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Trimethoprim resistance in commensal bacteria isolated from farm animals

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SUMMARY

Trimethoprim resistance was examined in faecal bacteria obtained from chickens, sheep, cattle and pigs. The incidence of trimethoprim resistance in porcine strains was 17% (157/922) and, whereas 15·8% (146/922) of these bacteria were highly resistant, only 4% (37/922) of the isolates possessed trimethoprim resistance plasmids. Highly resistant porcine strains were obtained from 44% of the pig farms (41/93) but transferable trimethoprim resistance was found in isolates from 11% (10/93) of the farms. There was an association between the carriage of trimethoprim resistance plasmids and certain farms. Most of the resistance plasmids were not identical with those found in human clinical bacteria but one porcine plasmid was the same as the most ubiquitous trimethoprim resistance plasmid in Edinburgh.

INTRODUCTION

Although the first discovery of R-plasmids conferring trimethoprim resistance was made in clinical bacteria (Fleming, Datta & Grüneberg, 1972), it was shortly followed by the isolation of trimethoprim R-plasmids in bacteria isolated from cattle (Fleming, 1973). These veterinary R-plasmids heralded a new dimension in trimethoprim resistance because the resistance gene was encoded on a transposon (Tn*7*). In clinical bacteria the proportion of trimethoprim R-plasmid bearing enterobacteria progressively increased (Amyes, Emmerson & Smith, 1978; Romero & Perducca, 1977; Acar *et al.* 1977; Towner *et al.* 1980). In 1978–79, the proportion of trimethoprim resistant urine isolates from some Edinburgh hospitals had reached 36% and the proportion of trimethoprim R-plasmid carrying strains had reached 11% (Amyes, McMillan & Drysdale, 1980). At that time, nearly every single strain that was highly resistant to trimethoprim (minimum inhibitory concentration [MIC] > 1000 mg/l) possessed a trimethoprim R-plasmid.

The spread of trimethoprim R-plasmids was slower in animal strains than clinical isolates (Smith, 1980). In a 29-month survey of Great Britain starting in May 1974, 0·6% of animal faecal strains tested were trimethoprim resistant and a quarter of these (56) were highly resistant (West & White, 1979). The highly resistant strains represented 61% of all trimethoprim resistance (MIC > 10 mg/l). During this period the incidence of trimethoprim resistance around Aberdeen was high and some trimethoprim R-plasmid containing strains were found. However,

only a few resistant strains were found amongst bacteria isolated from the area around Edinburgh and none of them possessed trimethoprim R-plasmids (West & White, 1979). On the other hand, in *Salmonella typhimurium*, the proportion of trimethoprim resistant strains has been rising. This was particularly evident in bovine strains isolated in 1979 and 1980 when the proportion of trimethoprim resistant strains rose from 1·5 to 22·9 % (Ward, Rowe & Threlfall, 1982). All of these trimethoprim resistant *S. typhimurium* transferred their trimethoprim resistance (Threlfall *et al.* 1983). At the same time, the proportion of trimethoprim resistant *S. typhimurium* in other animal isolates was increasing but to a much lesser extent (Ward, Rowe & Threlfall, 1982).

This present study examines the incidence and type of trimethoprim resistance amongst faecal bacteria isolated from animals reared in farms in southern Scotland in order to identify the reservoirs of trimethoprim resistance.

MATERIALS AND METHODS

Isolation of bacterial strains

Faecal specimens were obtained from sheep, cattle and pigs at an Edinburgh city market. The specimens were streaked onto modified Difco Mueller Hinton Agar plates containing trimethoprim (10 mg/l) and similar plates lacking the drug (Amyes & Gould, 1984).

Characterization and sensitivity testing

The MIC of trimethoprim for each trimethoprim resistant organism was tested by diluting an overnight broth culture 10000-fold into saline and plating approximately 40–100 organisms onto the surface of Oxoid Diagnostic Sensitivity Test Agar (DSTA) plates containing increasing concentrations of trimethoprim. The lowest concentration showing no visible growth after 18 h incubation at 37 °C was taken as the MIC. The sensitivity of bacteria to other antibacterial drugs was performed by plating a similar dilution onto Oxoid DSTA plates containing 10 mg/l of drug, except in the cases of sulphamethoxazole and spectinomycin where 100 mg/l was used.

Transfer of resistance plasmids

Bacterial strains, resistant to 10 mg/l trimethoprim were tested for their ability to transfer trimethoprim resistance by a modification of the method of Smith (1969) as described previously (Amyes & Gould, 1984). *Escherichia coli* K12 strains resistant to either nalidixic acid (strain J62-1) or rifampicin (strain J62-2) were employed as suitable recipients (Bachmann, 1972).

Mobilization of non-self-transmissible plasmids

Organisms in which no self-transmissible trimethoprim resistance plasmid (R-plasmid) could be demonstrated were further investigated for the presence of non-self-transmissible plasmids by mobilization with the transfer factor X^+ (Anderson, Pitton & Mayhew, 1968; Anderson & Threlfall, 1974) by the method previously described (Young *et al.* 1986).

Extraction and separation of plasmids

Plasmid sizes were determined by electrophoresis in 0·5 % agarose gels by the method of Birnboim & Doly (1979). Plasmid DNA was prepared for restriction by the method of Takahashi & Nagano (1984) and the fragments separated in 0·7 % agarose gels.

Transposon extraction

Transposable DNA sequences were extracted from the bacterial chromosome and transferred into plasmid RP4 by the method of Young & Amyes (1983). The subsequent increase in size of RP4 was measured by agarose gel electrophoresis.

RESULTS

Proportion of trimethoprim resistant strains

Specimens were collected weekly from farm animals at Olivers and Sons Ltd, livestock market in West Edinburgh from November 1981 until August 1984. The majority of animals were destined for slaughter at the nearby abattoir and the carcasses were then distributed to the various butchers and supermarket chains in the Edinburgh area. The specimens were collected immediately after voiding. Care was taken not to include specimens from animals from the same farm at the same visit. Specimens from chickens were obtained weekly from D. B. Marshalls Ltd, Newbridge and were derived from 46 farms in Scotland.

The incidence of chickens possessing trimethoprim resistant faecal enterobacteria (MIC > 10 mg/l) was just under 4 % (Table 1). None of these strains was highly resistant to the drug and none of them were able to transfer their trimethoprim resistance to *E. coli* J62-2. The proportion of sheep with trimethoprim resistant bacteria was very low (5/974) even though animals from a large number of farms had been examined. Three strains were highly resistant and two of these were able to transfer their trimethoprim resistance to the standard *E. coli* strain. The number of cattle possessing trimethoprim resistant bacteria was also very low (19/966), again this was in spite of the number of animals from different farms tested. Fourteen of the resistant strains were highly resistant to trimethoprim (73 %) and six of them were able to transfer this resistance.

The proportion of pigs carrying trimethoprim resistant bacteria (MIC > 10 mg/l) was very much higher (157/922). Ninety-three percent of these bacteria (146) were highly resistant to trimethoprim. When these resistant strains were conjugated with *E. coli* J62-2, 35 were able to do so when the transfers were performed at 37 °C. No further strains were able to transfer their resistance when the conjugations were repeated at 30 °C. However, two trimethoprim resistance plasmids were mobilized in the presence of the X^+ factor.

Distribution of resistant strains from porcine bacteria

The proportion of highly resistant strains amongst the porcine bacteria varied through the study. The percentage of high-level resistant strains was analysed in 6-month blocks (Fig. 1). In 1982, the incidence of high-level resistance was high. However, it fell in the following year but rose again the year after. It is uncertain

Table 1. *Trimethoprim resistance amongst faecal enterobacteria in farm animals reared near Edinburgh (1982–1984)*

Animal	No. of farms	No. of isolates	Percentage of animals possessing bacteria with MIC of trimethoprim (mg/l) > 10 mg/l	> 1000 mg/l	Trimethoprim R-plasmids
Chickens	46	1004	3·9	0	0
Sheep	294	974	0·5	0·3	0·2
Cattle	231	966	2·0	1·4	0·6
Pigs	93	922	17·0	15·8	4·0

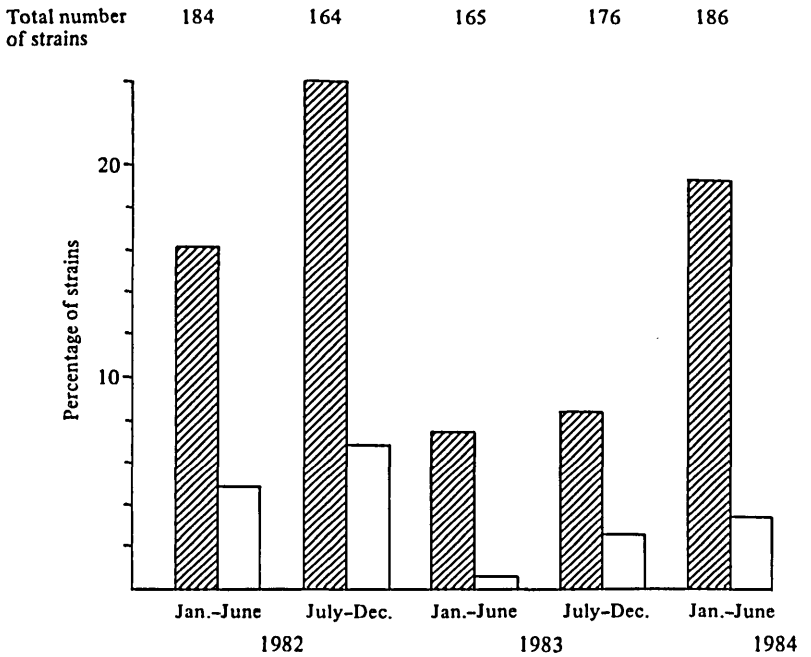

Fig. 1. The percentage of high-level trimethoprim resistance and transferable trimethoprim resistance in porcine strains. ▨, High-level trimethoprim resistance. □, Transferable high-level trimethoprim resistance.

why this change should have taken place but a corresponding variation was found with the high-level resistant strains which possessed trimethoprim R-plasmids. Thus, the proportion of highly resistant strains possessing trimethoprim R-plasmids remained fairly constant throughout the study period, except during the first 6 months of 1983.

Ninety-three farms in southern Scotland had provided pigs from whom these specimens were taken. The resistance results were collated for each farm and the farms classified according to the type of resistant strains which had been isolated. The distribution of the farms is shown in Fig. 2 (the location of six farms could

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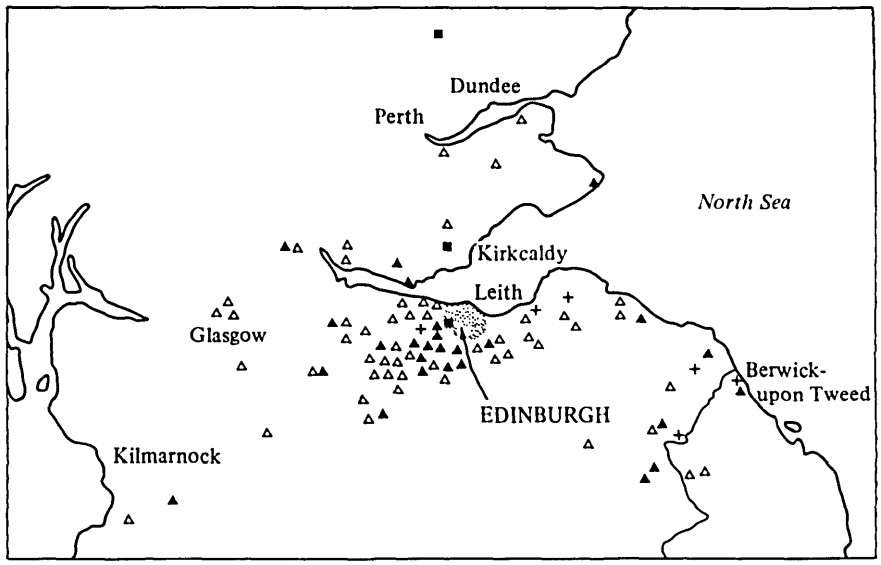

Fig. 2. The distribution of farms in southern Scotland providing high-level trimethoprim resistant bacteria. △, No strains with high-level trimethoprim resistance. ■, Some high-level trimethoprim resistance which was all transferable. ▲, Some high-level trimethoprim resistance none of which was transferable. +, Some high-level trimethoprim resistance some of which was transferable.

not be established: 3, △; 2, ▲; 1, ■). The majority of farms (55/93) provided no high-level resistant strains; however, most of those that did, produced strains which were unable to transfer trimethoprim resistance.

Although 15·8% of all strains were highly resistant to trimethoprim, these had come from 41 (44%) of the farms so high-level trimethoprim resistance was not confined to a specific group of farms. However, there was a cluster of 12 farms situated to the South-West of Edinburgh which had provided strains with high-level trimethoprim resistance. Over a quarter of all specimens (240) were obtained from these 12 farms and they also provided a third (47) of all the highly resistant strains.

Trimethoprim resistance plasmids

All the plasmids that carried trimethoprim resistance were classified according to their resistance pattern and size. A total of 17 plasmid types were identified (Table 2). Over half of the plasmid types (9/17) were found on only one isolation. Plasmid-containing strains were obtained from pigs in only 10 of the farms and thus there was a strong association between the presence of trimethoprim R-plasmids in porcine bacteria and certain farms. This close linkage was not between specific plasmid types and specific farms but rather between the predisposition towards trimethoprim R-plasmid containing bacteria in pigs from these farms. This is exemplified by the two plasmids found most frequently (pUK414 and pUK622) which were isolated from pigs from different farms on each occasion. Conversely, as many as five plasmid types were found in the porcine bacteria from the same farm.

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Table 2. *Resistance pattern and molecular size of trimethoprim R-plasmids from porcine strains*

Number	Resistance pattern	Molecular size (kbases)	Plasmid number	No. of farms
6	Sm/Sp Su Tp	90	pUK414	6
6	Su Tp	32	pUK622	6
5	Ap Sm Su Tc Tp	53	pUK348	2
3	Ap Km Sm/Sp Su Tc Tp	48	pUK350	2
2	Ap Km Sm/Sp Tc Tp	77	pUK351	1
2	Ap Km Su Tc Tp	56	pUK576	1
2	Ap Cm Km Tp	76	pUK620	2
2	Sm/Sp Tp	47	pUK425	2
1	Cm Km Sm Su Tc Tp	85	pUK640	1
1	Ap Sm/Sp Su Tp	74	pUK642	1
1	Km Sm Su Tc Tp	84	pUK643	1
1	Sm/Sp Su Tc Tp	106	pUK426	1
1	Km Sm/Sp Tp	115	pUK354	1
1	Sm/Sp Tc Tp	120	pUK415	1
1	Km Su Tp	67	pUK562	1
1	Km Tp	87	pUK588	1
1	Tc Tp	107	pUK555	1

Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Su, sulphamethoxazole; Tc, tetracycline; Tp, trimethoprim.
Total number of plasmids = 37.

Within the 17 plasmid types, spectinomycin resistance was found in nearly half of them. The linkage of trimethoprim and spectinomycin resistance is strongly suggestive of the presence of Tn7 in these plasmids.

Similarity of trimethoprim R-plasmids with human clinical plasmids

Most of these plasmids are quite unlike those that we have found in human bacteria (Amyes, Doherty & Young, 1986). However, two plasmids (pUK354 and pUK642) were similar in size and resistance pattern to two plasmids found in human urinary bacteria. The porcine plasmid pUK354 was similar to the human plasmid pUK458 but this plasmid was identified on only one occasion in both studies. On the other hand, the porcine plasmid pUK642 was similar to the most successful trimethoprim R-plasmid (pUK28) spreading through the clinical population. Plasmid DNA preparations of strain J62 (pUK642) and J62 (pUK28) were prepared by alkaline denaturation and digested by the restriction endonuclease *Hin* dIII. The restriction patterns of the porcine and human plasmids are identical (Fig. 3) indicating that they are the same plasmid.

High-level non-transferable trimethoprim resistance

The majority of the highly resistant strains possessed no transferable trimethoprim resistance and the majority of farms which provided highly resistant porcine bacteria had no trimethoprim R-plasmid containing strains. In most of the strains that were examined, there was an array of plasmids some of which were able to transfer resistance to antibacterial drugs other than trimethoprim. Four strains

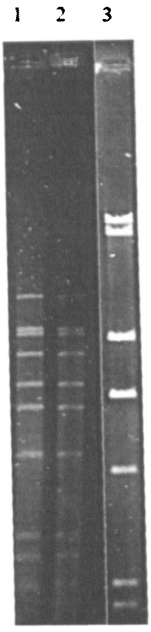

Fig. 3. The *Hin* dIII restriction pattern of the DNA of plasmids pUK28 and pUK642. Track 1, porcine plasmid pUK642. Track 2, clinical plasmid pUK28. Track 3, λ phage DNA.

devoid of any plasmids were examined for the presence of chromosomally located transposons. The *inc* P plasmid RP4 was introduced into the porcine strain and the transposon extracted. The RP4 plasmid was then transferred into strain J62-2 and the size of the transposon established.

In all four cases, the increase in size was about 12 kbases and RP4 had acquired resistance to trimethoprim and streptomycin. This suggests that the bacterial resistance derived from the insertion of Tn*7* into the chromosome of the porcine strain.

DISCUSSION

In this study, the only significant emergence of trimethoprim resistant faecal enterobacteria in farm animals has occurred in pigs. The lack of trimethoprim resistant strains in chickens and sheep is not surprising. Although chickens are intensively farmed and infections may be common, the finances of chicken rearing render it uneconomic to treat them with large numbers of antibacterial drugs. Sheep are not intensively farmed and, in Scotland, there is more space per animal than in the rest of the United Kingdom. Therefore the number of infections tends to be low. The proportion of cattle possessing trimethoprim resistant bacteria is also low compared with calves from England (Wise *et al.* 1985). Our results also contrast with observations that, in the past, trimethoprim R-plasmids in animals mainly derive from bovine bacteria (West & White, 1979). In Scotland, cattle are grazed more often and less densely than their English counterparts resulting in fewer infections and the concomitant need to use antibacterial drugs.

Pigs are generally reared in confined areas and the spread of infection is rapid

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amongst these animals. The proportion of isolates that were trimethoprim resistant in these strains was higher than those reported for strains responsible for diarrhoea in pigs around Nottingham (Wise *et al.* 1985). Most of the trimethoprim resistant porcine strains described in this study were highly resistant to trimethoprim (MIC > 1000 mg/l) and this is following the same trend of trimethoprim resistance that we have been observing in clinical strains both in Scotland (Amyes, Doherty & Young, 1986) and in India (Young *et al.* 1986). Similarly, amongst 75 trimethoprim resistant strains of mixed origin (bovine, porcine and ovine), Wise *et al.* (1985) found that 91 % were highly resistant to the drug. However, in contrast with our results, these workers found that over 50 % of highly resistant strains possessed trimethoprim R-plasmids and this mirrors the proportion of trimethoprim R-plasmid containing strains that they are finding in their clinical population Towner, Smith & Cowlishaw, 1983). On the other hand, our results show a lower proportion (25 %) and this reflects the proportion that we found in the final year (1984) of our most recent clinical study (Amyes, Doherty & Young, 1986). It is possible that some of the non-transferable trimethoprim resistance still resided on plasmids but it could not be mobilized by the methods used.

The surprising nature of transferable trimethoprim resistance was that it was confined to bacteria isolated from pigs which had derived from a small number of farms. We could not associate outbreaks of specific plasmids in porcine bacteria in these farms, merely a strong pre-disposition towards R-plasmid-containing bacteria. The reasons for this observation are unclear but they may hold the key to the cause of the shift towards non-transferability of high-level trimethoprim resistance which we have been observing in our clinical strains. It has proved impossible to establish reliable information of antibacterial drug usage in the farms providing the R-plasmid containing bacteria or, for that matter, in farms which had provided non-transferable high-level trimethoprim resistant strains which we had identified as controls.

The linkage between drug resistance in animal bacteria and clinical strains has long been suggested (Anderson *et al.* 1975; Bezanson *et al.* 1981; Mee & Nikoletti, 1983). Indeed contamination of carcasses by animal faecal flora is a common occurrence at slaughter (Linton *et al.* 1976). Our results do show that there was a 'hot-spot' of 12 farms to the south-west of Edinburgh that were supplying pigs, many with trimethoprim resistant faecal bacteria, to the city market. Moreover, these 12 farms were responsible for over one quarter of all the specimens that were collected in this study and this reflects the frequency with which they supply the city.

It is difficult to show if plasmids in animal strains are related to those in clinical isolates although it is certain that the transposons responsible are very similar (Richards *et al.* 1978). In this study, two plasmids from the porcine bacteria did appear similar to two plasmids we have found in clinical strains. One of them (pUK642) was the same size, gave the same restriction pattern and carried the same resistance markers as the clinical plasmid pUK28. We have shown, by molecular weight determinations, resistance profile and, latterly, restriction analysis, that pUK28 was the most successful clinical trimethoprim R-plasmid in this part of Scotland in the early 1980s (Amyes, Doherty & Young, 1986; Hales & Amyes, 1986). Certainly, the one identification of this plasmid in the porcine

strains does not reflect its ubiquitous spread through the clinical population. Indeed, it is possible that the pig had been infected with contaminated food. However, the shift of high-level trimethoprim resistance from R-plasmids to the bacterial chromosome is matched to the same extent in both porcine and clinical strains which may suggest an active link between the two populations.

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