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# Further studies on the antibiotic resistance of *Shigella sonnei*

## II. The acquisition of transferable antibiotic resistance *in vivo*

By JOAN R. DAVIES, W. N. FARRANT
and the late A. J. H. TOMLINSON
*Public Health Laboratory, County Hall, London, S.E.* 1

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## INTRODUCTION

It has been shown (Davies, Farrant & Tomlinson, 1968) that the antibiotic resistance of some strains of *Shigella sonnei* isolated in the London area can be transferred to a suitable recipient strain of *Escherichia coli*. Our previous study was confined to the examination of one strain, i.e. the first strain isolated, from each family incident. Transferable resistance would have been detected only if it was present in the infecting strain or if a sensitive strain had acquired resistance early in the course of infection in the individual. Lewis (1967) has shown that, in an outbreak of infection with *Sh. flexneri* type 2*a* in a mental hospital ward, strains isolated from different individuals showed different antibiotic resistance patterns and much of this resistance was transferable to *E. coli* K12. It seemed important to attempt to assess the extent to which strains isolated from individuals or families showed antibiotic resistance different from that of the infecting strain.

It is the policy of many of the Local Health Authorities in our area to collect specimens of faeces from known and suspected cases of Sonne dysentery and from their contacts both for diagnosis and for clearance. This provided an opportunity to examine all the *Sh. sonnei* strains from family incidents and to compare them with the first. It seemed reasonable to assume that, within a family, any strain with an antibiotic resistance pattern which differed from the index strain only by a single transferable resistance, or by several resistance determinants transferred as a single unit, represented acquired resistance rather than a separate source of infection.

Our previous experience suggested that:

(*a*) Most strains of *Sh. sonnei* would be resistant to sulphonamide and that this resistance would be transferable to *E. coli* K12.

(*b*) Many strains would be resistant to 800 $\mu$g./ml. of streptomycin and that this resistance would not transfer.

(*c*) Some strains might possess a low level transferable resistance to streptomycin.

(*d*) Tetracycline resistance would be relatively uncommon but would be transferable.

(*e*) Most strains would be resistant to ampicillin but only those resistant to 500 $\mu$g./ml. or more would transfer this resistance.

(*f*) That resistance to other antibiotics would be extremely uncommon.

It was decided therefore to examine all the strains isolated from family outbreaks for the following properties:

(*a*) Sensitivity to sulphonamides, streptomycin, tetracycline and ampicillin.
(*b*) Degree of resistance to streptomycin (800 $\mu$/ml.) and ampicillin (500 $\mu$/ml.).
(*c*) Ability to transfer resistance to streptomycin, tetracycline and ampicillin.
(*d*) Colicine type.

## MATERIALS AND METHODS

The isolation and identification of *Sh. sonnei*, antibiotic sensitivity testing by the disk diffusion method and the technique for colicine typing have been described previously (Farrant & Tomlinson, 1966). We continued to use these methods for the first strain isolated (index strain) from each family incident.

In order to examine further strains from the index case and from other members of the family it was necessary to mechanise the techniques of sensitivity testing, colicine typing and testing for the transfer of antibiotic resistance. Specimens of faeces were plated on deoxycholate citrate agar direct and from selenite broth in the usual way. A typical colony, which agglutinated with *Sh. sonnei* antiserum on a slide, was picked onto a segment of a MacConkey plate. After overnight incubation each strain was subcultured onto a nutrient agar slope and stored until a suitable number of strains to be tested had accumulated. (In practice about 50–100 strains were tested once a week.)

A bacteriophage typing machine (Lidwell pattern) was adapted with a special head carrying twelve stainless steel $\frac{1}{16}$ in. rods set at $\frac{3}{4}$ in. centres. The rods were arranged in four rows (2-4-4-2) so that they could be used to inoculate a $3\frac{1}{2}$ in. Petri dish. Nutrient broth was dispensed in round-bottomed $3 \times \frac{1}{2}$ in. agglutination tubes set in racks at the same centres and arrangement as the rods. A lid was fitted over each set of twelve broths.

The rods were sterilized by immersion in boiling water and were first used to mark nutrient agar templates. Strains of *Sh. sonnei* to be tested were then inoculated over the marks on the templates and incubated for 4 hr. An extra template was inoculated with *E. coli* K12 $F^-Met^-$ (K12) over each mark. Growth was then transferred from the templates to the broths using the rods. Two sets of broths were inoculated from each template of *Sh. sonnei* strains, one set to determine antibiotic sensitivity and the other, with K12, to determine antibiotic resistance transfer. At the same time two plates of colicine medium were inoculated from each *Sh. sonnei* template. The broths were incubated overnight at 35° C. and the colicine plates at 33° C.

### *Antibiotic sensitivity tests*

Plates of media containing suitable concentrations of antibiotics were inoculated from the overnight broth cultures using the rods. All plates were then incubated overnight at 35° C.

The concentrations of antibiotics were chosen after a number of strains, whose minimal inhibitory concentration (MIC) had previously been determined, had been examined using the rods to deliver the inoculum onto plates containing

various concentrations of antibiotic. It was possible to select, for each antibiotic, a concentration at which all strains previously classified as 'sensitive' would be completely inhibited but on which all 'resistant' strains would grow. The concentrations used were:

Sulphathiazole, 10 $\mu$g./ml.
Streptomycin sulphate, 10 $\mu$g./ml.
Terramycin hydrochloride, 5 $\mu$g./ml.
Ampicillin, 5 $\mu$g./ml.

An additional plate containing 500 $\mu$g./ml. of ampicillin was used to distinguish the highly resistant strains.

The medium used for these sensitivity tests was that described by Davies (1954) except that it contained 0·14% basic fuchsin. This dye was added merely to distinguish the plates from the minimal agar containing antibiotics used for transfer experiments and referred to later.

This medium was used for three reasons:

(*a*) It gave clear-cut results in sulphonamide sensitivity tests even with the relatively large inoculum deposited by the multiple inoculator.

(*b*) It permitted the recognition of strains of *Sh. sonnei* exacting for amino acids. These strains are infrequent but when they occur this character is a useful epidemiological 'label'. The sensitivity of such strains was determined by the disk method.

(*c*) It did not support the growth of K12 which is exacting for methionine. It was possible therefore to determine the antibiotic sensitivity of *Sh. sonnei* strains from the mixed broths although in practice a set of broths containing the *Sh. sonnei* strains alone was used for this purpose.

The test for high level resistance to streptomycin was performed on nutrient agar containing 800 $\mu$g./ml. of streptomycin sulphate.

The results were read after overnight incubation at 35° C. as growth or no growth on the appropriate medium.

### *Colicine typing*

The colicine plates which had been incubated overnight at 33° C. were exposed to chloroform and the growth was removed with a sterile slide. One plate of each set was inoculated with indicator strain 17 (Abbott & Shannon, 1958) and the other with indicator strain Row, using a sterile swab to spread the inoculum. The plates were then incubated overnight at 35° C. Where a strain of *Sh. sonnei* had produced colicine a circular area of inhibition of growth (approx. 1·5 cm. diameter) was seen with one or both indicator strains. With the indicator strains used, four results were possible:

| | |
|---|---|
| No inhibition | Colicine type 0 |
| Inhibition of strain 17 only | Colicine type 7 |
| Inhibition of strain Row only | Colicine type 6 or 12 |
| Inhibition of both strains | Colicine type other than 0, 7 6 or 12 |



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Since about 80% of the strains of *Sh. sonnei* isolated from the index cases were colicine type 0 or 7 the use of these two indicator strains was appropriate to check the identity of subsequent isolations from the same family incident. When the index strain was of some other colicine type, inhibition of both indicator strains was accepted as evidence of identity unless some change in antibiotic sensitivity had occurred. Then the colicine typing was repeated by the standard technique using nine indicator strains.

### *Resistance transfer*

The multiple inoculator was used to transfer the inoculum from sets of broths containing a mixed growth of *Sh. sonnei* and K12 to plates of minimal agar containing antibiotics. The minimal agar, described previously (Davies *et al.* 1968) would support the growth of K12 but was nutritionally deficient for *Sh. sonnei*. It was found that at the appropriate concentration of antibiotic (streptomycin 10 $\mu$g./ml., terramycin 10 $\mu$g./ml., ampicillin 25 $\mu$g./ml.) growth would occur on the minimal medium only if antibiotic resistance had been transferred to K12. A control plate of minimal agar containing no antibiotics was included since some strains of *Sh. sonnei* produced enough colicine in the mixtures to inhibit K12 so that transfer could not be demonstrated by this screening technique. When no growth occurred on the control plate or when resistance to more than one antibiotic was transferred the test was repeated. A loopful of the mixed culture was plated on minimal medium containing antibiotics so that the drug resistance pattern of single colonies could be investigated. The criteria for deciding whether resistance determinants were transferred as a single unit or independently are given in our previous paper (Davies *et al.* 1968).

## RESULTS

Between March and September 1967 952 strains of *Sh. sonnei* were examined. These represented the first strains isolated from 221 index cases and all subsequent strains from the index case and his family contacts. The index strain from 199 incidents had no transferable antibiotic resistance other than to sulphonamide; the remaining 22 index strains transferred resistance to streptomycin, tetracycline or ampicillin.

### *Changes in antibiotic resistance in the index case*

*Sh. sonnei* was isolated a second time from 127 of the 199 index cases originally excreting a strain with no transferable resistance other than to sulphonamide. Nine of these second isolates differed from the index strain. From these 127 patients 151 further strains were examined, and those from two differed from the previously isolated strains. The details are shown in Table 1.

It will be seen that all strains showing a change in antibiotic resistance could transfer the newly acquired resistance determinant to K12. In three incidents (nos. 3, 4 and 7) there was a change not only of antibiotic resistance but also of colicine type. In each case the ability to produce the specific colicine was also transferable and was linked to the new resistance determinant.

Twenty-three further strains were examined from fourteen index cases whose first strain transferred resistance to streptomycin, tetracycline or ampicillin. No changes in resistance pattern were observed.

It might be supposed that the acquisition of new resistance would be more common in those index cases whose first isolate was multiply resistant. This was not the case; 126 index strains were resistant to sulphonamide, streptomycin and ampicillin; the strains from four patients acquired further resistances (nos. 5, 6, 7 and 11). The forty-nine patients whose index strains were resistant to sulphonamide and ampicillin or to sulphonamide and streptomycin produced four 'new' resistances while strains from three of the twenty-four patients whose index strains were resistant only to sulphonamide or to ampicillin acquired resistance.

Table 1. *Changes in antibiotic resistance in strains of* Shigella sonnei *isolated from index cases*

| | Index strain | | Subsequent strains | | | |
|---|---|---|---|---|---|---|
| Incident no. | Resistance pattern | Colicine type | Resistance pattern | Transferable resistance* | Colicine type | Strain showing change† |
| 1 | SuAmp | 0 | SuStTeAmp§ | Su, (StTeAmp) | 0 | 2nd (5) |
| 2 | SuAmp | 0 | SuStTeAmp | Su, (StTe) | 0 | 2nd (20) |
| 3 | SuAmp | 0 | SuTeAmp | Su, Te | 2 | 2nd (9) |
| 4 | SuAmp | 0 | SuTeAmp | Su, Te | 2 | 2nd (6) |
| 5 | SuSt‡Amp | 0 | SuSt‡Amp§ | Su, (StAmp) | 0 | 2nd (7) |
| 6 | SuSt‡Amp | 0 | SuSt‡TeAmp | Su, (StTe) | 0 | 3rd (12) |
| 7 | SuSt‡Amp | 0 | SuSt‡TeAmp | Su, (StTe) | 4 | 2nd (6) |
| 8 | Amp | 1B | TeAmp | Te | 1B | 2nd (9) |
| 9 | Amp | 1B | StTeAmp | (StTe) | 1B | 4th (37) |
| 10 | Amp | 4 | StTeAmp | (StTe) | 4 | 2nd (8) |
| 11 | SuSt‡Amp | 4 | SuSt‡TeAmp | Su, Te | 4 | 2nd (12) |

Su = sulphonamide, St = streptomycin, Te = tetracycline, Amp = ampicillin.
* Parentheses, in this column, indicate resistances transferred as a single unit.
† Parentheses, in this column, indicate number of days after isolation of the index strain.
‡ Resistant to 800 μg./ml. streptomycin.
§ Resistant to 500 μg./ml. ampicillin.

*Changes in antibiotic resistance in other members of the family*

Members of the families of 165 index cases were found to be infected. A total of 651 strains from 350 infected family contacts were examined. Of these, 319 persons (605 strains) came from 148 families in which the index strain did not transfer resistance other than resistance to sulphonamide. From eighteen of these patients strains with 'new' resistances were isolated. The families of index cases whose strains initially possessed transferable resistance to streptomycin, tetracycline or ampicillin showed no changes in resistance pattern.

The details of the eighteen strains with 'new' resistances are shown in Table 2.

The ten patients from the first five incidents belonged to families in which strains from the index case also showed an altered resistance pattern. Sometimes the same change was observed in several members of the family (nos. 1, 7 and 11) but in other families members excreted organisms of different patterns (nos. 4 and

5). In some families it could be argued that change had occurred in one individual who then infected others with the altered strain. This is possible in incident no. 1 where either the index case or patient '*a*' could have infected patient '*b*' with the altered strain. In incident no. 5, however, patients '*a*', '*b*' and '*c*' were all infected with the index strain since the changes were not observed until the second or subsequent strain from these patients.

All members of the family were not necessarily involved when a change was observed. In incidents nos. 5, 7, 14 and 16 some individuals continued to excrete organisms with the characteristics of the index strain.

As with the index cases all newly acquired characters were transferable and where multiple characters were acquired these were transferred as a single unit.

Table 2. *Changes in antibiotic resistance in strains of* Shigella sonnei *isolated from family contacts*

| | Index strain | | Strains from members of family | | | |
|---|---|---|---|---|---|---|
| Incident no. | Resistance pattern | Colicine type | Resistance pattern | Transferable resistance* | Colicine type | Strain showing change† |
| 1 | SuAmp | 0 | (*a*) SuStTeAmp§ | Su, (StTeAmp) | 0 | 1st (0) |
| | | | (*b*) SuStTeAmp§ | Su, (StTeAmp) | 0 | 1st (5) |
| 4 | SuAmp | 0 | SuStTeAmp§ | Su, (StTeAmp) | 0 | 2nd (12) |
| 5 | SuSt‡Amp | 0 | (*a*) SuSt‡Amp§ | Su, (StAmp) | 0 | 2nd (9) |
| | | | (*b*) SuSt‡Amp§ | Su, (StAmp) | 0 | 4th (21) |
| | | | (*c*) SuSt‡TeAmp | Su, (StTe) | 4 | 4th (22 |
| 7 | SuSt‡Amp | 0 | (*a*) SuSt‡TeAmp | Su, (StTe) | 4 | 1st (2) |
| | | | (*b*) SuSt‡TeAmp | Su, (StTe) | 4 | 2nd (8) |
| 11 | SuSt‡Amp | 4 | (*a*) SuSt‡TeAmp | Su, Te | 4 | 1st (3) |
| | | | (*b*) SuSt‡TeAmp | Su, Te | 4 | 1st (11) |
| 12 | SuSt‡Amp | 0 | SuSt‡TeAmp | Su, (StTe) | 0 | 2nd (7) |
| 13 | SuSt‡Amp | 0 | SuSt‡TeAmp | Su, Te | 0 | 1st (5) |
| 14 | SuSt‡Amp | 0 | (*a*) SuSt‡TeAmp | Su, (StTe) | 0 | 1st (0) |
| | | | (*b*) SuSt‡TeAmp§ | Su, (StTeAmp) | 0 | 3rd (7) |
| 15 | SuAmp | 7 | SuStTeAmp | Su, (StTe) | 7 | 1st (7) |
| 16 | SuSt‡Amp | 7 | SuSt‡Amp | Su, St | 7 | 1st (13) |
| 17 | Amp | 4 | (*a*) SuAmp | Su | 4 | 1st (8) |
| | | | (*b*) SuAmp | Su | 4 | 1st (14) |

Su = sulphonamide; St = streptomycin; Te = tetracycline; Amp = ampicillin.
* Parentheses in this column indicate resistances transferred as a single unit.
† Parentheses in this column indicate number of days after isolation of the index strain.
‡ Resistant to 800 μg./ml. streptomycin; § Resistant to 500 μg./ml. ampicillin.

## DISCUSSION

The examination of strains of *Sh. sonnei* from convalescent patients and their family contacts showed some to be excreting strains with antibiotic resistance patterns different from those of the strains with which they were initially infected. The acquisition of new characters was not, however, a very frequent event, occurring in about 5 % of infected persons (11 out of 199 index cases and 18 out of 319 contacts) or in 17 out of 199 incidents (8·5 %).

These calculations are based on those incidents in which the index strain

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transferred no resistance other than that to sulphonamide, and undoubtedly underestimate the real frequency of acquired resistance. Where the index strain possessed transferable resistance this could indicate initial infection with a strain with this character, or it could mean that resistance had been acquired by a relatively sensitive strain before the first isolation was made. There were twenty-two families in which the index strain possessed transferable resistance to streptomycin, tetracycline or ampicillin. Four of these families were epidemiologically related, the index strain being isolated from the same borough in the same week and having the same characters and three other pairs of families were similarly related, so that sixteen epidemiological incidents were caused by these strains. These sixteen incidents were widely distributed in time and space, appearing as isolated examples of a strain with these characters in the boroughs concerned. However, in eleven instances there had been isolated from the same borough at the same time a possible 'parent' strain, i.e. a strain with a resistance pattern and colicine type lacking only the transferable resistance of the relevant index strain. In one case the evidence was strong, for the index strain had transferable resistance to sulphonamide and tetracycline and non-transferable resistance to ampicillin, it was colicine type 0 and exacting for tryptophan. Four other incidents had occurred in the borough during the previous two months from which identical strains—apart from the tetracycline resistance—had been isolated. Tryptophan-dependence is a very unusual character of *Sh. sonnei*, these five incidents being the only examples which were observed in this series. It seems unlikely that the tryptophan-dependent tetracycline-resistant strain was unrelated to the tetracycline-sensitive strains.

If these incidents are included in the calculation then of a total of 215 incidents there was evidence for acquired, transferable antibiotic resistance in 28 families (13 %).

The effect which transferable antibiotic resistance has on the proportion of strains of *Sh. sonnei* in the population resistant to various antibiotics will be determined by a number of factors. The most important of these may be the stage of infection at which the resistance is acquired since this, among other things, will determine the probability of infection with the new resistant strain being passed on to start a local epidemic. A good example of what may happen was observed in two day nurseries (not included in the family outbreaks described here).

At the beginning of November a strain of *Sh. sonnei* colicine type 9 (uncommon in this area), resistant to sulphonamide, streptomycin and ampicillin, was isolated from a number of cases in the borough of Camden. During the first 3 weeks of November a total of twenty-two persons was found to be infected with this strain including nine children attending Day Nursery 'A' and four of their home contacts. On 22 November nine children attending Day Nursery 'B' were found to be excreting *Sh. sonnei*. The strains from four of these children were identical with that known to be present in the borough, viz. colicine type 9, sensitive to tetracycline, but the other five children were excreting a strain of the same colicine type but resistant to tetracycline. Up to the end of the month a total of

twenty-one children attending Day Nursery 'B' and fifteen of their family contacts were found to be infected, seven with the tetracycline-sensitive strain and twenty-nine with the tetracycline-resistant strain. Up to the end of the year eight further persons in the borough with no obvious connection with Day Nursery 'B' were infected with *Sh. sonnei* colicine type 9. In four of these cases the strain was resistant to tetracycline. We do not know whether the acquisition of tetracycline resistance occurred only once or whether it occurred in a number of individuals. In either case the strain with the new character, arising at the beginning of an epidemiological incident, spread to produce a local outbreak.

The strains examined in this investigation were single-colony picks from specimens shown to contain *Sh. sonnei*. It seemed possible that many more persons were excreting organisms with acquired characters than were detected by this technique. Unless such organisms predominated we would have been unlikely to pick them. To estimate the extent of this source of error, 117 specimens of faeces which had been shown by the standard technique to contain tetracycline-sensitive strains of *Sh. sonnei* were plated on deoxycholate citrate agar containing 10 $\mu$g./ml. terramycin hydrochloride. From two patients a single colony of *Sh. sonnei* resistant to tetracycline was isolated. In neither case was *Sh. sonnei* isolated on a subsequent occasion so it was not possible to determine whether the resistant strain could have become predominant. On the other hand the 117 specimens contained two from individuals from whom a subsequent strain showed acquired tetracycline resistance. Plating on tetracycline-containing medium did not enable us to anticipate this change.

No information was sought on the antibiotic treatment, if any, that individual patients received. The presence of antibiotics in the gut might well favour the survival and continued multiplication of organisms with appropriate acquired resistance. But strains from patients infected initially with multiply resistant strains appeared no more likely to acquire new resistance than those from patients whose initial strain was relatively sensitive. The chance of *Sh. sonnei* acquiring resistance would also depend upon the presence in the gut of a suitable donor. Further studies on the incidence of coliform organisms with transferable antibiotic resistance in the faeces of patients infected with *Sh. sonnei* are in progress.

## SUMMARY

A method is described for the testing of strains of *Sh. sonnei* for antibiotic resistance, colicine type and drug resistance transfer using a multiple inoculator.

The examination of 731 strains of *Sh. sonnei* isolated from convalescent cases and their family contacts showed that about 5% of individuals were excreting organisms which differed from the infecting strain by a single transferable antibiotic resistance determinant. It is suggested that the acquisition of such resistance may occur in 13% of family incidents.

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