the liquid buffer with the electrophoresis agar.

ELECTROPHORESIS AGAR Nutrient infusion broth (prepared in this department) was solidified with 1.1% w/v Davis New Zealand agar with a final pH of 7.4.

BUFFER Sorensen's phosphate buffer pH 7.4 M/15.

BUFFER AGAR Buffer was solidified with 2% w/v Davis agar.

ELECTROPHORESIS DISHES Phoenix glass drying dishes² 12 in. × 12 in. (chosen for their flat bottoms) with aluminium lids were used.

METHOD

Quantities of nutrient agar, each of 75 ml., were poured into sterile dishes which had previously been warmed to

37°C. to ensure an even layer of agar. When the agar was set the plates were dried and possible contamination was reduced by exposure to chloroform vapour. Excess chloroform was removed by opening the plates, agar surface down, in the 37°C. incubator for a few minutes.

COLICINE PRODUCTION The dried plates were inoculated with 10 colicine producers (these were applied with the tip of a straight wire) from Dorset egg stock cultures using a template. An alternative method of inoculating the producer strains was used when several sensitive indicator strains were subsequently to be applied to each plate and for this another template was used. The plates, inoculated with producer strains, were incubated for approximately 24 hours at 37°C. and then removed to the refrigerator (4°C.) to allow prediffusion of colicine. After 18 hours in the refrigerator the plates were allowed to come to room temperature. They were inverted onto the buffer agar blocks (resting in phosphate buffer in the trays) on the electrophoresis stand.

ELECTROPHORESIS The power supply was set to give 60 volts (the current taken was approximately 250 mA), and electrophoresis allowed to take place for 18 hours. After electrophoresis the plates were removed and the sites of inoculation were punched out using a no. 4 cork borer. The little plugs of agar were inverted, thus preventing the bacterial colony from being broken up and dispersed over the agar surface when the sensitive indicator strain was applied.

INDICATOR STRAINS On the same day that electrophoresis was begun, 5 ml. of nutrient broth was inoculated with the sensitive indicator strain(s).

Single sensitive indicator strain After electrophoresis and the treatment described above, the plates were flooded with the sensitive indicator strain broth culture (diluted to 20 ml. with normal saline) and allowed to dry. The dried plates were incubated for about 18 hours (overnight) at 37°C.

Many sensitive indicator strains An alternative method to that described above was the use of many sensitive strains applied to a single plate (up to a maximum of eight). The cultures of the sensitive strains were prepared as above but the plates were placed on the template once again and filter paper strips, saturated with the cultures, were drawn across the plate. Whether the strips were drawn to the anode or the cathode ends of the plate made no difference to the resultant pattern.

RECORDING THE RESULTS After preliminary examination of the results, the plates were photographed using a standard procedure of oblique transmitted light and Recordak Microfile film (35 mm.). When processed the negative film was cut, and mounted as 2 in. × 2 in. slides.

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RESULTS

Experiments have been carried out to see whether the results of electrophoresis could be repeated. Fredericq's colicines A-J (Fredericq, 1948) were subjected to electrophoresis on six separate days. The position of the components of the colicine complexes was revealed by using the same sensitive indicator strain (C6 of Fredericq, 1948) on each occasion. The conditions of the six runs were, as near as possible, identical. The electrophoretic patterns were photographed and the distances travelled by the components measured. It is necessary here to define what is meant by 'electrophoretic' pattern. The pattern is made up of the distance travelled by each component of the complex, the widths of the zones of inhibition, the direction of travel (to anode or cathode), together with the actual appearance of each zone (sharp or diffuse edge, presence or absence of resistant colonies, etc.).

The distances travelled by certain of the components were measured and these are shown in Table I. The ratios of distance travelled by a reference inhibition zone (colicine C) to the distances travelled by the other zones were measured, and are also given in Table I. Fig. 1 shows a typical plate of the type from which the measurements were taken.

Bearing in mind the limitations of the technique (variation in room temperature, length of incubation, size of inocula of producing and sensitive strains), there was little apparent variation in the electrophoretic patterns, both in the distances travelled by the same zones and consequently in the ratios mentioned above.

While carrying out runs to test the apparatus it was noticed that different batches of the same type of medium produced a slight alteration in the distances moved by

FIG. 1. Typical electrophoresis plate. Fredericq's colicines A, B, C, D, E, F, G, H, and I as shown by his C6 sensitive indicator strain.

individual components of the colicine complexes, which could not be accounted for by the experimental variations mentioned above. There was no alteration in the direction of travel or of the actual appearance of the zones of inhibition. That slight variations did occur was not surprising in view of the method that was employed in the preparation of the nutrient agar medium. The nature of the nutrient broth used in the preparation of this medium is dependent upon factors such as the quality and availability of the meats used.

TABLE I

DISTANCES TRAVELLED BY CERTAIN COMPONENTS OF COLICINE COMPLEXES AND RATIO OF DISTANCES TO REFERENCE COMPONENT OF COLICINE C

Run No.		Colicine Type											
		B	C	D	E	F	G		H		I	J	
		Da ⁺	Da ⁻ only	Sa ⁺	Sa ⁻	Sa ⁻	Da ⁻	Da ⁺	Da ⁻	Da ⁺	Da ⁻	Sa ⁻	Db ⁻
10	Distance Ratio	23 0.7	32 —	30 0.9	39 1.2	57 1.8	36 1.1	21 0.7	30 0.9	22 0.7	57 1.8	38 1.2	57 1.8
11	Distance Ratio	19 0.6	31 —	24 0.8	40 1.3	60 1.9	42 1.3	21 0.8	33 1.1	20 0.6	63 2.0	44 1.4	64 2.0
12	Distance Ratio	21 0.6	34 —	26 0.8	45 1.3	66 1.9	38 1.1	20 0.6	32 0.9	19 0.6	65 1.9	42 1.2	65 1.9
13	Distance Ratio	25 0.6	43 —	37 0.9	54 1.2	70 1.6	— —	— —	— —	— —	72 1.7	51 1.2	71 1.6
2	Distance Ratio	21 0.5	38 —	28 0.7	48 1.2	64 1.7	38 1.0	22 0.6	34 0.9	23 0.6	64 1.7	— —	— —
Harting	Distance Ratio	18 0.6	31 —	25 0.8	— —	— —	35 1.1	18 0.6	31 1.0	18 0.6	59 1.9	— —	— —

B to J = Fredericq colicine type

S = sharp-edge inhibition zone

+ = zone on the anode side of the starting line

- = zone on the cathode side of the starting line

D = diffuse-edge inhibition zone

a = zone nearest the starting line

b = the next zone after 'a'

FIG. 2

FIG. 2. Electrophoresis patterns of strains of *E. coli* 0.26:B.6 isolated from two outbreaks of infantile gastroenteritis. Reading from left to right: 1 to 5 from one outbreak (note the pattern produced by 3) and 6 to 10 from another outbreak.

FIG. 3

FIG. 3. Electrophoretic patterns produced by strains of *Shigella sonnei* isolated at different times representing four family outbreaks of dysentery which were unconnected epidemiologically. All were Abbott and Shannon type 7 and the colicine was shown by the Abbott and Shannon sensitive strain no. 17.

It was hoped that this problem could be overcome by using a standardized medium such as Difco dehydrated nutrient broth, which has been used in the phage typing of *Staphylococcus aureus* (Anderson and Williams, 1956). This broth is made in such large quantities and so rigidly controlled in preparation that only small variations occur from batch to batch. However, use of this medium produced drops of liquid on the surface of the agar during electrophoresis ('sweating'). As soon as the affected plate was moved the drops of moisture ran across the plate and quite frequently these drops contained colicine so that when the plate was developed with a sensitive indicator strain, patches of inhibition were produced which bore no relationship to the starting points.

As a result of these findings 15 litres of nutrient agar were prepared from a single batch of nutrient broth. This quantity was sufficient for the programme of experiments that was due to be carried out. This ruled out the repeated examination of the standard set of colicine producers, which was necessary each time a new batch of medium was used.

An investigation of two outbreaks of institutional gastroenteritis caused by *Escherichia coli* 0.26:B.6 (Chapple, 1959) showed that all isolates from each outbreak had identical serological and biochemical reactions. In addition a group of eight phages showed the same qualitative action on the isolates from each outbreak. However, it was apparent that there was a quantitative difference between isolates 1, 2, 4, and 5 and isolate 3. Not much attention was paid to this slight difference until the strains were examined electrophoretically when it became immediately apparent that there was a significant difference between isolates 1, 2, 4, and 5 and isolate 3 (Fig. 2). No satisfactory explanation can be given for

this observation as it was most unlikely that there could have been two completely different strains in this particular section of the isolation hospital concerned. A tentative suggestion is that the 'aberrant' strain was derived from the other or vice versa.

The patterns obtained with the isolates from the two *E. coli* outbreaks are shown in Fig. 2.

All the strains belonging to a particular Abbott and Shannon *Sh. sonnei* type (Abbott and Shannon, 1958) so far collected were examined. These strains were isolated at different times and from different people and it was unlikely that there was any connexion between strains. There were 12 such strains belonging to the Abbott and Shannon type 7. They all showed identical patterns when examined electrophoretically using the Abbott and Shannon sensitive indicator strain no. 17. Altogether 20 isolates of the Abbott and Shannon type 7 were examined as there was more than one isolation from some individuals. Figure 3 shows 10 of these isolates.

DISCUSSION AND CONCLUSION

The Abbott and Shannon technique requires the production of a strip of growth across the centre of an agar plate. At the end of 48 hours the bacterial growth is removed and sensitive indicator organisms are grown, as thin streaks, at right angles to the original inoculum. The pattern of inhibition of these indicator organisms is used to determine the type of the organism under test. This method employs the activity of the colicine complex as a whole and requires a large number of plates and sensitive indicator organisms. Also, it is time consuming and the result is obtained on the fourth day. The electrophoretic technique has certain advantages; it provides

a method whereby any colicinogenic bacterium can have the colicine complex examined. There is no necessity for using and maintaining a large collection of sensitive indicator strains and the actual result of the test can be recorded and retained on microfilm so that errors of interpretation are cut to a minimum and in case of doubt it is possible to refer back to the actual experimental results. The result is obtained on the third day. In addition it is possible to detect differences between strains by the electrophoretic method which were missed by the Abbott and Shannon technique whereas the converse does not occur. This method is being used at the present time for the examination of strains of *Sh. sonnei* which have been typed by the Abbott and Shannon method at the Dysentery Reference Laboratory, London, and which have then been sent to this Department.

From an examination of about 100 such strains it appears that the method can help in cases where it is difficult to interpret the results obtained by the Abbott and Shannon technique. Bearing in mind these facts, together with the fact that the electrophoresis method requires more complex and more expensive apparatus than the Abbott and Shannon technique, it is suggested that it is more likely that electrophoresis will complement rather than supersede present typing methods.

In addition to the practical applications of the electrophoresis method there is the possibility of using the technique for the study of the colicines.

Electrophoresis has given information with regard to the nature of the sensitivity of strains to colicine complexes. For example, the organism producing colicine C is sensitive to colicine D. On electrophoresis colicine D shows a complex of probably three components (using C6 as the indicator strain). However, when the organism which produces colicine C is used as the indicator strain, only one component of the D complex is visible. Work is being carried out to show how the various Abbott and Shannon sensitive indicator strains differ in their sensitivity to the same colicines. A search is being made for sensitive strains that will reveal the composition of colicine complexes. At the present time it is difficult to say exactly how many components there are in a given complex, because of the amount of tailing which is present in the electrophoresis plates. By finding strains and the conditions in which they are only sensitive to one component,

FIG. 4. Theoretical diagram of the breakdown of a colicine complex by the use of several different sensitive indicator organisms. A = most sensitive indicator available, and B, C, and D = indicators of differing sensitivity.

which shows as a spot with the minimum of tailing, it should then be possible to build up a picture of the complex like the theoretical one shown in Fig. 4.

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REFERENCES

- Abbott, J. D., and Shannon, R. (1958). *J. clin. Path.*, **11**, 71.
 Anderson, E. S., and Williams, R. E. O. (1956). *J. clin. Path.*, **9**, 94.
 Chapple, P. J. (1959). Ph. D. thesis, University of Bristol.
 Fredericq, P. (1948). *Rev. Belge. Path.*, **19**, suppl. 4, 1.
 Ludford, C. G., and Lederer, M. (1953). *Aust. J. exp. Biol. Med. Sci.*, **6**, 553.