High and low level tetracycline resistance in *Shigella sonnei*

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SUMMARY

The results presented in this paper confirm the existence of two types of tetracycline resistance in *Shigella sonnei*. One group of strains had a high level of resistance to tetracycline and oxytetracycline, with a variable level of minocycline resistance. The second group had a lower level of tetracycline resistance and were sensitive to minocycline. After conjugation with *E. coli* K12 the selected *E. coli* transconjugants had the same levels of resistance as the parent *Sh. sonnei* strain, with one exception. *Sh. sonnei* 87 was resistant to a high level of tetracycline, but was able to transfer only low level resistance. It is suggested that *Sh. sonnei* 87 carries two plasmids: pSU1, a conjugative plasmid conferring a low level of tetracycline resistance, and pSU2, a non-conjugative plasmid which confers a high level of resistance to tetracycline.

INTRODUCTION

Strains of *Shigella sonnei* can be identified for epidemiological purposes by means of colicin typing and the pattern of antibiotic resistance (Farrant & Tomlinson, 1966; Davies, Farrant & Tomlinson, 1968*a*, *b*), including testing for high and low levels of resistance to tetracycline.

Kabins & Cohen (1968) postulated the existence of two classes of plasmid-mediated tetracycline resistance. These have been termed *tet* A and *tet* B (Scavizzi, 1972; Chabbert & Scavizzi, 1976). The *tet* B gene confers a high level of tetracycline resistance, and also resistance to minocycline. Strains carrying *tet* A have at most a very low level of resistance to minocycline. In this paper, we report the occurrence of these two classes of tetracycline resistance among a number of *Sh. sonnei* strains isolated between 1972 and 1975.

MATERIALS AND METHODS

Bacterial strains

The *Sh. sonnei* strains were selected from those sent to Guildford for typing during the years 1972–5. They were stored on nutrient agar slopes in sealed tubes at room temperature. Other strains referred to in the text are listed in Table 1.

Table 1. *Bacterial strains*

Strain no.	Plasmids	Relevant characters	Source
		Sh. sonnei	
87	pSUI, pSU2	Sm Tc (high)*	Clinical isolate
JD179	—	Sensitive	Clinical isolate
JD196	pSU1	Tc (low)	JD195 × JD179
JD199	pSU1, pSU2⁻	Sm Tc (low)	Acridine treatment of 87
		E. coli	
K12	—	Met⁻, F⁻, sensitive	
JD195	pSU1	Tc (low)	87 × K12

* Sm, Tc denote resistance to streptomycin and tetracycline respectively.

Media

The minimal medium used was that described by Davies *et al.* (1968 *a, b*). For *E. coli* K12 *met⁻*, it was supplemented with lactose (0·2 %), DL-methionine (5 μg/ml) and 0·02 % nutrient broth. For *Sh. sonnei*, the supplements were glucose (0·2 %) and nicotinic acid (trace), plus a small amount of basic fuchsin which was added to colour the medium.

Yeast extract agar and diagnostic sensitivity test agar (DST) were obtained from Oxoid.

Antibiotics

Oxytetracycline hydrochloride was obtained from Pfizer Ltd; tetracycline hydrochloride and minocycline were kindly donated by Lederle Ltd.

Screening methods

The methods used for screening strains for antibiotic resistance patterns, resistance transfer, and colicin typing have been described previously (Davies *et al.* 1968 *b*). Strains with low and high levels of tetracycline resistance are differentiated by their ability to grow on minimal agar containing 4 and 35 μg/ml of oxytetracycline, respectively.

Determination of minimum inhibitory concentrations (*MIC*)

The spot test for determining MIC values was adapted from the screening technique mentioned above. Using a multiple inoculator, broth cultures of the different strains were spotted on the surface of yeast extract agar plates containing a series of doubling dilutions of the antibiotic under test.

For a more sensitive determination of the MIC, colony counts were performed on DST agar containing different concentrations of antibiotics.

Resistance transfer

Transfer of tetracycline resistance was by the method of Smith (1969), selecting transconjugants on appropriately supplemented minimal medium containing 4 μg/ml of oxytetracycline.

Table 2. *Incidence of tetracycline resistance in* Shigella sonnei

Year	Number of strains	Non-conjugative		Conjugative		Number of tetra-cycline resistant strains	% of total
		Low	High	Low	High		
1972	277	85	20	44	14	163	59
1973	389	114	37	26	18	195	50
1974	444	45	0	31	19	95	21
1975	462	16	8	22	50	96	21

RESULTS

Of the 1572 strains of *Sh. sonnei* that were sent to Guildford for typing between 1972 and 1975, 549 (35%) were resistant to tetracycline. Table 2 shows the incidence of low and high levels of tetracycline resistance for each year, and whether the resistance was conferred by a conjugative plasmid. The proportion of strains that were tetracycline resistant declined during this period from 59 % to 21 %. This seems to be due mainly to a decline in the number of strains with non-conjugative resistance, whereas the percentage, of the total number of strains, from which tetracycline resistance was transferred by conjugation showed very little change, being 21 % in 1972 and 16 % in 1975. In a retrospective study there is always the danger that apparent trends can be produced by gradual changes in technique or interpretation of test results. In this case, however, retesting a number of these strains showed that very few had been wrongly characterized as either conjugative or non-conjugative.

For this study 130 tetracycline resistant strains were chosen, taking care to choose only one representative strain where a number with the same characteristics were isolated from the same area. Strains isolated from patients known to have travelled abroad were excluded.

Of the strains selected, 101 were originally recorded as having conjugative tetracycline resistance; of these, 44 were high level resistant strains, and 57 were resistant to a low level of tetracycline. There were 29 strains with non-conjugative resistance: 11 were high level resistant and 18 were low level only.

Four of the conjugative strains had died on storage and were therefore excluded. The remaining 97 conjugative strains were retested by the screening methods for antibiotic resistance and transferability and for colicin type. In no case was any change in colicin type observed.

Eight strains originally found to have high level tetracycline resistance, and 12 with low level resistance, had become sensitive to tetracycline. Fourteen of these 20 strains had also lost resistance to at least one other antibiotic. This was presumably due to plasmid instability during storage.

Thirteen strains originally resistant to a high level of tetracycline were found on retesting to possess only low level resistance. Apart from two strains which had also changed from high level streptomycin resistance to low level, none of the other

Table 3. *MIC values for* Shigella sonnei *isolates and derived* E. coli *strains*

Shigella strain no.	Values for *Sh. sonnei*		Values for *E. coli*	
	Oxytetra-cycline	Minocycline	Oxytetra-cycline	Minocycline
9	250	23	250	12
27	250	23	250	23
29	250	23	125	12
15	250	6	125	23
10	250	3	62	1·4
7	250	1·4	62	1·4
87	250	1·4	16	0·7
25	125	6	125	6
34	125	12	250	12
46	125	3	62	1·4
61	125	1·4	62	3
92	125	12	250	12
5	31	0·4	16	0·7
32	31	0·4	31	0·7
43	31	0·4	16	0·7
2	16	0·4	31	0·7
21	16	0·7	16	0·7
57	16	0·4	16	0·7
59	16	0·4	16	0·7
69	16	0·4	16	0·7
74	16	0·4	31	0·7
91	16	0·4	16	0·7
97	16	0·4	16	0·7
95	16	0·4	16	0·7
Sensitive controls	< 1·9	0·4	< 1·9	0·7

The MIC values were determined by a spot test on yeast extract agar. The values given are in μg/ml.

strains had lost any other antibiotic resistance marker. This could be explained either by loss of the high level tetracycline resistance gene from an unstable plasmid cointegrate, or by errors in reading the original results due to difficulties in distinguishing the two levels of tetracycline resistance. It is interesting to note that in only three cases did the reverse change, low to high level, occur. This presumably gives an estimate of the likely error rate.

Four strains had lost the ability to transfer their tetracyline resistance; this is thought to be due to experimental techniques, since five originally recorded as having non-conjugative tetracycline resistance had apparently acquired this ability. If transfer frequencies are low, it is quite likely that transfer will not be seen every time, using a spot test.

In order to study the levels of tetracycline resistance specified by particular plasmids in both the original *Sh. sonnei* host, and after transfer to *E. coli* K12, 24 strains of *Sh. sonnei* with conjugative tetracycline resistance plasmids were selected. Each strain had a different antibiotic resistance pattern, thus ensuring that each strain was an independent isolate. These were mated with *E. coli* K12, and the transconjugants selected for tetracycline resistance. After purifying the

transconjugants, the minimum inhibitory concentration (MIC) of oxytetracycline and minocycline was determined for each transconjugant and its parent *Sh. sonnei* strain.

The results in Table 3 confirm the division of the Shigella strains into two groups with respect to their tetracycline resistance. One group had a high level of resistance to oxytetracycline and a variable level of minocycline resistance; strains of the second group had a lower level of tetracycline resistance and were essentially sensitive to minocycline. Results for tetracycline (not shown) correlate well with those for oxytetracycline, although the tetracycline MIC values were somewhat lower. With the exception of strain 87 (see below for further discussion of this strain), the oxytetracycline resistance levels of the *Sh. sonnei* donors were paralleled very closely by the levels in the derived *E. coli* strains.

The nature of tetracycline resistance in strain 87

Strain 87 was isolated in 1972 in Irving (Scotland). It is resistant to streptomycin, and tetracycline, sensitive to sulphonamide, ampicillin and neomycin, and is colicin type 4. Strain 87 was mated with *E. coli* K12 for 6 h and tetracycline resistant transconjugants were selected; these were found at a frequency of 8×10^{-4} per donor cell, if $4 \mu g/ml$ of tetracycline was used. If selection was made at a concentration of $62 \cdot 5 \mu g/ml$, the apparent transfer rate was much less, about $1 \cdot 7 \times 10^{-6}$. Five colonies were purified from each tetracycline concentration, and their tetracycline resistance levels were checked by Stokes' method using a $50 \mu g$ tetracycline disk on DST agar, with *Sh. sonnei* 87 as the control organism. In every case, the *E. coli* strains were less resistant than the original donor, even when they had been selected on the high concentration of tetracycline. Thus the high level resistance determinant had not been transferred, and this confirms the previous result that *Sh. sonnei* strain 87 is able to transfer only a low level of tetracycline resistance. The plasmid mediating this resistance is designated pSU1. No streptomycin resistant transconjugants were obtained.

In order to eliminate the possibility that the tetracycline resistance gene of the plasmid was inefficiently expressed in *E. coli*, pSU1 was transferred by conjugation from JD195 to a sensitive *Sh. sonnei* strain JD179. The resulting isolates were shown by a disk sensitivity method to have low level tetracycline resistance only, i.e. their resistance was identical with that in the *E. coli* strains, and less than that of the original *Sh. sonnei* donor, strain 87.

This was confirmed by the more sensitive technique of colony counts on DST agar containing a range of concentrations of tetracycline. The cut-off point was quite sharp in each case giving the following values for the MIC:

> *Sh. sonnei* 87 (original donor) $25 \mu g/ml$
> JD195 (*E. coli* K12 (pSU1)) $6 \mu g/ml$
> JD196 (*Sh. sonnei* (pSU1)) $6 \mu g/ml$
> sensitive controls, JD179 and K12 less than $0 \cdot 8 \mu g/ml$

It should be noted that these values are much less than those quoted in Table 3 because of the different medium used and the more sensitive method employed.

When tetracycline resistance was induced in strain 87 by adding $2 \mu g/ml$ of tetracycline to the growing culture 3 h before testing, the MIC as determined by single colony formation was increased to $100 \mu g/ml$. This treatment had, however, little effect on JD196, hence the tetracycline resistance mediated by pSU1 appears to be non-inducible. The inducibility of the original donor strain, *Sh. sonnei* 87, must therefore be due to other genes present in this strain, not associated with the pSU1 plasmid but with the genes responsible for the high level tetracycline resistance.

In order to elucidate the nature of the antibiotic resistance determinants in strain 87, attempts were made to cure the strain of the plasmids it contains, firstly by growing it at $44°$, and secondly by growth at $37°$ in the presence of acridine orange. Colonies obtained on minimal medium were replicated onto plates containing tetracycline ($4 \mu g/ml$ or $62·5 \mu g/ml$) or streptomycin ($10 \mu g/ml$). No colonies sensitive to streptomycin were obtained; since this marker has therefore been neither transferred by conjugation nor eliminated by these methods, its plasmid location remains in doubt.

There were no colonies showing full sensitivity to tetracycline, but approximately 10% were unable to grow on minimal agar containing $62·5 \mu g/ml$ of tetracycline. Sixteen of these colonies (4 from growth at $44°$ and 12 from acridine orange treatment) were spot tested for their level of tetracycline resistance, and 13 were shown to have MIC values lower than their parent strain but close to that of JD196, i.e. they had lost their high level resistance but retained their low level determinant. (The other three colonies still had a high level of resistance and were not tested further.) This result indicates that the high level tetracycline resistance determinant of strain 87 is also plasmid borne; this non-conjugative plasmid is designated pSU2.

One of the pSU2-colonies, JD199, was purified and mated with *E. coli* K12. Low level tetracycline resistance was transferred at a frequency similar to that obtained with strain 87 as the donor. JD199 therefore still contains the pSU1 plasmid, apparently unchanged.

It has not been possible, unfortunately, to identify colonies that have lost pSU1 (low level) but retained pSU2 (high level). It remains a possibility, therefore, that pSU2 carries not a gene that by itself confers a high level of tetracycline resistance, but merely one that modifies the behaviour of the tetracycline resistance gene carried by pSU1.

DISCUSSION

The results presented here support the notion of two classes of plasmid mediated tetracycline resistance in *Shigella sonnei*, and the usefulness of these as epidemiological markers, although as with any plasmid mediated character the possibility of loss or acquisition of a plasmid during an outbreak must be borne in mind. Tetracycline resistance genes of the two classes occurred in similar numbers among both conjugative and non-conjugative strains.

The existence of two classes of plasmid mediated tetracycline resistance has been described by other workers previously (Scavizzi, 1972; Robertson & Reeve, 1972;

Foster & Walsh, 1974; Chabbert & Scavizzi, 1976). One class has a comparatively low level of tetracycline resistance and is almost fully sensitive to minocycline; this type is referred to as *tet* A by Chabbert & Scavizzi (1976) and as class II by Foster & Walsh (1974). The high level tetracycline resistance described here, which carries also a variable level of resistance to minocycline, is equivalent to that designated *tet* B by Chabbert & Scavizzi (1976) and class I by Foster & Walsh (1974). It is noticeable that whereas there is a reasonable correlation between the levels of resistance to tetracycline and oxytetracycline, these levels do not correlate with those for minocycline. Del Bene & Rogers (1975) have proposed that minocycline and tetracycline are taken up by the cell by different transport mechanisms.

Sh. sonnei strain 87 appears to possess two tetracycline resistance genes: a low level gene of the *tet* A type, which is located on a conjugative plasmid pSU1, and a gene of the *tet* B type which is not transferred and we suggest is located on a non-conjugative plasmid designated pSU2. It has not yet proved possible to mobilize pSU2, using for example *Salmonella typhimurium* 36Δ+ in a triple mating (Anderson & Lewis, 1965). Plasmid-mediated tetracycline resistance has been shown to be inducible by growth in low levels of tetracycline (Izaki, Kiuchi & Arima, 1966; Unowsky & Rachmeler, 1966; Franklin, 1967). The *tet* A gene carried by pSU1 was not induced under the conditions used here, whereas the tetracycline resistance of *Sh. sonnei* 87 was induced, presumably by induction of the *tet* B gene on PSU2. Chabbert & Scavizzi (1976) state that the increase in resistance level, by induction, appears to be much higher in *E. coli* harbouring *tet* A than in *tet* B strains. That would certainly not seem to be the case here, and in fact the results of other workers (for example Foster & Walsh, 1974), while showing a variation in induction ratios between different R factors, do not support the idea of a simple relationship to the type of tetracycline resistance.

The discovery of a naturally occurring strain of *Sh. sonnei* carrying both types of tetracycline gene is a reminder that the possible presence of a *tet* A gene together with, and masked by, a *tet* B marker in a bacterial strain should not be overlooked, for example when investigating the mechanisms of resistance. In this case, the presence of the two genes was disclosed by the failure of one of the plasmids to be transferred during conjugation. If the second plasmid had been a conjugative one, or worse still if the two genes had been linked on the same plasmid, the presence of the *tet* A gene would probably not have been noticed, and indeed the possibility of its presence would be very difficult to eliminate.

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