COLICINE TYPING OF *ESCHERICHIA COLI* IN PIGS

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THE classification of *Escherichia coli* strains is largely dependent upon serological techniques. However, for epidemiological investigations these techniques are time-consuming and dependent on the availability of an extensive range of typing antisera rarely available outside major typing centres. Bacteriocine typing, a simpler technique, has proven to be a valuable method for classifying strains of *Shigella sonnei* (Abbott and Shannon, 1958; Farrant and Tomlinson, 1966; Naito *et al.*, 1966) and *Pseudomonas aeruginosa* (Darrell and Wahba, 1964; Gillies and Govan, 1966) in epidemiological investigations. A limited amount of work has indicated that colicine typing may be a useful technique for differentiating strains of *E. coli* (Shannon, 1957; Linton, 1960; Prakash, 1962).

During oecological studies of the *E. coli* flora of healthy swine it was found that 53 per cent. of 5527 strains of *E. coli* were colicine-producers and that the proportion of colicine-producers in individual investigations ranged from 21 to 67 per cent. (Craven and Barnum, 1971*a* and *b*). Either all the isolates belonging to a particular serological O group produced colicine or all failed to do so, suggesting that isolates belonging to a particular serological type may all belong to the same colicine type. Thus, it was considered worth while to apply a colicine typing system in epidemiological studies of *E. coli*.

MATERIALS AND METHODS

Initial studies were directed at evaluating techniques and comparing the effect of cultural conditions on colicine production.

Test organisms. The following standard colicine producer and indicator strains were obtained from Professor P. Fredericq (University of Liège, Belgium).

A. Indicator strains: *E. coli* C6, sensitive to all colicines; *E. coli* ROW, a derivative of *E. coli* K12 susceptible to all colicines and giving resistant mutants easily. Resistant mutants: R/V, resistant to colicines V and I; R/E, resistant to colicines of group E; R/K, resistant to colicine K; R/B, resistant to colicine B.

B. Standard colicinogenic strains: CA18, *E. coli* producing colicine B; CA23, *E. coli* producing colicine D; CA31, *E. freundii* producing colicine A; CA38, *E. coli* producing colicines E+I; CA42, *E. coli* producing colicine F; CA46, *E. coli* producing colicine G; CA53, *E. coli* producing colicine I; CA62, *E. coli* producing colicines J+I; P1, *Shigella paradyserteriae* producing colicine S1; P9, *Shigella sonnei* producing colicines S3+I.

The cross-streak technique. This method, which is attributed to Fredericq, Thibault and Gratia (1946, cited by Linton), has been modified by various workers (Shannon; Abbott and Shannon; Abbott and Graham, 1961; Gillies and Govan). Each producer organism was streaked diametrically across an agar plate to give a line of confluent growth 5–8 mm wide. After incubation, the growth was scraped off the plate and the residual cells were killed by exposure to chloroform vapour for 30 min. The indicator organisms were streaked across the medium at right-angles to the original producer streak and incubated overnight. When colicines were produced, growth of one or more of the indicator strains was inhibited in a zone around the original streak.

Four producer organisms (CA23, CA38, CA42 and CA46) and six indicator organisms (C6, ROW, R/V, R/E, R/K and R/B) were studied.

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The overlay technique. This method, described by Fredericq (1957) as a means of demonstrating colicine production, does not appear to have been used for colicine typing. Colicinogenic strains were stabbed into agar plates, incubated for 24 or 48 hr and killed by exposure to chloroform vapour for 30 min. The indicator organism was added to melted semi-solid agar in sufficient amount for confluent growth and poured over the surface of the plate. Growth of the indicator organism was inhibited around colonies producing colicines to which it was sensitive. Seven producer strains (CA18, CA23, CA31, CA38, CA42, CA46, CA53) and four indicator strains (ROW, R/V, R/E and R/K) were studied.

Experimental design. The effect of several parameters on the diameter of the zone of inhibition was studied, using both the cross-streak and the overlay techniques. The two techniques were not compared. For each technique an experiment based on a block design was set up. The effect of the following on each of the resulting producer-indicator combinations was studied: (a) media: tryptic soy agar with and without 5 per cent. bovine blood; (b) time of incubation of producer organisms: 24 and 48 hr; (c) temperature of incubation: 37° and 40°C. Each block was replicated four times.

Application of colicine typing to oecological studies of porcine coliflora

The overlay technique was used for these investigations, by stabbing up to 15 *E. coli* isolates into trypticase agar plates, and incubating at 37°C for 24 hr.

Colicine typing based on colicine production by field isolates of E. coli. The 1059 colicine-producing *E. coli* isolates under study were collected from pigs in the same barn during the course of three different investigations. In the initial study, 269 colicine-producers were obtained from 95 pigs sampled on the same day. A similar sampling on the next batch of pigs in this barn yielded 302 colicine-producers. The remaining 488 *E. coli* isolates were collected from six litters of pigs over a 6-wk period. All these colicine-producing *E. coli* isolates were typed serologically. The methods of isolation, identification and serological classification have been described (Craven and Barnum, 1971a).

Colicine typing by the sensitivity of field isolates to standard colicinogenic strains. Four hundred and fifty-four *E. coli* isolates collected from 95 young pigs in the same barn and on the same day were tested for their sensitivity to the colicines produced by nine standard colicinogenic strains: CA18, CA23, CA31, CA38, CA42, CA53, CA62, P1 and P9.

RESULTS

Effect of cultural conditions on colicine production

In each of the 28 combinations of producer/indicator organisms the indicator strain was consistently sensitive or resistant. The degree of inhibition of the indicator organisms ranged from a completely clear zone to a zone where the growth was slightly less than elsewhere on the plate. The degree of inhibition appeared to be characteristic of particular combinations of colicine producer and indicator strains and was not altered appreciably by the different media or by differences in the duration and temperature of incubation. The mean diameter of the zone of inhibition was not altered by the addition of 5 per cent. bovine blood to the media. The diameter of the zone for all tests incubated at 37°C was 16.09 mm and at 40°C was 17.13 mm. This difference is statistically significant at the 1 per cent. level of probability. Similarly the mean diameter of the zone of inhibition was increased significantly from 14.04 mm when incubated for 24 hr to 19.18 mm after 48 hours' incubation.

The two-way and three-way interactions between time of incubation, temperature of incubation, and media were calculated. There was no significant interaction between these factors.

There was no difference in the results obtained between the cross-streak and the overlay techniques.

Colicine typing based on colicine production by field isolates of E. coli

Colicine types were defined in terms of the indicator strains they were able to inhibit. Results were read as positive when the indicator strain was inhibited by a colicine and a

TABLE I
Comparison of serological type and colicine type of 1045 isolates of E. coli

Sero- logical type	Number of strains of colicine type*											
	+++++	+++	++	+	—	++	+	—	++	+	—	++
O116	14	209	0	0	0	1	0	0	0	0	0	0
O114	153	0	0	0	0	0	0	0	0	0	0	0
O146	14	0	0	0	0	105	0	0	0	0	0	0
P831	4	41	0	0	54	0	0	1	5	0	0	0
O108	0	0	73	0	0	2	0	0	0	0	0	0
O98	0	0	72	0	0	0	0	0	0	0	0	0
D881	46	0	0	0	0	14	3	0	0	0	0	0
O145	0	0	60	0	0	0	0	0	0	0	0	0
AF241	34	0	0	0	0	1	0	0	0	0	0	0
T361	0	0	33	0	0	0	0	0	0	0	0	0
T421	28	0	0	0	0	0	0	0	0	0	0	0
AA611	21	0	0	0	0	0	0	0	0	0	0	0
C171	0	0	5	0	0	0	0	5	0	4	0	0
AA663	12	0	0	0	0	1	0	0	0	0	0	0
O69	8	0	0	0	0	0	0	0	0	0	0	0
O2	0	0	0	0	0	0	0	0	0	0	0	0
O76	2	0	1	0	0	0	8	0	0	0	0	3
D882	0	0	0	0	0	0	0	0	0	0	0	0
C2R1	2	0	0	0	0	0	4	0	0	0	0	0
C1101	0	0	0	0	0	0	0	2	0	0	0	0

* Based on colicine production. The results for the following sequence of indicator strains comprised a colicine type: R/V; R/E; R/K; R/B; R/CA31 and R/CA62.

colicine type was the pattern obtained when the results for six indicator organisms were recorded in a predetermined order. It was sometimes difficult to determine whether or not the growth of an indicator strain was inhibited although, in general, the degree of inhibition of an indicator strain was similar for different isolates belonging to the same serological type. The results are shown in table I. Five other colicine types were encountered but as each was represented by only one isolate the results were excluded from the table. In addition, nine isolates that were the sole representatives of nine serological types were also omitted. In general, most of the isolates belonging to a particular serological type belonged to one or sometimes two colicine types. Many serological types were included in most of the colicine types and, in fact, over 90 per cent. of the isolates belonged to only four colicine types. The diameter of the zone of inhibition and the degree of inhibition were recorded in all cases, but these did not vary appreciably between serological types.

TABLE II
Correlation of serological and colicine typing on the basis of sensitivity to known colicines

Serological type	Number of isolates tested	Number of colicine types	Number of isolates within each colicine type
O116	115	4	104; 6; 4; 1.
O98	53	12	13; 10; 8; 6; 3; 3; 3; 2; 2; 1; 1; 1.
O114	29	1	All resistant to colicines.
C161	26	9	7; 5; 3; 2; 2; 2; 2; 2; 1.
O36	25	2	14; 9.
P831	18	2	17; 1.
O146	14	1	14.
O8	14	2	8; 6.
C171	9	1	All resistant to colicines.
AA611	8	2	7; 1.

Colicine typing on the basis of sensitivity to standard colicinogenic strains

Three hundred and fifty-seven (79.3 per cent.) of the 450 *E. coli* isolates were sensitive to one or more of the colicines used in this study. All but one of the 93 isolates that were not sensitive to any of the colicines were colicine-producers.

Four hundred and thirteen (91.8 per cent.) of the isolates had previously been classified serologically into 22 types and the results of serological and colicine typing were compared. In general, there were large numbers of colicine types within each serological type and, in some cases, isolates belonging to particular serological types included isolates that were sensitive to colicines and others that were not (table II). In two instances all of the isolates belonging to particular serological types (O114 and C171) were not sensitive to any of the colicines.

DISCUSSION

In the experiments designed to study the effect of media, temperature, and duration of incubation each combination of a producer and an indicator organism consistently resulted either in inhibition or growth of the indicator organism when the overlay technique was used. The diameter of the zone of inhibition was not influenced by the addition of 5 per cent. bovine blood to tryptic soy agar. Gardner (1950) proposed that 5–10 per cent. of blood should be included to provide a source of catalase to eliminate any hydrogen peroxide that may be formed. Gillies (1964) reduced the concentration of blood to 2.5 per cent. without

altering the size of the zone of inhibition and the present results suggest that blood may be omitted from the media without affecting the results.

Increasing the temperature of incubation to 40°C has been reported to increase the width of the zone of inhibition (Vasenius, 1967). The present results confirmed this finding, but the increase in zone size was too small to be of significance for colicine typing.

The optimum time for incubating colicine producers is not clear; different authors have recommended from 24 hr (Gillies, 1964; Farrant and Tomlinson, 1966) to 3 days (Abbott and Shannon, 1958). All attempts at colicine typing *E. coli* have involved 48 hours' incubation of the producer organisms (Shannon, 1957; Linton, 1960; Prakash, 1962). In the present study, the diameter of the zone of inhibition was significantly greater when the organisms were grown for 48 hr than when grown for 24 hr. The smaller zone size is an advantage when typing large numbers of organisms, because it permits more colicine-producers to be grown on an agar plate without having the zones of inhibition overlapping.

The aim of these preliminary studies was to evolve a simple technique for the colicine typing of large numbers of *E. coli* isolated from oecological investigations. The use of the overlay technique and of growth of the producer organisms on tryptic soy agar for 24 hr at 37°C was a simple and effective way of achieving this aim.

Application of this system to typing *E. coli* isolated from swine demonstrated that isolates of a particular serological type usually all belonged to one or two colicine types when typing was based on colicine production by field isolates. However, the value of colicine typing was limited because isolates belonging to different serological types often all belonged to a single colicine type. The results suggest that isolates belonging to different serological types produce the same colicine and it appears, therefore, that colicine typing would not make a suitable substitute for serological typing of *E. coli*. The extent to which colicine production may be used to supplement serological typing will vary according to the proportion of isolates that produce colicines and the number of colicine types encountered.

The proportion of *E. coli* isolates sensitive to colicine is variable, but is generally higher than the proportion of strains that are colicine-producers. The proportion of sensitive organisms has been reported to vary from 50 to 73 per cent. (Linton; Prakash; Vosti, 1968).

In the present study almost 80 per cent. of the organisms were sensitive to one or more colicines. However, the value of a system of typing based on sensitivity to colicines was severely limited by the large number of colicine types included in each serological type. We concluded that the different colicine types represented inadequacies of the test rather than differences between organisms. Shannon examined *E. coli* strains from diverse human sources and concluded that sensitivity to colicines was not a reliable basis for a typing method. Even though the strains used in the present study were derived from one source, the results were too unreliable to serve as the basis for a colicine typing system.

SUMMARY

The effect of cultural conditions on the size of the zone of inhibition surrounding growth of colicine-producing organisms was examined. The size of the zone was not altered by the addition of 5 per cent. bovine blood to tryptic soy agar, but was slightly increased by raising the temperature of incubation from 37° to 40°C and substantially increased by extending incubation of producer organisms from 24 to 48 hr. A colicine typing system based on colicine production by field strains of *E. coli* was evolved and compared with serological typing in epidemiological investigations of *E. coli* on pigs. Colicine-producing isolates belonging to particular serological types generally belonged to the same colicine type. However, isolates belonging to a number of serological types frequently belonged to the same colicine type. A colicine typing system based on the sensitivity of field strains of *E. coli* to nine standard colicinogenic strains was too unreliable to serve as the basis for a colicine typing system.

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