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Trimethoprim-resistance and its transferability in *E. coli* isolated from calves treated with trimethoprim-sulphadiazine: a two year study

By M. P. FLEMING

*Wellcome Veterinary Research Station, Frant, Tunbridge Wells, Kent**

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SUMMARY

Regular examination of rectal swabs revealed the presence of very low numbers of trimethoprim resistant *Escherichia coli* in the faeces of 10 batches of calves successively reared in the same shed and none of these strains transferred trimethoprim resistance to *E. coli* K12. All the calves had received oral doses of 30 mg/kg day of trimethoprim-sulphadiazine for 5 consecutive days. From two subsequent batches of calves reared in the same shed, however, several isolations were made of *E. coli* with transmissible R factors determining trimethoprim and streptomycin resistance. Shortly before these strains were detected, isolations of *E. coli* with similar properties had been made from other calves, in a different shed, which had been fed much higher doses of trimethoprim-sulphadiazine. Serological evidence indicated that all the *E. coli* isolated carrying this R factor belonged to the same strain, which had apparently spread from the second shed to the first. No evidence of 'in vivo' transfer of the R factor to other enteric bacteria was obtained.

INTRODUCTION

Trimethoprim was first made available for general use in human medicine in 1968 and released for use in veterinary medicine in 1969. It is a synthetic antibacterial drug which inhibits the enzyme dihydrofolate reductase, necessary for the essential synthesis, by bacteria, of folinates. Therapeutic preparations of trimethoprim are always combined with a sulphonamide because of the strong synergistic action exhibited by their combination (Bushby, 1969). The combination of trimethoprim and sulphonamide has proved useful in the treatment of a wide range of bacterial infections in man (Hughes, 1969; Seboulet, 1971; Kabbage, Ben Brahim & Amine, 1971) and in animals (Rehm & White, 1970; Rail & Kaller, 1971; Scholl *et al.* 1971).

One important aspect of the use of the combination in human medicine has been the low incidence of trimethoprim-resistant coliform bacilli encountered since its introduction 4 years ago (McAllister *et al.* 1971; Gillespie, Lee, Linton & Rowland, 1971; Fleming, Datta & Grüneberg, 1972; Lacey, Gillespie, Bruten & Lewis, 1972). Recently, however, a number of strains of *Klebsiella* spp. and

* Present address: Product Evaluation Section, The Wellcome Research Laboratories, Beckenham, Kent.

E. coli, of human origin, were discovered harbouring transmissible R factors determining high levels of resistance to both trimethoprim and sulphonamide (Fleming *et al.* 1972; Datta & Hedges, 1972). Little information has been published on the incidence and transferability of trimethoprim-resistance in bacteria of animal origin. This report describes the incidence of trimethoprim-resistant strains of *E. coli* encountered in batches of calves treated with trimethoprim-sulphadiazine, together with the isolation and epidemiology of a strain of *E. coli* harbouring a transmissible R factor determining resistance to both trimethoprim and streptomycin.

MATERIALS AND METHODS

Source of samples

During the period between 1 October 1970 and 9 August 1972 twelve successive batches of 28 one-week-old Friesian calves were kept in the same building (Shed 1) in adjacent individual pens and fed milk replacer once daily. Each batch of calves was reared in Shed 1 for 6 weeks and then moved out into nearby covered yards for fattening and subsequent sale. After the removal of each batch of calves, the interior of Shed 1 was thoroughly cleaned, disinfected with caustic soda solution and left empty for 2 weeks. All calves in each batch received trimethoprim-sulphadiazine,* prophylactically, in their milk replacer for five consecutive days beginning 48 hr. after their arrival. The dose, 30 mg/kg day, was that normally recommended for the treatment of infection. Rectal swabs were taken from each calf upon arrival and at weekly intervals until removal.

During the period between 14 April 1972 and 16 May 1972 an additional batch of 18 one-week-old Guernsey calves (Batch A) were monitored. All calves in Batch A were housed in a building (Shed 2) which was separated from Shed 1 by a distance of approximately 100 yards. All calves were kept in adjacent individual pens, fed milk replacer for 5 weeks, and then sold. On arrival, and as part of an experimental study, Batch A was divided in three groups (Groups 1, 2 and 3). Groups 2 and 3 received high doses of trimethoprim-sulphadiazine† corresponding to 60 mg/kg day and 150 mg/kg day respectively for eight consecutive days. Group 1 did not receive any treatment and the animals were used as controls. Rectal swabs were taken from each calf in all groups, every 2 days, until treatment ended and from then on at weekly intervals.

Trimethoprim-resistant E. coli

Strains of trimethoprim-resistant *Escherichia coli* were isolated by streaking each rectal swab on a plate of 'Wellcotest' Sensitivity Testing Agar‡ (W.S.T.), containing 1 % (w/v) lactose, 0·005 % (w/v) neutral red and 1·0 μg. trimethoprim per ml. All plates were incubated at 37° C. for 24 hr. and examined for lactose-fermenting colonies. One lactose-fermenting colony, having typical morphological features, was picked off each plate and initially identified by streaking on MacConkey agar. Each culture was then subjected to further biochemical tests.

* (Tribrissen Dispersible Powder, Burroughs Wellcome & Co.)
† (Tribrissen Bolus – Burroughs Wellcome & Co.)
‡ (Wellcome Reagents Ltd., Beckenham, Kent.)

All cultures identified as *E. coli* were tested for their sensitivity, by means of Oxoid 'Multodisks' to ampicillin (A); streptomycin (S); chloramphenicol (C); tetracycline (T); neomycin (N); furazolidone (F); sulphonamide (Su); trimethoprim-sulphamethoxazole (1:20; 25 μg.) and with individual disks containing 1·25 μg. trimethoprim (TMP). The medium used was W.S.T. agar. Strains with no zone of inhibition around the trimethoprim disks were recorded as resistant. The minimum inhibiting concentrations (M.I.C.) of trimethoprim for these strains were then determined by streaking small inocula on plates of W.S.T. agar containing serial dilutions of the drug. The inocula were standardized by diluting broth cultures sufficiently to produce about 20 colonies per inoculum.

Demonstration of R factors in strains of trimethoprim-resistant Escherichia coli

Each strain of trimethoprim-resistant *E. coli* was grown in mixed broth culture with a nalidixic acid-resistant *E. coli* K12 recipient. The mixtures were then plated on a selective medium of W.S.T. agar containing 1 % (w/v) lactose, 0·005 % (w/v) neutral red, nalidixic acid (100 μg./ml.), and either streptomycin (25 μg./ml.), chloramphenicol (25 μg./ml.), ampicillin (25 μg./ml.) or trimethoprim (5·0 or 1·0 μg./ml.). The recipient used was *E. coli* K12, non-lactose fermenting and sensitive to antibacterial drugs other than nalidixic acid. Neither donor nor recipient grew on the selective medium. Colonies developing from the mixed cultures were streaked on MacConkey agar plates, identified as *E. coli* K12 by their colonial morphology and inability to ferment lactose, and tested for their sensitivity to a range of antibacterial drugs including trimethoprim.

Serological tests

All cultures of *E. coli*, harbouring transmissible R factors determining trimethoprim-resistance, were serologically tested for antigenic similarity.

Four representative cultures were chosen and used to prepare OK antisera in rabbits (Sojka, 1965). All cultures, including the four immunizing cultures, were then tested for agglutination by each of the four antisera, using conventional tube methods with either live, boiled or autoclaved suspensions of each culture (Sojka, 1965). The titres of each set of agglutinations were recorded.

The four immunizing cultures were serotyped by Dr B. Rowe, Central Public Health Laboratory, Colindale.

Enterotoxin tests

The four immunizing cultures were tested for their ability to produce enterotoxin using the ligated gut test in calves (Smith & Halls, 1967). In addition, following transfer of the R factor determining trimethoprim-resistance, a culture of *E. coli* K12 and of a known recipient of enterotoxin plasmids *E. coli* O8:K$^-$:H$^-$ (referred to as strain D282 by Smith & Linggood, 1972) were tested using the same technique, to determine the possibility of a link between enterotoxin production and the R factor.

E. coli O8:K$^-$:H$^-$ with and without the R factor determining trimethoprim-resistance, was also tested by Mr W. J. Sojka, Central Veterinary Laboratory,

Table 1. *Trimethoprim-resistant* Escherichia coli *isolated from Batch* 10 *in Shed* 1 *between* 1 *March and* 12 *April* 1972

No. of weeks after arrival of calves	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	M.I.C. of TMP for each culture (μg/ml)	Drug resistance transferred to *E. coli* K12 from each culture
0	0	—	—	—	—
1*	8	S, C, T, A, TMP, Su	5	16·0	C, T, Su
		S, T, N, A, TMP, Su	1	3·0	None
		S, T, A, TMP, Su	1	12·0	None
		S, A, TMP, Su	1	4·0	None
No further trimethoprim-resistant *E. coli* isolated					

* End of treatment period.

S, Streptomycin; C, chloramphenicol; T, tetracycline; N, neomycin; A, ampicillin; F, furazolidone; Su, sulphonamide; TMP, trimethoprim.

Weybridge, for antigen 'KCO', whose presence is correlated with enteropathogenicity in calves (Smith & Linggood, 1972).

RESULTS

Incidence and epidemiology of trimethoprim-resistant Escherichia coli *encountered in the calves*

No trimethoprim-resistant *E. coli* were isolated from nine successive batches of calves reared and monitored in Shed 1 during the first 17 months of the study. Trimethoprim-resistant *E. coli* were isolated, however, from the next three batches of calves (Batch Nos. 10, 11 and 12) reared in Shed 1 and details of these strains are summarized in Tables 1, 2 and 3 respectively. Tables 2 and 3 show that the majority of cultures isolated from Batches 11 and 12 transferred trimethoprim-resistance to *E. coli* K12. These were very prevalent in the faecal flora of the calves shortly after the end of treatment, and remained so even when samples were taken 3 or 4 weeks later. There were no serious outbreaks of scouring in any of the batches monitored in Shed 1.

No trimethoprim-resistant *E. coli* were isolated from the calves in Batch A until shortly after the end of treatment. Details of the trimethoprim-resistant *E. coli* isolated from calves in this batch, after treatment are summarized in Table 4. No trimethoprim-resistant *E. coli* were isolated from the untreated group of calves (Group 1). A serious outbreak of scouring occurred in all groups of calves in Batch A together with some deaths; bacteriological findings, however, did not implicate trimethoprim-resistant *E. coli* as the causative pathogen.

Strains of *E. coli* with transferable trimethoprim-resistance were first isolated from calves in Batch A which had received 150 mg. trimethoprim-sulphadizine/kg day (Group 3). These calves were reared in Shed 2 at approximately the same time as Batch 11 in Shed 1. It therefore seems likely that these strains were, in some way, transferred from Shed 2 to Shed 1 where they subsequently re-emerged

Table 2. *Trimethoprim-resistant* Escherichia coli *isolated from Batch* 11 *in Shed* 1 *between* 25 *April and* 6 *June* 1972

No. of weeks after arrival of calves	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	M.I.C. of TMP for each culture (μg/ml)	Drug resistance transferred to *E. coli* K12 from each culture
0	2	C, A, TMP, F	1	3·0	None
		S, C, T, A, TMP, Su	1	2·0	None
1*	3	S, C, A, TMP, Su	1	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
		S, TMP, Su	1	4·0	None
2	17	S, C, A, TMP, Su	7	> 1000	All
		S, C, T, A, TMP, Su	9	> 1000	All
		S, A, TMP, Su	1	3·0	None
3	9	S, C, A, TMP, Su	3	> 1000	All
		S, C, T, A, TMP, Su	6	> 1000	All
4	2	S, C, A, TMP, Su	1	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
5	4	S, C, A, TMP, Su	1	> 1000	All
		S, C, T, A, TMP, Su	3	> 1000	All
6	4	S, C, A, TMP, Su	2	> 1000	All
		S, C, T, A, TMP, Su	2	> 1000	All

* End of treatment period.

Table 3. *Trimethoprim-resistant* Escherichia coli *isolated from Batch* 12 *in Shed* 1 *between* 28 *June and* 9 *August* 1972

No. of weeks after arrival of calves	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	M.I.C. of TMP for each culture (μg/ml)	Drug resistance transferred to *E. coli* K12 from each culture
0	1	C, A, TMP, Su	1	15·0	None
1*	25	S, C, A, TMP, Su	24	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
2	17	S, C, A, TMP, Su	16	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
3	8	S, C, A, TMP, Su	8	> 1000	All
4	6	S, C, A, TMP, Su	5	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
5	3	S, C, A, TMP, Su	3	> 1000	All
6	2	S, C, A, TMP, Su	2	> 1000	All

* End of treatment period.

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Table 4. *Trimethoprim-resistant* Escherichia coli *isolated from Batch A in Shed 2 between 14 April and 16 May 1972*

No. of weeks after end of treatment period	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	Dose rate of TMP/Su given to calves from cultures originated (mg/kg day)	M.I.C. of TMP for each culture (μg/ml)	Drug resistances transferred to *E. coli* K12 from each culture
0*	5	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	2	150	> 1000	All
		TMP, Su	1	60	4·0	None
		T, TMP, Su	1	150	4·0	None
1	5	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	2	150	> 1000	All
		S, A, TMP, Su	1	60	4·0	S, Su
		T, TMP, Su	1	150	4·0	None
2	2	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	1	150	> 1000	All
3	2	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	1	150	> 1000	All

* 24 hr after last dose of trimethoprim-sulphadiazine administered.

in the faeces of calves in Batch 11 after treatment. Disinfection of Shed 1, after the removal of Batch 11, did not prevent the re-emergence of these strains in the following batch of calves (Batch 12) after treatment.

Transfer of trimethoprim-resistance

The transferable drug resistances possessed by each strain of trimethoprim-resistant *E. coli* are shown in Tables 1–4. The only strains with transferable trimethoprim-resistance were those resistant to at least 1000 μg. trimethoprim per ml. These strains had either of two resistance patterns, S, C, T, A, Su, TMP or S, C, A, Su, TMP. When these cultures were used as donors of drug resistances and selection was made for either streptomycin, chloramphenicol or ampicillin resistance, every colony of converted recipient tested had all the donor's drug resistant markers *except TMP*. When trimethoprim resistance was selected for, however, all colonies of converted recipients tested had one of three resistance patterns: S, C, T, A, Su, TMP; S, C, A, Su, TMP or S, TMP. When recipient colonies received either S, C, T, A, Su, TMP or S, C, A, Su, TMP their M.I.C.'s of trimethoprim and streptomycin were always > 1000 μg./ml. and > 25 μg./ml. respectively. When recipient colonies received S, TMP, however, their M.I.C.'s of trimethoprim and streptomycin were always > 1000 μg./ml. and 10 μg./ml. respectively. These results suggest that each wild strain of *E. coli* had several different transmissible R factors, one of which determined resistance to both trimethoprim and streptomycin.

Frequencies of transfer of trimethoprim-resistance

The frequency of transfer of trimethoprim-resistance from the wild strain of *E. coli* to *E. coli* K12 during overnight incubation of mixtures was approximately 5×10^{-8} per potential donor cell.

Serological tests on cultures of Escherichia coli *with transferable trimethoprim-resistance*

All live, boiled, and autoclaved suspensions of each culture tested, agglutinated to titres of 1/100, 1/100 and 1/400 respectively in all four OK antisera. All four immunizing cultures belonged to the same serotype; O101:K?(A):H37. It therefore seems likely that all the cultures tested belonged to the same serotype as the immunizing cultures.

Enterotoxin tests

All four immunizing cultures dilated segments of calf intestine and were therefore considered to be capable of enterotoxin production. Both *E. coli* K12 and *E. coli* O8:K$^-$:H$^-$ each containing the R factor determining trimethoprim-streptomycin resistance did not dilate segments of calf intestine. It was therefore assumed that the R factor was not directly associated with enterotoxin production.

No extra antigenic component was detected in *E. coli* O8:K$^-$:H$^-$ on acquisition of the trimethoprim-streptomycin R factor (W. J. Sojka, personal communication).

DISCUSSION

The incidence of trimethoprim-resistant *E. coli* encountered during the first 18 months of the study was very low and in no case was trimethoprim resistance transmissible. During the remaining 5 months of the study, however, a large number of isolations of trimethoprim-resistant *E. coli* were made. The majority of these strains grew on a medium containing 1000 μg. trimethoprim/ml. All the strains which did so transferred similar degrees of trimethoprim resistance to *E. coli* K12 in mixed culture. In each of the wild strains trimethoprim and sulphonamide resistance was determined separately by two different transmissible R factors. The R factor determining trimethoprim-resistance also determined streptomycin resistance. This R factor was designated a prototype of a new compatibility class, B (Hedges, Datta & Fleming, 1972); however, its designation has subsequently been changed to compatibility class Iβ, since it determines I pili (Hedges & Datta, 1973).

The hosts for the R factors determining trimethoprim-streptomycin resistance and sulphonamide resistance were all typical strains of *E. coli*. All had similar drug resistance patterns and had similar serological reactions. All were isolated from animals on one experimental station. It therefore seems likely that all were members of a single clone. This strain first emerged in a group of calves treated with large doses of trimethoprim-sulphadiazine. It seems likely that the strain was present in some of these calves' intestines before treatment, but in numbers

so low as to be rendered undetectable by the method of sampling. The subsequent proliferation of the strain in these calves was probably due to a strong selective pressure created by the high levels of drug administered. The strain subsequently spread to a different building where it became prevalent and persistent for several weeks in the faeces of a group of calves treated with normal doses of trimethoprim-sulphadiazine. This evidence, therefore, demonstrates how R factors determining trimethoprim and sulphonamide resistance in a host bacterium can lead to the rapid proliferation of that host when the combination is present in the environment. Such a situation would be expected to increase the chances of both R factors spreading to other strains of bacteria, for example *E. coli*. There was no evidence, however, to suggest that the latter had occurred in this study. This result may have been connected with the host strain's in vitro ability to transfer the R factor determining trimethoprim-streptomycin resistance to *E. coli* K12 at only very low frequencies.

It appears that R factor-determined trimethoprim-resistance in *E. coli* isolated from animals is, at present, a very rare phenomenon. The widespread use of trimethoprim in animals may encourage the spread of these R factors, but until now no such spread has become apparent in *E. coli*. I hope that other workers will continue to monitor the situation and report any changes which may occur in the prevalence of trimethoprim-resistance and its transferability in bacteria of animal origin.

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