SHORT ARTICLES

A NOTE ON BACTERIOCINE TYPING TECHNIQUES

J. N. MACPHERSON AND R. R. GILLIES

Bacteriology Department, University of Edinburgh Medical School

PLATE X

THE investigation of outbreaks of infection is often greatly facilitated by the use of laboratory methods that allow detailed characterisation of the causal microorganism, e.g., bacteriophage typing of staphylococci and serological identification of salmonellae. Some other bacterial species cannot be readily marked for epidemiological purposes and, until recently, *Shigella sonnei* and *Pseudomonas pyocyanea* (*Ps. aeruginosa*) were in this category. Within the last decade, however, the introduction of bacteriocine typing techniques has allowed these and certain other species to be typed. The techniques depend on the ability of the bacteria to produce bacteriocines that give different patterns of inhibition against a stock set of passive, indicator strains of related bacteria.

Abbott and Shannon (1958) introduced a method of colicine typing of *Sh. sonnei*, which with some modifications gives a rapid and reliable identification of at least 17 colicine types in this species (Gillies, 1964; Gillies and Brown, 1966). Similar methods have been used to type strains of *Escherichia coli* (Shannon, 1957; McGeachie, 1965) and *Ps. pyocyanea* (Darrell and Wahba, 1964; Gillies and Govan, 1966). Kohn (1966) reported that the typing of *Ps. pyocyanea* by pyocine production could be simplified if, instead of streaking the strain under test directly on to the surface of the medium, the inoculation was made on a strip of microporous cellulose acetate laid on the medium. He obtained satisfactory results with this modification of the method, which eliminated the need to remove the macroscopic growth of the producer strain with a glass slide.

The purpose of this note is to report our experience with Kohn's modified method.

MATERIALS AND METHODS

Producer strains. Three hundred and seventy-nine strains of *Pseudomonas pyocyanea* were acquired from two hospital laboratories; they had been isolated from a wide variety of lesions as well as from apparatus and other inanimate materials in the hospital environment; 89 strains of *Shigella sonnei* isolated from cases of bacillary dysentery in Edinburgh and Leeds were also examined.

Identification of strains. Each strain of *Ps. pyocyanea* was examined for Gram reaction, oxidase production (Kovacs, 1956), motility in semi-solid agar, pigment production, utilisation of glucose and ability to liquefy gelatin (Kohn, 1953). Strains of *Sh. sonnei* were identified biochemically in composite media (Gillies, 1956) and then by serological agglutination.

Indicator strains. The indicator strains used in the pyocine typing of *Ps. pyocyanea* were the 8 strains used by Gillies and Govan, and those used in the colicine typing of *Sh. sonnei* were the 15 indicator strains of Abbott and Shannon.

Culture media. The medium used in typing both *Ps. pyocyanea* and *Sh. sonnei* was Tryptone Soya Agar (Oxoid) to which 5 per cent. of horse blood was added; this tryptone soya blood agar (TSBA) was used for growing the producer strains. Infusion broth (Cruickshank, 1965) was used to grow the sets of indicator strains.

Received 20 June 1968; accepted 27 Aug. 1968.

Pyocine typing of *Ps. pyocyanea*. The strain to be typed was streaked diametrically across the surface of a TSBA plate so that the width of the inoculum was *c.* 1 cm. The plate was then incubated at 32°C for 14 hr. The macroscopic growth was removed with a glass slide, 3 ml of CHCl_3 was placed in the lid of the petri dish, and the medium-containing portion of the dish was then replaced on the lid for 15 min. so that microscopic remnants of the culture were killed. The plate was opened, the residual CHCl_3 was decanted, and traces of CHCl_3 vapour were eliminated by exposing the plate to the air for a few minutes.

Cultures of the eight indicator strains grown in infusion broth for 4–6 hr at 37°C were streaked on to the surface of the CHCl_3 -treated medium at right-angles to the line of the original inoculum, five of the strains to the left side of the line and the other three to the right. The plate was then incubated at 37°C for 18 hr.

Colicine typing of *Sh. sonnei*. The technique was similar to that described for *Ps. pyocyanea*, except that incubation of the potential colicine-producing strain was at 35°C and for 24 hr. The fifteen indicator strains were plated out with eight of them on the left and seven on the right of the original inoculum line.

Cellulose acetate strip modification. Strips of pure microporous cellulose acetate (Oxoid) were cut so that their length fitted the internal diameter of petri dishes and their width allowed inoculation of the test bacterium to be made with a wide margin of uninoculated strip. The strips were autoclaved at 115°C for 20 min. before they were applied to the surface of TSBA plates. After inoculation and incubation under the relevant conditions, the strip, along with the confluent bacterial growth on it, was removed with sterile dissecting forceps and discarded into a beaker containing CHCl_3 . Otherwise, the methods of typing were identical to those described above.

RESULTS

Each strain of *Pseudomonas pyocyanea* and *Shigella sonnei* was examined in parallel for bacteriocine production by the normal method and the acetate strip method. With the exception that the inoculum was made on a strip of cellulose acetate in the latter method, all the conditions of the two tests were identical.

The results for 379 strains of *Ps. pyocyanea* are given in the table. It will be noted that in only six types (determined by the normal method) did all the strains give identical results by the two methods. These six types were type 2 (2 strains), type 13 (4 strains), type 17 (2 strains), type 29 (4 strains), type 32 (1 strain) and type 36 (1 strain). All of 20 "untypable" strains also gave the same results by the normal and strip methods. Among 345 other strains in 13 types and the unclassifiable group, only 142 gave identical patterns of inhibition by the two methods. Thus, in all, only 176 (46.4 per cent.) of the 379 strains gave the same results by the normal and strip methods of testing.

Fig. 1 shows the inhibition pattern given by pyocine type-1 strains of *Ps. pyocyanea* by the normal method of testing; all the indicator strains, except no. 6 (top right) are inhibited. By contrast, as shown in fig. 2, the same producer strain, when tested on the acetate strip, does not affect any of the indicator strains.

Thirty-six strains of *Ps. pyocyanea* belonging to pyocine type 1 were submitted to sub-typing (Govan and Gillies, 1969) by the normal and acetate-strip methods; 17 of these strains showed patterns of inhibition by the strip method that differed from those obtained by the normal method.

Of 89 strains of *Sh. sonnei*, representing 7 different colicine types, only 6 strains gave different inhibition patterns on the 15 indicator strains when tested by the normal and strip techniques. These 6 strains were all of colicine type 3A when tested by the normal method, but yielded type-11 patterns of inhibition when tested by the strip method.

DISCUSSION

Bacteriocine typing methods have proved useful in epidemiological studies of bacillary dysentery caused by the serologically homogeneous species *Shigella sonnei*, and pyocine

BACTERIOCINE TYPING TECHNIQUES

FIG. 1.—Inhibition pattern given by pyocine type-1 strains of *Pseudomonas pyocyanea* when tested by the normal typing procedure; all the indicator strains, except no. 6 (top right), are inhibited.

FIG. 2.—Inhibition pattern given by the same pyocine producer strain as in fig. 1 when tested by streaking on a cellulose acetate strip laid on the surface of the medium. The absence of inhibition of any of the indicator strains contrasts strongly with the clear-cut pattern of inhibition in fig. 1.

TABLE
Pyocine type patterns of 379 strains of *Pseudomonas pyocyanea* tested by the normal and cellulose acetate strip methods

Type by normal method	No. of strains tested	No. of strains giving, by acetate strip method, the type pattern:																other type patterns *	u/t †	u/c ‡			
		1	2	3	5	8	10	11	13	16	17	18	22	25	27	29	32				35	36	37
1	111	23	3	1	4	0	0	1	0	0	5	2	0	0	0	0	0	0	0	0	43	6	23
2	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	40	0	0	1	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	2	0
5	42	0	0	0	38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
8	29	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	18
10	22	0	0	0	0	0	6	2	0	0	0	0	0	0	0	0	0	1	0	0	3	0	10
11	17	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	1	0	1
13	4	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
16	7	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	1
17	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1
18	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
25	5	0	1	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	1
27	11	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
29	4	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
32	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0	0	0	0	0	0	0
35	10	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	3
36	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
37	4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
u/t †	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
u/c ‡	44	0	0	0	3	2	0	0	0	1	1	0	0	0	0	0	0	0	0	9	26	1	0

* When tested by the strip method, six of the (normal-method) type-1 strains gave patterns of types 4, 6, 9, 14, 24 and 28; two type-3 strains gave type-30 patterns; one type-8 strain gave a type-19 pattern; one "unclassifiable" strain gave a type-14 pattern.

† u/t = Untypable strains, i.e., strains not producing pyocines detectable with our indicators.

‡ u/c = Unclassifiable strains, i.e., strains giving patterns of inhibition differing from those of the 37 accepted pyocine types.

typing of *Pseudomonas pyocyanea* is now in use in many countries and appears to be more universally acceptable than the bacteriophage or serological methods that have been described by many workers over the years. Although the bacteriocine typing techniques presently in use are simple to perform, any modification that could speed up the processing of plates before the application of the indicator strains would be valuable.

Kohn (1966) reported a simplification of pyocine typing in which a sheet of cellulose acetate was applied to the medium and the producer strain inoculated on to it; after incubation, the growth of the producer strain could be removed along with the cellulose acetate strip. Kohn reported favourably on this modification of pyocine typing, but he did not state the number of strains examined nor did he indicate their pyocine types.

We have found that less than half of the strains of *Ps. pyocyanea* we tested gave identical patterns of pyocine production by the acetate strip and normal methods of pyocine typing. It is impossible to reconcile our findings with those of Kohn, but it should be noted that Wahba (1963) also commented that not all pyocines were capable of passing through cellulose acetate.

Since we autoclaved the cellulose acetate strips prior to use, it might be considered that they suffered physical damage that influenced the results of the tests. However, additional tests with strips that were not autoclaved showed no better correlation with the results obtained by the normal pyocine typing method.

As can be seen in the table, the 345 strains of *Ps. pyocyanea* (301 giving recognised pyocine type patterns and 44 unclassifiable) that produced pyocines by the normal method of typing, included 83 (24.1 per cent.) that did not produce pyocines capable of passing through cellulose membranes and appeared to be untypable when tested by the strip method. Similarly, a further 119 strains when tested on cellulose acetate membranes gave patterns of pyocine production that differed from those given by the normal typing technique. In all of these instances the patterns obtained when the producer strains were grown on cellulose acetate strips showed a loss of inhibition of one or more of the indicator strains that were inhibited in tests made by the usual method; this indicates that pyocines differ in their size or physical state, and that some can pass through the acetate membranes whereas others are retained.

The much closer correlation of the results obtained by the two methods in the colicine typing of the 89 strains of *Sh. sonnei* was not unexpected, since the majority of the bacteriocines produced by *Sh. sonnei* diffuse more easily and more widely in TSBA than do those produced by *Ps. pyocyanea*, and it is apparent that the molecule size of the former allows them to pass through the acetate membrane (average pore size *c.* 0.5 μ m).

In view of our findings we cannot recommend the use of cellulose acetate strips in the two bacteriocine typing methods tested, but such material may have some value in separating bacteriocines from each other in a mixed preparation if some are retained by the strips and others can pass through them. Even if the suggested modification had not led to loss of inhibition patterns by the majority of strains tested, it is doubtful whether its use could have been justified, since it required the added expense of the membranes and, in our hands, did not save any time; it was, indeed, found to be more time-consuming to ensure that the cellulose acetate strips were smoothly and completely applied to the surface of the medium than it was to remove the macroscopic growth of the producer strain with a glass slide as in the normal typing procedure.

SUMMARY

Strains of *Pseudomonas pyocyanea* and *Shigella sonnei* were tested for bacteriocine production both by the normal method and by a modification of it in which the producer strains were inoculated on to cellulose acetate membranes that had been placed on the surface of the culture medium. In only 43.7 per cent. of 379 strains of *Ps. pyocyanea* were the results identical by the two methods. A higher correlation was noted in the case of *Sh. sonnei* and only 6 out of 89 strains of this species gave different results by the two methods. We cannot recommend the use of cellulose acetate membranes in these bacteriocine typing techniques.

We are grateful to Mr D. O. Brown and Dr J. R. W. Govan for their interest and support during this work which was made possible by a grant from the Scottish Hospital Endowments Research Trust.

REFERENCES

- ABBOTT, J. D., AND SHANNON, R. . . . 1958. *J. Clin. Path.*, **11**, 71.
 CRUICKSHANK, R. 1965. Medical microbiology, 11th ed., *Edinburgh and London*, p. 740.
 DARRELL, J. H., AND WAHBA, A. H. . . . 1964. *J. Clin. Path.*, **17**, 236.
 GILLIES, R. R. 1956. *Ibid.*, **9**, 368.
 " 1964. *J. Hyg., Camb.*, **62**, 1.
 GILLIES, R. R., AND BROWN, D. O. . . . 1966. *Ibid.*, **64**, 305.
 GILLIES, R. R., AND GOVAN, J. R. W. . . 1966. *J. Path. Bact.*, **91**, 339.
 GOVAN, J. R. W., AND GILLIES, R. R. . . 1969. *J. Med. Microbiol.*, **2**, 17.
 KOHN, J. 1953. *J. Clin. Path.*, **6**, 249.
 " 1966. *Ibid.*, **19**, 403.
 KOVACS, N. 1956. *Nature, Lond.*, **178**, 703.
 MCGEACHIE, J. 1965. *Zentbl. Bakt., Abt. I, Orig.*, **196**, 377.
 SHANNON, R. 1957. *J. Med. Lab. Technol.*, **14**, 199.
 WAHBA, A. H. 1963. *J. Hyg., Camb.*, **61**, 431.

METABOLISM OF THE LIPOPROTEINS OF SERUM BY LEPTOSPIRES: DEGRADATION OF THE TRIGLYCERIDES

L. B. KASÁROV AND LAURA ADDAMIANO

Laboratori di Microbiologia, Istituto Superiore di Sanità, Rome, Italy

PLATE XI

THE studies of Schneiderman *et al.* (1951), Woratz (1955), Fulton and Spooner (1956), Helprin and Hiatt (1957), Johnson (1958), Ivler (1960), Johnson and Wilson (1960) and Mifuchi, Hosoi and Yanagihara (1961) have shown that the lipids are an important energy source for leptospires and that the major function of the serum as a component of nutrient media for leptospires is to supply lipids. Helprin and Hiatt, Woratz (1957), Ivler (1960), Vaneseltine and Staples (1961), Johnson and Gary (1963), Ellinghausen and McCullough (1965), Shenberg (1965, 1967) and Shenberg and van der Hoeden (1961) have proved the role of fatty acids as growth factors for leptospires by showing that they will replace the serum in a nutrient medium. Johnson and Gary showed that one strain belonging to serotype *pomona* utilised palmitic acid.

Almost all (c. 95 per cent.) of the fatty acids in the serum are found in the lipoproteins as esters of glycerides and cholesterol and as esters or amides of phospholipids. The capacity of the leptospires to utilise the lipoproteins depends on their possession of enzymes catalysing degradation of lipoproteins. The present paper describes observations on the degradation of the triglycerides of the serum by leptospires.

MATERIALS AND METHODS

Organisms. Observations were made with both parasitic and saprophytic strains of *Leptospira* taken from the collection of WHO/FAO *Leptospira* Reference Laboratory, kept in the Istituto Superiore di Sanità in Rome. The parasitic strains were: *copenhageni* Monika, *copenhageni* Wijnberg, *copenhageni* Ratto 67, *icterohaemorrhagiae*

Received 11 Mar. 1968; accepted 29 July 1968.