

A survey of trimethoprim resistance in the enteric bacterial flora of farm animals

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(Received 4 August 1978)

SUMMARY

For 29 months the Veterinary Investigation Centres, covering the whole of Great Britain, forwarded trimethoprim-resistant gram negative enteric bacteria to the Wellcome Research Laboratories. These were examined for degree of resistance, presence and type of R factors. Trimethoprim resistance was found in 0.6% of the total number of strains examined by the Veterinary Investigation Centres. Trimethoprim R factors were demonstrated in one quarter of the resistant strains, and R factors were found in two strains of *Salmonella typhimurium*. It was concluded that while the incidence of trimethoprim resistance revealed by the survey gave no cause for alarm, the detection of resistant strains, and particularly R factors, indicated that the drug should continue to be used only for specific therapeutic purposes.

INTRODUCTION

Trimethoprim (TMP) is a relatively new antibacterial agent in the veterinary field, having been available for the treatment of livestock since 1969. Because of the mutual potentiation exhibited in the presence of sulphonamides, commercial TMP veterinary products always contain a sulphonamide, usually sulfadoxine or sulphadiazine. Although bacterial resistance to TMP was initially negligible, recent reports have indicated the occurrence of resistant strains in man and farm animals (Fleming, Datta & Grüneberg, 1972; Jobanputra & Datta, 1974; Fleming, 1973), in some cases carrying resistance transfer plasmids.

The potential danger to human health of widespread resistance to medically valuable antibacterial drugs in the bacterial flora of animals was emphasized by the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine (Swann *et al.* 1969), and one of the recommendations of this committee was that greater effort should be made to study the epidemiology of bacterial resistance in livestock. To assess the prevalence of TMP resistance, and also to investigate the incidence and type of R factors, a survey was instigated in collaboration with the Veterinary Investigation Services of the Ministry of Agriculture, Fisheries and Food.

MATERIALS AND METHODS

Source of strains

It was arranged that each of the twenty-eight Veterinary Investigation Centres (V.I.C.) in England, Scotland and Wales would send to the Wellcome Research Laboratories, Berkhamsted, all strains of gram-negative bacteria which appeared resistant to TMP/sulphonamide in routine disk sensitivity tests, over a twenty-nine month period starting in May, 1974. Cultures were transported on Dorset Egg slopes.

Identification of strains

After plating out, a single colony inoculum in 5 ml sterile quarter strength Ringer's solution was used to inoculate a set of identification media (API 20E). Identification was based on the API 20E chart with reference to Cowan & Steele (1974).

Disk sensitivity testing

A standard method was used throughout the study, following the guidelines recommended by Garrod & Waterworth (1971). The medium used was 'Wellcotest' sensitivity testing agar, which contains less than 0.03 $\mu\text{g/ml}$ of thymidine, a necessary condition for TMP sensitivity testing (Koch & Burchall, 1971). Bacterial inocula were prepared to give a semi-confluent growth, by inoculating one colony into 5 ml quarter strength Ringer's solution and spreading over the plate in three directions using a cotton wool swab. After drying, a 'Mastring' composite sensitivity test preparation was applied containing the following antibacterial agents: ampicillin 10 μg ; chloramphenicol 30 μg ; co-trimoxazole 25 μg (TMP/sulphamethoxazole 1:20); furazolidone 50 μg ; neomycin 10 μg ; sulphadiazine 100 μg ; and tetracycline 30 μg . Plates were incubated at 37 °C for 18–20 h. After every 50 tests a standard sensitive strain of *Staphylococcus aureus* (NCTC 6571) was treated in the same way as a check on technique. Bacteria were regarded as sensitive if the zones of inhibition were similar in size to or larger than those of the control strain, or resistant if there was little or no zone.

Determination of minimum inhibitory concentration (MIC)

A series of dilutions of TMP, sulphadiazine, or a 1/20 mixture of the two drugs was made in Wellcotest agar as follows:

TMP ($\mu\text{g/ml}$)	Sulphadiazine ($\mu\text{g/ml}$)	TMP/Sulphadiazine ($\mu\text{g/ml}$)
1	10	21
10	100	210
100	1000	2100
1000		21000

Inocula were prepared to give a semi-confluent growth, as for disk sensitivity testing, and 20 strains were inoculated per plate using a multipoint inoculator, equipped with 2 μl inoculating pins.

The plates were incubated for 18–20 h at 37 °C. The minimum inhibition concentration was recorded as the lowest concentration of antibacterial agent which inhibited normal growth of the organism. Normal growth was assessed by simultaneous inoculation of a Wellcotest plate containing no antibacterial agents.

Determination of strains carrying R factors

Datta & Hedges (1972) reported that only strains resistant to 1000 µg TMP/ml or over carried transferable R factors. In this survey a random sample of 50 resistant strains which were inhibited by less than 1000 µg TMP/ml were tested for the presence of R factors, and these proved not to transfer TMP resistance thus agreeing with the findings of Datta and Hedges. Subsequently, only strains that were resistant to 100 µg TMP/ml or more were tested for R factors.

The method described by Datta & Hedges (1972), was used for detection of R factor transfer using a selective agar, comprising Wellcotest agar with neutral red, 1% lactose, 10 µg/ml TMP and 30 µg/ml nalidixic acid, for isolation of recombinants. Recombinant clones were tested by the disk sensitivity method to detect other resistance factors transferred with TMP resistance.

Incompatibility testing of R factors

The R factors isolated were tested for incompatibility by the method of Coetzee, Datta & Hedges (1972), with compatibility groups: A(RAI-IB), C(R40a), FI (R386, R455-2), FII (R1-1b), H (R27, R726-1), 1α (R144, JR66a, R621a), W(s-a-1), J (R391), K (R387), M (R69), 0 (R805a), P (RP4), S (R478) and 1δ (R821a).

RESULTS

In the 29 months of the survey a total of 386 strains of Gram negative bacteria were received from the Veterinary Investigation Centres. The majority, 348 strains, were *E. coli*, 31 were *Salmonella* spp., 3 *Pseudomonas aeruginosa*, 2 *Alcaligenes faecalis* and 2 *Proteus* spp. Although all these strains were alleged to be TMP resistant, 206 of the strains proved to be TMP sensitive when retested using the method described above. These sensitive strains consisted of 182 strains of *E. coli* and 24 strains of *Salmonella* spp. and originated from 17 Veterinary Investigation Centres. The disk sensitivity results also indicated that TMP resistance was invariably accompanied by sulphonamide resistance and usually with resistance to three or four other antibacterial agents in addition. Of the 180 resistant strains, 139 were of bovine origin, 12 were of ovine origin, 21 porcine, 2 canine, 2 avian, 2 equine and 2 came from unidentified hosts. This suggests that cattle are the chief source of TMP resistant strains among farm animals.

The areas of origin of the resistant strains are indicated on the map, Fig. 1, which shows that some laboratories, predominantly in the south-west, south-east and East Anglia, detected no TMP resistant organisms. The number of resistant strains increased towards northern England and Scotland, with an exceptionally large number originating from the east coast region of Scotland.

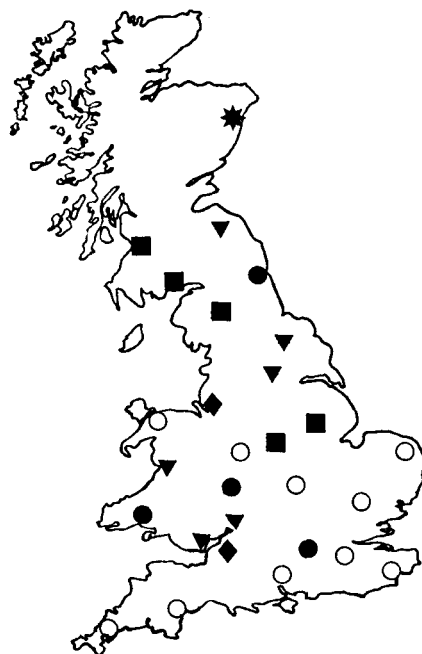


Fig. 1. Distribution of resistant strains. ○, No strains; ▼, 1-4; ●, 5-9; ■, 10-14; ◆, 15-19; ★, 20 plus.

Table 1. *Minimum inhibition concentrations of strains received in survey on ten-fold dilution series*

Trimethoprim MIC ($\mu\text{g/ml}$)	1	10	100	1000	Over 1000	Total
No. of strains	226	68	36	2	54	386
Sulphadiazine MIC ($\mu\text{g/ml}$)	10	100	1000	Over 1000		
No. of strains	21	32	6	327		386
Sulphadiazine/ Trimethoprim (20/1) MIC ($\mu\text{g/ml}$)	21	210	2100	Over 2100		
No. of strains	234	66	33	53		386

The MIC of TMP and sulphadiazine for all the strains received are shown in Table 1. The large number sensitive to the lowest dilution groups includes all the strains sensitive on a disk test. The $1 \mu\text{g/ml}$ TMP MIC group also incorporates strains which produced a narrow zone of inhibition, in comparison with a standard sensitive strain, and this result is usually classified as resistant. The other two largest MIC groups were at $10 \mu\text{g/ml}$, and at $1000 \mu\text{g/ml}$ or over. Most of the strains were highly resistant to sulphadiazine.

The 56 strains resistant to $1000 \mu\text{g/ml}$ or more were all tested for TMP R factors. Eleven of these strains, despite repeated attempts, failed to produce any demonstrable R factor transfer when mixed with the standard *E. coli* K12 recipient. All these strains were *E. coli* but had no common factors of origin.

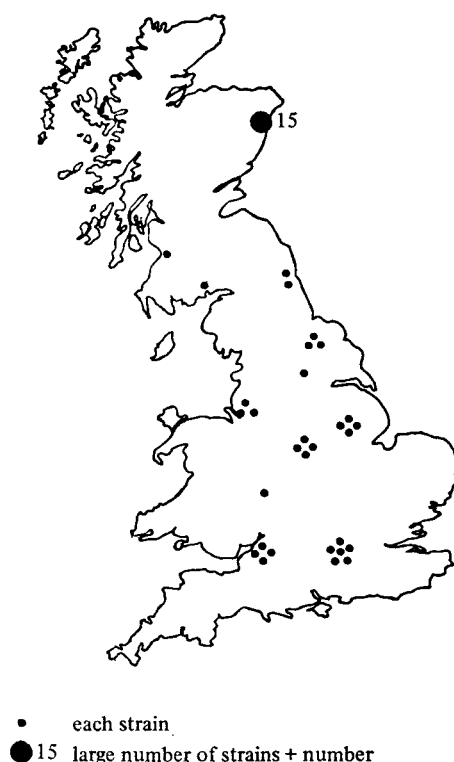


Fig. 2. Distribution of strains carrying R factor received in survey.

Table 2. Resistances transferred by strains carrying R factor

Resistance transferred	Number of strains	
	Strain IDs	Count
Tm	R187, R202, R301, R302, R328	5
Tm, Su	R149, R388, R414	3
Tu, Su, Cm	R26, R75, R113, R163, R169, R248, R271, R272, R281, R293	10
Tm, Su, Amp	R200	1
Tm, Su, Sm	R115, R151, R307, R396, R398	5
Tm, Su, Km	R220, R259	2
Tm, Su, Cm, Km	R392	1
Tm, Su, Amp, Tc, Cm, Km, Sm	R105, R150	2
Tm, Su, Cm, Km, Sm	R106	1
Tm, Su, Amp, Tc, Cm	R125, R132, R135, R241, R389	5
Tm, Su, Sm, Km	R144, R380	2
Tm, Su, Cm, Km, Sm	R164	1
Tm, Su, Amp, Sm	R298	1
Tm, Su, Cm, Sm	R304	1
Tm, Su, Tc, Sm	R306	1
Tm, Su, Amp, Tc, Cm, Km	R413	1
Tm, Tc, Sm	R303	1

Key; Tm, trimethoprim; Su, sulphonamide; Cm, chloramphenicol; Amp, ampicillin; Sm, Streptomycin; Km, Kanamycin; Tc, tetracycline.

Forty-five of the strains with high MIC of TMP were shown to carry transmissible R factors conferring TMP resistance, but in some cases transfer was only achieved after several attempts. These 45 strains came from diverse areas (Fig. 2) and no R factor-carrying strains were isolated in the south west or south east of England, East Anglia or Wales. TMP R factors were fairly evenly distributed throughout northern England, and the largest number originated from the east of Scotland. The distribution of TMP R factor strains thus showed good correlation with the distribution of all TMP resistant strains.

All recombinants were disk sensitivity tested to determine which drug resistance factors were transferred with TMP resistance. The strains carrying R factors, and the resistance transferred are shown in Table 2. No attempt was made to determine whether all resistance was transferred as a result of a single R factor or by transfer of more than one R factor simultaneously.

Table 2 shows that sulphonamide resistance was usually transferred along with resistance to TMP, and that resistance to chloramphenicol, ampicillin, streptomycin or kanamycin was frequently transferred in addition. Only one combination of R factors, transferring resistance to TMP, sulphonamide, ampicillin, tetracycline and chloramphenicol, had a common source, all from the same VIC. No other correlations were seen.

Forty of the R factor carrying strains were *E. coli* and five were *Salmonella typhimurium*. Three of the *Salmonella typhimurium* strains, all phage type 49, came from one VIC and these had been isolated from different calves in the same outbreak. The other two strains, from a different VIC, were known to have been isolated from two calves on the same farm and were both phage type 204. The fact that two different phage types of *Salmonella typhimurium* were involved suggests that the two strains were unrelated and that the TMP R factors arose independently.

The R factors were incompatibility-tested where possible. Only two R factors were found to belong to one of the groups listed in the method description above. R factors from strains R396 and R398, both conferring resistance to TMP, sulphonamide and streptomycin were found to be incompatible with R821a, group I δ . These were from different sources.

DISCUSSION

The particular problems of disk sensitivity testing with TMP and sulphonamides were illustrated by the fact that more than half of the strains received proved to be TMP sensitive when retested under carefully controlled conditions. A common cause of reports of false resistance is the use of medium containing thymidine, which permits bacteria to by-pass the anti-metabolic action of TMP, but the medium used by the Veterinary Investigation Service laboratories, 'Sensitest Agar', was screened for the absence of significant thymidine content, and therefore false appearances of resistance must have resulted from occasional technical irregularities. Zones of inhibition can be reduced or obscured by partially inhibited colonies by the use of a heavy inoculum producing a dense confluent

growth, or even by preparing an inoculum by suspending colonies in nutrient broth which may have a high thymidine content. However, in relation to the total number of sensitivity tests on Enterobacteriaceae carried out by the VIS, approximately 12000 per annum (E. T. Davies, M.A.F.F., personal communication), the 206 erroneous results comprised only about 0.7%.

Also in relation to the total number of sensitivity tests, the incidence of true TMP resistance (180 strains) was approximately 0.6%. In view of the fact that specimens submitted to Veterinary Investigation Centres invariably originate from problem cases on the farm, this low detection rate of TMP resistance in diagnostic laboratories probably represents an extremely low level of resistance in the general livestock population. The erratic geographical distribution of the sources of TMP resistant strains (Fig. 1), may to some extent have reflected differences in farming practices, with predominantly arable areas such as the Midlands and East Anglia apparently remaining free of TMP resistance, whereas the largest number of strains originated from the east of Scotland, an area with many intensive livestock units.

Resistance to TMP was shown to be mediated by transmissible R factors in 25% of the resistant strains but as it was only possible to classify two plasmids by incompatibility testing, both placed in Group 18, no conclusions could be drawn with regard to the epidemiology of the TMP R factors detected. Although TMP R factors of Group 1 α had been frequently demonstrated in *E. coli* isolated from calves on a farm in S.E. England in 1972 (Fleming, 1973), failure to detect this plasmid in this survey indicates that it has not since become widely disseminated. The isolation of two unrelated strains of *S. typhimurium* possessing TMP R factors was disquieting, particularly as TMP-resistant salmonellas from farm animals had not previously been reported in Great Britain (Sojka, Hudson & Slavin, 1974; Sojka & Hudson, 1976; Sojka, Wray & Hudson, 1977).

The relation between the detection of R factors among the resistant strains and the level of TMP resistance confirmed the finding previously reported by Datta & Hedges (1972), that TMP R factors were only found in strains resistant to at least 1000 $\mu\text{g}/\text{ml}$. However, of the 54 strains with this degree of resistance, no evidence of resistance transfer was obtainable with 11 strains in spite of repeated attempts. These 11 strains were all *E. coli* but had no other factors in common to suggest a common origin. The range of resistance to other antibiotics transferred with TMP resistance (Table 2) varied considerably. In some cases the resistances transferred varied between different colonies of a recombinant strain, indicating the possibility that more than one R factor was present, although no tests for multiplicity of R factors were carried out. In no case was any correlation apparent between distribution, source and types of resistances transferred.

In the United Kingdom, the use of veterinary products containing TMP is restricted by law to control by the veterinary profession, and all products currently available are recommended for therapeutic use only. Low level administration to large groups of animals for extended periods has probably not been carried out to any significant extent. This policy may well be associated with the low level of TMP resistance revealed by the survey. The discovery that resistant

strains, including some carrying TMP R factors, were widely distributed, albeit in small numbers, suggests that the potential exists for resistance to become a serious problem if favoured by continuous selective pressure, which would result from long term oral administration. The present regulation and manner of use of veterinary TMP products appears so far to have resulted in no resistance problems of real significance either in the treatment of animals or with regard to the exposure of humans to resistant strains, but it is suggested that it would be valuable to carry out a similar survey after an interval of about 5 years in order to detect any possible changes in the extent or character of TMP resistance.

The cooperation of the staff of all the VICs who collected and dispatched the many strains of bacteria to this laboratory is gratefully acknowledged. We also thank Dr B. Rowe for phage typing the *Salmonella typhimurium* strains.

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