SYMBIOTIC BACTERIOPHAGE AS A 'MARKER' IN THE IDENTIFICATION OF STRAINS OF *SALMONELLA TYPHI-MURIUM*

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Symbiotic bacteriophage is present in a large percentage of strains of *Salmonella typhi-murium*. A recent study (Boyd, 1950) has shown that this phage comprises a number of different types which can be identified by appropriate methods. These types occur separately, or sometimes in pairs, in different strains of the organism. Bacteria and phage divide in harmony, so that each daughter cell contains the same phage as does the parent. Thus, phage infection of a strain of bacteria is passed on indefinitely, and the phage constitutes an additional character or 'marker' by which a particular 'line' of bacteria can be recognized. Identification of the symbiotic phage in cultures of *S. typhi-murium* can therefore be used for tracing the origin and following the spread of an outbreak of infection, as cultures from related cases and carriers will all contain the same type. It is of course possible that in its peregrinations a strain of bacteria, if originally phage-free, might become infected, or one infected with a single type of phage might acquire a second, or indeed might cease to be phage-infected, but laboratory experiments suggest that the chance of any of these things happening is remote, at any rate as far as *S. typhi-murium* is concerned. As eleven types of phage have already been found in *S. typhi-murium*, and there is every reason to believe that others will be discovered, an ample range of subdivisions is furnished.

Though this method of identifying different races achieves the same results as that devised by Craigie and his colleagues for typing typhoid bacilli (Craigie & Yen, 1938), the approach to the problem is fundamentally different. Craigie's method turns on the use of a series of conditioned 'lytic' phages, each of which has a specific action on the bacterial type having the particular Vi antigen to which it is adapted. These subtle variations in the composition or structure of the Vi antigen are permanent hereditary characters of the bacterium, and the method is therefore one of great precision. The test is easily carried out if a battery of reliable type phages is available. Its only disadvantage is that the results may occasionally be obscured by the presence of symbiotic phage in the bacteria, but this occurs in a relatively unimportant number of cases. Felix & Callow (1943) have evolved a similar technique for typing *S. paratyphi* B and *S. typhi-murium*.

Yet another method is used for typing staphylococci (Wilson & Atkinson, 1945). A series of phages with an action directed against the O antigen is used. None of these is specific for any one bacterial type, but together they give varied patterns

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of positive and negative reactions by which different types can be recognized. This method is less satisfactory than the other, for apart from some definite types which are relatively common, many others occur showing a variety of combinations, and classification has proved difficult. A similar method has been used for *S. typhi-murium* (Lilleengen, 1948) and is suggested for *Pseudomonas pyocyanea* (Warner, 1950).

In all these earlier methods typing is carried out by ascertaining the action of known phages on the cultures which are being tested. In the method used in the investigation now being recorded, typing is achieved by identifying the symbiotic phage which the different strains carry.

First experiments with cultures from diverse sources gave results which showed that the method was feasible but, as the majority of the cases were scattered and unrelated to each other, the information provided was insufficient to assess its value. It was accordingly decided to examine a series of related cultures. This investigation is the basis of the present paper.

ORIGIN OF THE CULTURES

A collection of *S. typhi-murium* strains isolated during 11 months (February 1950–January 1951) in or near Manchester was selected for the experiment. The cultures were all derived from human cases or symptomless excreters living in the urban area which extends for 10–15 miles in each direction from the centre of Manchester and which contains a population of about two million people. The series included the majority of strains isolated in the area during 1950, and we were fortunate in being able to obtain epidemiological information about all the cases, including those diagnosed bacteriologically by other laboratories. It is therefore possible to relate our findings to the total of ascertained infection in the area.

Two hundred and sixty-one persons are known to have been infected with *S. typhi-murium* during the period under review. Two hundred and thirty-eight infections were diagnosed bacteriologically, 215 by the Manchester Public Health Laboratory and the rest by other laboratories. A further 23 were diagnosed clinically and were all closely associated with bacteriologically confirmed cases. The distribution of cases followed the familiar pattern: there were a few large outbreaks and a large number of apparently sporadic cases. In all, 100 separate 'incidents' of infection were recorded.

Not all the strains isolated at the Public Health Laboratory were kept, as the decision to carry out this investigation was not taken until late in 1950: 28 of 32 strains from one outbreak and 10 others were therefore not examined. The remaining 177 strains contained representatives of 83 of the 100 incidents and 126 of them were from 32 incidents in which two or more strains were available for comparison.

The cultures, on Dorset egg medium, were sent in three batches to the Wellcome Laboratories of Tropical Medicine, where they were examined. The labels bore only the index numbers under which the cultures had been isolated. To avoid any subconscious bias which might have an influence when cultures with consecutive numbers were being examined, the culture bottles were 'scrambled' and given

a new serial index number under which they were tested. The results were reported back to the isolating laboratory where, for the first time, they were correlated with the cases from which the specimens were derived. In a few instances, where apparent discrepancies were noted, the strains concerned were re-tested. This will be discussed later in the paper.

METHOD OF EXAMINATION

The methods and culture media used in isolating and identifying the symbiotic phage were those already described (Boyd, 1950). Two indicator strains were used, *S. typhi-murium* 1404 and *S. typhi-murium* 1411. A culture was tested by inoculating it into two tubes of broth. One of these was then lightly sown with 1404 and the other with 1411. Thereafter both tubes were incubated overnight at 37°C. A small portion from each tube was pipetted off and heated to 58°C. for 30 min. The remainder was passed through a Chamberland L3 filter candle. Thus, two samples were obtained from each tube, one sterilized by heat and the other by filtration. On marked sections of an agar plate, using a loopful of a growing broth culture, two areas about an inch in diameter were sown with 1404 and two with 1411. On a properly prepared plate these dried off almost at once. On one patch of 1404 was placed a loopful of the heated mixed culture of the test organism and 1404, and on the other a loopful of the filtrate of the same culture. These were deposited as drops and were not spread, so that they covered the centre of the underlying patch but did not extend to its margin. The two portions of the mixed culture of test organism and 1411 were similarly spotted on the patches of 1411. When these drops had dried off, the plate was incubated overnight.

Next morning the patches were examined with a binocular plate microscope fitted with lenses giving a magnification of about 20 diameters, using as illuminant a strong beam of light directed obliquely from above. As experience was gained it usually became possible to recognize at once, from the appearance of the patches or plaques which developed, the type of phage (if any) which was present. Occasionally it was necessary to dilute the filtrate (serial decimal dilutions) and to repeat the 'patch' test, in order to see the characters of individual plaques. Wherever there was any doubt, confirmation was sought by carrying out a neutralization test with specific antisera. In the present investigation this was done in the majority of cases to acquire experience: it was obvious, however, that in most instances it was unnecessary. In certain cases a special test was done. For example, Types B 4 and B 6 have the property—not shared by any of the others—of producing large plaques on certain strains of *S. paratyphi* C, and their identity can be confirmed by this test.

The type of symbiotic phage was therefore determined by the patch and plaque characters, by the effect of heating to 58°C. for 30 min., by the selective action of two slightly different indicator strains, by antigenic structure, and in some cases by range of action.

NEW TYPES

Since the publication of the previous paper (Boyd, 1950) three new types of phage have been discovered, one belonging to subgroup A and two to subgroup B.

Type A 4 (*Type strain S* 22). Plaques round, with a comparatively small central disk surrounded by a granular zone which fades almost imperceptibly into a weak halo. Diameter across halo up to 2 mm. The plaques are shallow and there is no clear marginal rim such as is seen in the other members of this group. Propagates on indicator strain 1404, and weakly on 1411. Resists heating to 85°C. for 30 min. Antigen is distinctive.

Type B 6 (*Type strain C* 156). This type has the same general characters as Type B 4, but differs in that it will only propagate on indicator strain 1411, and not on 1404. It has the same lytic action on certain strains of *S. paratyphi* C.

Type B 7 (*Type strain S* 176). Only one strain of this type has been found. Both plaques and antigen are distinctive, but as detailed investigation has not yet been completed, a full description will be reserved for a later publication.

From further work, which is now in progress, there is reason to suspect that the term 'phage-free' used in previous papers on this subject is a misnomer, and that it will ultimately be possible to isolate phage from every strain of *S. typhi-murium*. It is therefore proposed to change the designation 'phage-free' to 'phage-undetected'. This does not invalidate past or present findings, but predicts a further break-down in the erstwhile 'phage-free' series.

INCIDENCE OF STRAINS OF SYMBIOTIC PHAGE IN THE SERIES

The Manchester series contained representatives of ten different phages or combinations of phages. Thus, with the phage-undetected group, it was possible to recognize eleven varieties of *S. typhi-murium*. It is most important that these should not be unwittingly confused with the 'types' of Felix & Callow. For this reason it is proposed to eschew the use of the more accurate and descriptive word 'type' and to use instead the term 'mark'. Thus, a strain of *S. typhi-murium* infected with phage Type *A* 1 will be referred to as '*S. typhi-murium* Mark A 1,' or more briefly 'Mark A 1,' a strain with phages A 2 and B 2 will be 'Mark A 2 + B 2, and one in which no phage has been found will be simply 'Phage undetected'.

An analysis of the different marks in this series is given in Table 1. Four previously described marks (A 1, A 2, A 3 and B 5) and one combination (A 1 + A 2) were not found, but the three new marks (A 4, B 6 and B 7) and a new combination B 2 + B 4, are now recorded for the first time. One of the two strains of A 4 came from a case of gastroenteritis in a child and the other from a symptomless excreter in the same family. B 6, B 7, and B 2 + B 4 were from sporadic cases. The common varieties were B 1, B 4 and A 2 + B 2.

COMPARISON OF THE TYPING RESULTS WITH THE EPIDEMIOLOGICAL FINDINGS

Before using the comparison of strains from the same incident as a check on the consistency of the typing results, it was necessary to consider the strength of the epidemiological evidence for the association between cases in the different sorts

of outbreak. For example, it would be reasonable to expect all strains from explosive outbreaks in closed communities to be of the same type. In 'open' groups, such as families, the different individuals may come into contact with more than one source of infection, and it would not be surprising if two types were occasionally found. The incidents were therefore classified into the following epidemiological types:

(1) *Successive infections in closed communities* (hospital outbreaks).
(2) *Explosive outbreaks.*
(3) *Explosive family incidents.*
(4) *Successive family incidents.*
(5) *Sporadic cases.*
(6) *Isolated symptomless excreters.*

Table 2 shows the relative frequency of the different types of outbreak and the number of strains available for comparison in each.

Table 1. *Frequency of varieties of symbiotic bacteriophage in Manchester strains of* Salmonella typhi-murium

Marks	No. of incidents	No. of cultures
A4	1	2
B1	18	35
B2	2	3
B3	5	10
B4	8	50
B6	2	2
B7	1	1
A1+B2	2	2
A2+B2	30	46
B2+B4	1	1
Phage undetected	16	25

(1) *Successive infections in closed communities*

Outbreaks occurred among young children in two hospitals and although they have been described as separate incidents there is reason to believe that they were connected.

Hospital A. On 1 July 1950 a child with diarrhoea was admitted to a cubicle ward, and a few days later three other cases occurred almost simultaneously in this and an adjacent ward. In all, ten cases occurred within 3 weeks. Examination of the faeces of the staff was not made until several weeks after the beginning of the outbreak, and all specimens were negative. It was later found that the strain isolated from the case admitted with diarrhoea carried the phages A2+B2 and that nine strains from subsequent cases all carried B4. Therefore, it would appear that, contrary to expectations, the first case was not the source of the outbreak.

The Public Health Laboratory took no further part in the investigation, but it was learned later that sporadic cases of diarrhoea continued to occur in these

Table 2. Salmonella typhi-murium *infections in the Manchester area from February* 1950 *to January* 1951

Type of incident	No. of incidents	No. of persons involved: Cases	No. of persons involved: Symptomless excreters	No. of strains examined for symbiotic phage	No. of incidents in which multiple strains from same incident were examined for symbiotic phage: Incidents	No. of strains in each incident	Phage
(1) Successive infections in closed communities (hospital outbreaks)	2	36	9	44	2	(*a*) 10	B4
						(*b*) 34	B4 (33), A2+B2 (1)
(2) Explosive outbreaks	3	48	9	6	2	(*a*) 4	B3
						(*b*) 2	B2
(3) Explosive family incidents	6	22	—	22	6	(*a*) 5	B1
						(*b*) 4	B1
						(*c*) 2	B1
						(*d*) 5	A2+B2
						(*e*) 3	B1
						(*f*) 3	B1
(4) Successive family incidents	8	20	7	14	3	(*a*) 4	Phage undetected
						(*b*) 2	B1
						(*c*) 4	A2+B2
(5) Sporadic cases	73	73	28	82	1	6	A2+B2
					2	3	Phage undetected
					4	2	A2+B2
					3	2	B1
					2	2	B3
					2	2	Phage undetected
					1	2	A4
					1	2	B4
					1	2	One B1 One phage undetected
						2	One A2+B2 One phage undetected
(6) Isolated symptomless excreters	8	—	9	9	1	2	B1
Total	100	199	62	177	32	126	

wards for some months. In December one strain of Mark B4 was obtained from a child infected in the hospital and discharged while the faeces were still positive.

Hospital B. Between 13 and 29 July, five of the cases infected in hospital A were transferred to hospital B while still excreting the organism in their faeces. They were placed in an open gastroenteritis ward with children suffering from a variety of types of diarrhoea. The last of the transferred cases ceased to be infectious on 11 September. In the meantime two symptomless cross-infections with Mark B4 were detected on 8 and 12 August. The next cross-infection in this ward occurred on 25 October. It was caused by Mark B4. By this time a large outbreak was in progress in the neighbouring whooping-cough ward. A second cross-infection occurred in the gastroenteritis ward on 2 November and was due to Mark A2+B2. A case infected with Mark A2+B2 had been admitted to the ward on 15 September and was known to be infectious up to 27 October.

Cases began to occur in the whooping-cough ward in mid-September and continued for many months. Thirty strains of *S. typhi-murium* were isolated from patients and excreters up to the middle of January 1951, and all were Mark B4. The outbreak was still in progress at the end of the period of the investigation. Repeated bacteriological examinations failed to reveal a persistent bacterial carrier among the staff, although two persons excreted the organism transiently and one suffered a clinical attack later in the outbreak. It was concluded that a series of patient-to-patient infections had taken place.

In all, strains of forty-four of the persons infected in hospital were examined: forty-three were Mark B4 and there was evidence that they all formed part of one large outbreak. One was Mark A2+B2 and was probably a sporadic cross-infection from another patient in the same ward. The typing results were, therefore, in full agreement with the epidemiological evidence.

(2) *Explosive outbreaks*

An explosive outbreak is defined as the simultaneous occurrence of two or more cases in persons not of the same family. The group includes the classical 'food-poisoning' outbreaks due to the consumption of a common meal or article of food. Only one such outbreak occurred in or near Manchester, but persons from the area were involved in two others in the course of trips to holiday resorts. Representative strains from two of the outbreaks were available.

(*a*) Twenty-nine cases of gastroenteritis occurred among guests at a wedding reception. *S. typhi-murium* was isolated from twenty-four cases, from seven symptomless excreters who had also consumed the suspected meal and from the person who prepared the food but did not eat any of it. Four strains were typed, three from sufferers and one from the food-handler, and all were Mark B3.

(*b*) A large outbreak followed the consumption of meat pies at a holiday resort. Two strains obtained from convalescents on their return to the Manchester area were both Mark B2.

(3) *Explosive family incidents*

When two or more members of one family become ill on the same day and have shared a meal in the previous 48 hr. the occurrence is described as an

explosive family incident. All of the twenty-two strains derived from six such incidents were typed. In every case the organisms isolated from all the affected members of one family were of the same type. Five incidents were due to Mark B 1 and one to Mark A 2+B 2.

(4) *Successive family incidents*

A successive family incident is one in which two or more cases of clinical infection occur in a family with an interval exceeding 24 hr. between each case. Eight occurred in the year, but multiple strains were only available from three of them. The findings were consistent, four phage-undetected strains being obtained from one, two of Mark B 1 from another, and four of Mark A 2+B 2 from a third.

(5) *Sporadic cases*

A sporadic case is defined as a single clinical infection in which inquiry reveals no recent contact with another person with similar symptoms. Of the 100 incidents in this series seventy-three were sporadic cases: seventy-two occurred in persons living at home, and one involved a patient in a mental hospital. Bacteriological examination of the faeces of contacts were made in connexion with forty-seven of the cases, and one or more symptomless excreters were found in twenty-one incidents. Multiple strains were available for comparison from eighteen incidents. In sixteen of these (thirty-eight strains in all), organisms carrying the same type of phage were isolated from the case and the corresponding contacts. In two instances there were apparent discrepancies, and as they are the only ones in the entire series they will be described in more detail.

Case A. A child of 11 years had gastroenteritis due to a phage-undetected strain, and a strain of Mark B 1 was isolated from his younger brother 11 days later. At this time there was a prevalence of sporadic infection due to Mark B 1 in the district in which they lived.

Case B. A child of 2½ years was infected with a phage-undetected strain, and in the routine examination of the family contacts a strain of Mark A 2+B 2 was isolated 13 days later from his grandfather who lived in the same house.

Both of these incidents occurred in early September at the peak of the *S. typimurium* 'season', and it is not surprising that two such incidents were encountered in a series of this size.

(6) *Isolated symptomless excreters*

An isolated symptomless excreter is defined as a person who has no known connexion with clinical infections due to the organism, though he may have been examined because of diarrhoea due to some other cause. Seven of the nine discovered in the year were apparently unconnected isolations. One pair of strains from a mother and her child were alike (Mark B 1).

GEOGRAPHICAL DISTRIBUTION AND SEASONAL PREVALENCE

The geographical distribution and seasonal prevalence of the various types were examined in detail in the hope that they might throw some light on the source of infection and channels of spread of disease in the community. Little of impor-

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tance emerged, although in a few instances a suspicious grouping of small numbers of cases infected with similar strains was noted. It seems probable that with a widely distributed infection such as *S. typhi-murium* it will be necessary to examine a much larger sample before results of epidemiological significance are obtained.

DISCUSSION

The following are the discrepancies already mentioned which were rectified by a re-test.

Six strains, which were first reported as A 2, were found to have been isolated in association with other strains carrying A 2 + B 2. In all six, phage B 2 revealed itself after the culture had been passed two or three times. In another instance, only B 2 was found at the first test, and both A 2 and B 2 were detected on re-test. It would appear that when a strain is infected with both these phages there is a tendency for one to be suppressed: when either is found alone, repeated passing is, therefore, advisable to ensure that there is not a double infection.

One of the strains carrying the new phage A 4 was at first labelled A 1, and was not identified until a second strain of A 4 was isolated and worked out.

A strain of B 2 was at first wrongly identified as B 1.

A strain reported as B 1 was later found to be phage-undetected. This, without doubt, was attributable to faulty marking during the test.

Apart from the mistakes in the A 2 + B 2 series, which are unlikely to be repeated now that the pitfall is known, the discrepancies are insignificant and will become even fewer as experience accumulates.

When these were rectified, the results of typing seventy-two strains from ten incidents in which infection occurred simultaneously or where successive infections had taken placed in a closed community (Table 2, groups 1–3) showed complete agreement with the epidemiological evidence.

In twenty-two incidents in which the evidence for association between the affected persons was not so strong (groups 4–6) the results were consistent in twenty (fifty out of fifty-four strains). In two incidents the results were not consistent with the most obvious epidemiological explanation, but it may well be that the most obvious explanation was not the right one.

This method can, therefore, be accepted as a reliable means of identifying strains of *S. typhi-murium*. It has the disadvantage of being more time-consuming than Craigie's method (as developed by Felix & Callow for *S. typhi-murium*), mainly because in each test two cultures have to be passed through Chamberland filters. For this reason the test is not, in its present form, suitable for everyday use in routine work. There is little doubt, however, that these same principles could be applied to other groups of organisms, and the method might have its uses where no other means of typing exist.

Attempts are being made to devise a technique in which filtration is unnecessary. Fredericq (1950) has reported that when a bacterium-bacteriophage culture is treated with chloroform the bacteria are killed but the phage is unaffected. In cases where chloroform has this action such treatment followed by spinning would be equivalent to filtration. Unfortunately certain of these symbiotic phages—

notably B3 and B4—are markedly susceptible to the action of chloroform, and there is no doubt that if sterilization were carried out in this way many of these strains would be missed. Further work is being done along these lines in an attempt to find an easy method of getting rid of the bacteria. Recently, a method which appears to be satisfactory has been devised. Details will be published if more extensive trials prove that it is reliable. If it is successful, the test will then take on a new importance, and its advantages and disadvantages *vis-à-vis* the Craigie method will call for more careful consideration.

As far as it has gone, this investigation has been little more than an academic exercise. Because of the widespread incidence of the infection no important epidemiological data are to be expected from such a limited survey. If, however, extensive typing were to be carried out throughout the country, there can be little doubt that the information it afforded would be of value in tracing the course of outbreaks which would otherwise remain undifferentiated.

SUMMARY

(1) A collection of *S. typhi-murium* strains, isolated during 11 months, in or near Manchester, was examined for the presence of symbiotic bacteriophage. In all, 177 strains derived from eighty-three incidents were investigated.

2. The different phages, or combinations of phage, allowed these strains to be divided into eleven varieties. Three hitherto undescribed types of phage were discovered.

3. With two minor exceptions, all groups of strains isolated from associated cases of infection carried the same type of phage, so that the 'typing' was in agreement with epidemiological expectations. In the two exceptions, the relationship of the cases was not intimate, and infection from different sources was an obvious possibility.

4. It is concluded that this is a reliable method of typing. It is probably applicable to other groups of bacteria. Its value would be enhanced by the discovery of a simpler method than filtration for sterilizing the lysate without damaging the phage.

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REFERENCES

Boyd, J. S. K. (1950). *J. Path. Bact.* **62**, 501.
Craigie, J. & Yen, C. H. (1938). *Canad. Publ. Hlth J.* **29**, 448.
Felix, A. & Callow, B. R. (1943). *Brit. med. J.* **2**, 127.
Fredericq, P. (1950). *C.R. Soc. Biol., Paris*, **144**, 295.
Lilleengen, K. (1948). *Acta path. microbiol. scand.* **24**, 501.
Warner, P. T. J. C. P. (1950). *Brit. J. exp. Path.* **31**, 112.
Wilson, G. S. & Atkinson, J. D. (1945). *Lancet*, i, 647.

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