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Antibiotic resistance in *Escherichia coli* causing generalized infections in chickens in the UK in 1982: the relationship between the results of *in vitro* and *in vivo* furazolidone sensitivity tests

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SUMMARY

Compared with a similar survey conducted ten years previously, a survey conducted in 1982, eleven years after the implementation of legislation forbidding the routine use of feeds containing 'therapeutic' antibiotics, revealed a decreased incidence of resistance to tetracyclines, furazolidone and sulphonamides in *Escherichia coli* strains causing generalized infections in chickens in the UK; the decrease was particularly marked in the case of tetracycline resistance, 17·9% of strains in 1982 being resistant to this antibiotic compared with 31·2% in 1972.

Giving furazolidone to groups of chickens inoculated intramuscularly with O2:K1 strains of *E. coli* of differing degrees of furazolidone sensitivity indicated that great care is required in the performance and interpretation of laboratory tests for sensitivity to this antibiotic. Infections caused by strains that required as little as 1·25 μg/ml of furazolidone to inhibit their multiplication in laboratory tests responded poorly to furazolidone treatment; those that were inhibited by less responded well, better than to treatment with tetracycline, chloramphenicol, ampicillin or trimethoprim.

INTRODUCTION

In a study of antibiotic resistance in *Salmonella gallinarum*, instigated by reports of the failure of furazolidone to control recent outbreaks of fowl typhoid in the Middle East, most of the strains examined from such outbreaks appeared slightly less sensitive to this agent than others as judged by disk tests (Smith, Tucker & Lovell, 1981). This was confirmed by minimum inhibitory concentration (MIC) determinations, the MIC for the less sensitive strains being 1·3–2·5 μg/ml and for the fully sensitive ones 0·3 μg/ml. The difference was important because furazolidone effectively controlled experimental infections in chickens caused by the fully sensitive strains whereas it failed to control those caused by the less sensitive ones.

Furazolidone is often used to control outbreaks of generalized *Escherichia coli* infection in broiler chickens, an economically important disease in the UK. It seemed of interest, therefore, to determine *in vitro* the furazolidone sensitivity of *E. coli* strains from such outbreaks and to correlate the findings with the results of treating chickens experimentally infected with a selection of these strains as we

had done in the case of the *S. gallinarum* strains. Added impetus was engendered by the fact that antibiotic resistance in *E. coli* strains isolated from similar sources in 1972 had been surveyed by Heller & Smith (1973), one year after the routine use of 'therapeutic' antibiotics as feed additives was banned in the UK as a consequence of the report of the Swann Committee (Report, 1969). Assessing the resistance of a new set of strains to furazolidone and to other antibiotics would give an impression of the effect of the ban and of general antibiotic use during 1972–82 on the incidence of antibiotic-resistant *E. coli* pathogenic for chickens. Because strains isolated in the 1972 survey were still available, their furazolidone sensitivity was re-examined, particularly to identify those with slightly reduced sensitivity. The results are reported in this paper.

MATERIALS AND METHODS

Escherichia coli *strains*

These had been isolated at diagnostic laboratories in the UK from chickens that had died from generalized *E. coli* infection; all had originated from different outbreaks of infection. They were maintained at this laboratory on Dorset's egg medium at 5 °C. For use, they were grown in 10 ml of nutrient broth (Oxoid, CM67) in a shaking waterbath at 37 °C for 24 h. When necessary, the numbers of viable organisms in these cultures were estimated by the method of Miles & Misra (1938).

Serological examination

All *E. coli* strains were examined to see whether they belonged to the two main serogroups that cause generalized infections in chickens, O2:K1 and O78 (Sojka & Carnaghan, 1961; Harry, 1964). Glucose agar cultures were first submitted to slide-agglutination tests with O78 antiserum and those that were agglutinated were accepted as of this serogroup. The remainder were tested for K1 antigen by the antiserum–agar technique of Sarffe *et al.* (1975) using equine meningococcal group B antiserum kindly supplied by Dr J. B. Robbins. Boiled suspensions of those judged to be $K1^+$ were then submitted to slide agglutination tests with O2 antiserum.

Antibiotic sensitivity and minimum inhibitory concentration (MIC) determinations

These were performed by the methods described by Smith, Tucker & Lovell (1981), disk tests being employed for assessing sensitivity to all the antibiotics except furazolidone. Because of difficulty in interpreting the results of such tests with furazolidone, those recorded for that drug were obtained from MIC determinations, strains with an MIC of 1·25 μg or more being recorded as resistant; this necessitated a re-examination of the Heller & Smith (1973) strains because their furazolidone sensitivities had been classified according to the results of disk tests.

Transfer of antibiotic resistance in vitro

Nutrient broth in 10 ml amounts was inoculated with 0·03 ml each of broth cultures of an antibiotic-resistant *E. coli*, the donor strain, and a spontaneous nalidixic acid-resistant mutant of a *lac* auxotrophic *E. coli* K12, the recipient

strain, and incubated at 37 °C for 24 h. After holding at room temperature for an additional 24 h to facilitate conjugation by temperature-sensitive plasmids, the mixed culture was inoculated on to a plate of solid medium containing sodium nalidixate and one of the antibiotics to which the donor was resistant. The plate was then incubated at 37 °C for 24 h and examined for colonies of the recipient strain. The solid medium was Sensitest Agar (Oxoid) when recipient organisms resistant to sulphonamide or trimethoprim were being selected, and MacConkey's agar when recipient organisms resistant to other antibiotics were being selected. Sulphafurazole, ampicillin, sodium nalidixate, trimethoprim and furazolidone were added at 75, 30, 20, 7·5 and 0·6 μg/ml respectively; other antibiotics were added at 15 μg/ml.

Isolation of furazolidone-resistant mutants of E. coli

Broth cultures were treated with 100 μg/ml of nitro-nitrosoguanidine by the method of Glover (1968) and then cultured on plates of MacConkey's agar containing 4 μg/ml of furazolidone. Any colonies that grew on them were then checked for furazolidone resistance.

Chickens

These were specified-pathogen-free Light Sussex, aged 7 days. They were kept in groups on wire-meshed floors in identically constructed pens in an animal house maintained at 21 °C. Additional heating was provided by an infra-red lamp suspended over each pen. They were fed *ad libitum* on a diet of the following composition: wheat meal, 40 %; maize meal, 40 %; British white fish meal, 20 %; mineral and vitamin supplement, 0·25 %. When required, antibiotic pre-mixes were incorporated in the diet by means of a mechanical mixer.

Antibiotic treatment of experimentally infected chickens

Groups of chickens were inoculated via the left gastrocnemius muscle with 0·1 ml of broth culture of an *E. coli* strain diluted to provide the required median lethal dose (LD50), which was calculated by the method of Reed & Muench (1938), the diet of all groups except control groups having been changed one day previously from a normal to an antibiotic-containing one. The latter diet was then fed continuously for nine days, when the experiment was terminated. The number of chickens that died on each day was recorded.

RESULTS

The incidence of antibiotic resistance amongst strains of Escherichia coli *causing generalized infections in chickens*

The antibiotic resistance found in strains of *E. coli* that had caused different outbreaks of generalized infection in chickens in the UK in 1982 is illustrated in Table 1. The results of the survey carried out by Heller & Smith (1973) in 1972 are included for comparison. Antibiotic resistance was commonly found, most of it being to sulphonamides and furazolidone; resistance to trimethoprim, chloramphenicol, sodium nalidixate and polymixin was low or non-existent.

Table 1. *Incidence of antibiotic resistance in strains of* Escherichia coli *causing generalized infections in chickens in* 1982

	Percentage resistant to:		
Sulphonamides	= 69·3 (83·8)	Neomycin	= 6·4 (0)
Furazolidone	= 67·1 (84·9)	Trimethoprim	= 2·9 (0)
Tetracyclines	= 17·9 (31·2)	Chloramphenicol	= 0·7 (0)
Streptomycin	= 20·7 (18·5)	Sodium nalidixate	= 0 (0)
Ampicillin	= 12·9 (1·2)	Polymixin	= 0 (0)
Spectinomycin	= 7·1 (2·9)		
	Percentage resistant to:		
Six antibiotics	= 1·4 (0)	Two antibiotics	= 36·1 (34·1)
Five antibiotics	= 5·7 (2·4)	One antibiotic	= 25·0 (18·3)
Four antibiotics	= 12·1 (15·1)	One or more antibiotics	= 87·9 (95·2)
Three antibiotics	= 7·1 (25·4)		

Results for strains isolated in 1972 (Heller & Smith, 1973) are in parentheses. No. of strains examined = 140 (173). % serogroup O78 = 17 (24·8), O2:K1 = 21·1 (36·4); others = 61·8 (38·8).

Table 2. *The incidence of transferable antibiotic resistance in the* Escherichia coli *strains isolated in* 1982

Antibiotic	Number of strains resistant	Percentage from which it was transferred
Sulphonamides	97 (145)	10·3 (16·5)
Furazolidone	94 (36)	0 (0)
Tetracycline	25 (51)	44·0 (49·0)
Streptomycin	29 (31)	65·5 (51·6)
Ampicillin	18 (2)	100 (100·0)
Spectinomycin	10 (4)	20·0 (80·0)
Neomycin	1 (0)	100
Trimethoprim	4 (0)	75
Chloramphenicol	1 (0)	100

Number of strains examined = 140 (193).

Results for strains isolated in 1972 are in parentheses.

The incidence of transferable antibiotic resistance in the Escherichia coli *strains*

The incidence of transfer of most kinds of antibiotic resistance was high but that for sulphonamide was low; furazolidone resistance was not transferred from any of the 130 strains resistant to this antibiotic that were tested (Table 2).

Classification of strains according to the degree of their resistance to furazolidone

No difficulty was experienced by means of disk tests in classifying the *E. coli* strains into sensitive or resistant to all the antibiotics except furazolidone in that either a wide zone surrounded the disks or the bacteria grew right up to them. The situation was different with furazolidone. A wide or a non-existent zone of inhibition was certainly noted when some strains were tested: many others, though, yielded zones of varying sizes between these extremes. The tests on 148 strains were repeated using disks containing 75, 15 (the amount normally used)

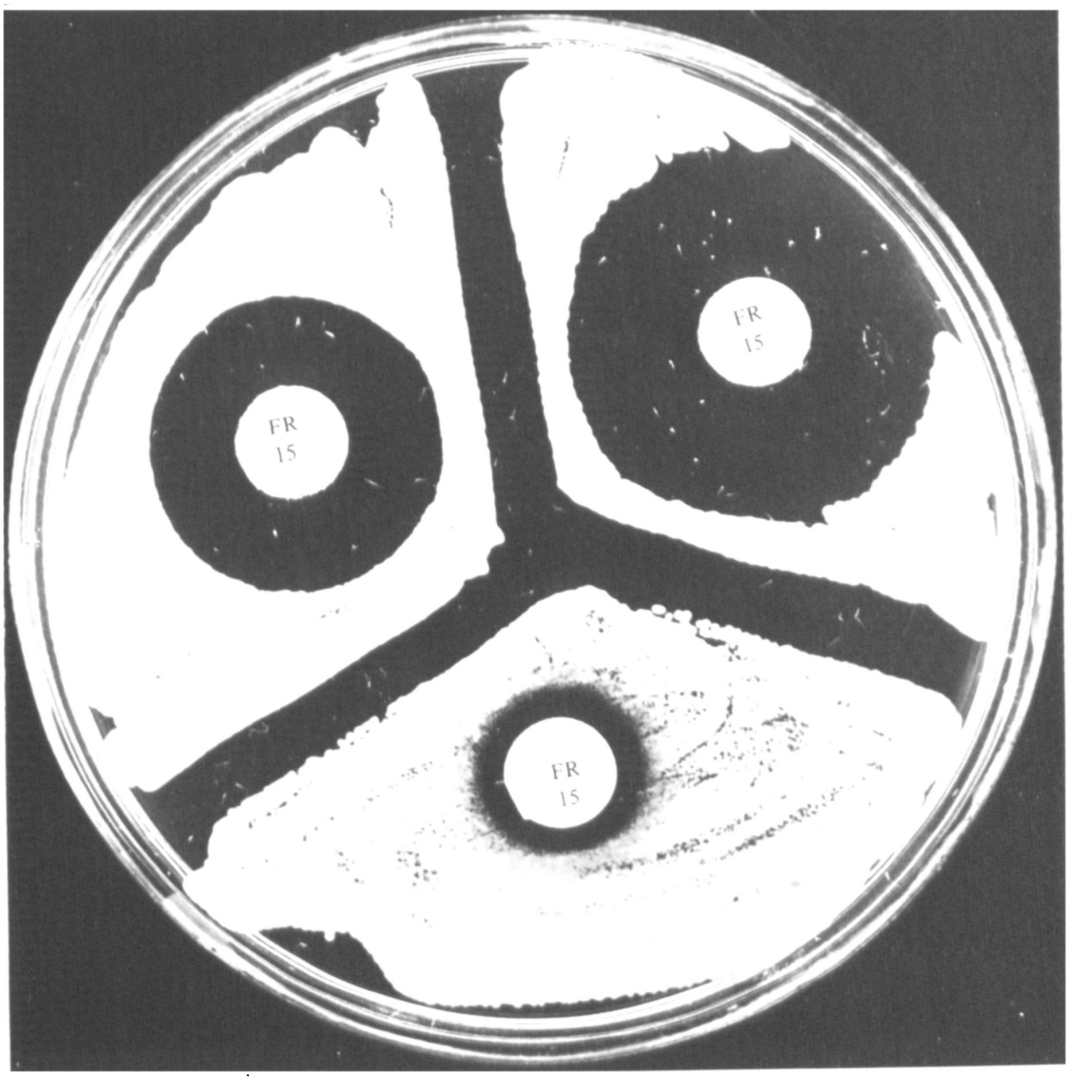

Fig. 1. Furazolidone sensitivity disk test (15 μg disk) against a fully sensitive strain of *Escherichia coli* (MIC = 0·15 μg/ml), an 'intermediate' resistant strain (MIC = 1·25 μg/ml) and a fully resistant strain (MIC = 20 μg/ml). $\times 1\frac{1}{3}$.

and 3 μg of furazolidone, but the results were substantially the same; the testing of a highly and a slightly sensitive strain and an 'intermediate' strain with a 15 μg disk is illustrated in Fig. 1.

The results of determining the minimum inhibitory concentration (MIC) of furazolidone for 289 strains of *E. coli* from the present survey and from the survey of Heller & Smith (1973) revealed, as had the disk tests, that the strains did not fall clearly into two groups, resistant and sensitive, but rather into a number of groups of varying degrees of sensitivity or resistance (Table 3).

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Table 3. *The minimum inhibitory concentration (MIC) of furazolidone for* 289 *of the strains of* Escherichia coli

MIC (μg/ml)	Number of strains
0·16	35
0·32	27
0·63	17
1·25	29
2·5	88
5·0	22
10·0	32
20·0	39

Table 4. *The effect of feeding diets containing* 400 *mg/kg of furazolidone to chickens inoculated intramuscularly with different doses of O2:K1* Escherichia coli *strains of differing furazolidone sensitivity (MIC)*

MIC for strain (μg/ml)	Strain no.	No. of LD50 inoculated	No. of 30 chickens that died	
			Treated	Untreated
0·16	1	10	1	25
	2	10	1	25
		100	10	30
		1000	29	30
0·32	3	10	1	29
	4	3	0	26
		10	0	24
		100	13	30
		1000	30	30
0·63	5	1	0	17
		3	0	21
		10	15	28
		100	29	30
1·25	6	1	5	12
		3	12	23
		10	17	25
	7	1	15	21
		10	28	28
2·5	8	1	2	14
		3	14	22
		10	27	28
	9	3	23	24
	10	3	25	27
10·0	2*	10	27	28
	4*	10	23	24
20·0	11	1	12	11
	12	1	14	10
		10	23	24
	13	10	29	30
	14	10	25	28

* Furazolidone-resistant mutants of strains 2 and 4 isolated in the laboratory.

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Table 5. *The effect of feeding diets containing different concentrations of furazolidone to chickens inoculated intramuscularly with O2:K1* Escherichia coli *strains of differing furazolidone sensitivity (MIC)*

MIC for strain (μg/ml)	No. of strain	Concentration of furazolidone in the diet (mg/kg)	No. of 15 chickens that died
0·16	2	400	0
		200	3
		100	2
		50	6
		25	10
		12	13
		0	10
1·25	7	1600	12
		800	11
		400	12
		0	13

Strains 2 and 7 were used at 10 and 3 LD50 respectively. For other details see Table 4.

The effect of feeding diets containing furazolidone to chickens inoculated with different doses of O2:K1 strains of Escherichia coli *of differing degrees of furazolidone resistance*

Very little mortality occurred in groups of 30 chickens whose diet was changed from a non-medicated one to one containing 400 mg/kg of furazolidone one day before they were inoculated intramuscularly with 10 or less LD50 of *E. coli* O2:K1 strains whose furazolidone MIC was 0·16 or 0·32 (numbers 1–4 in Table 4); a considerable reduction in mortality also occurred in groups inoculated with 100, but not 1000, LD50 of these strains. The furazolidone-containing diet was effective in controlling the mortality in groups of chickens inoculated with one or three LD50 of a strain whose furazolidone MIC was 0·63 μg (number 5 in Table 4), but it was only partially effective in a group inoculated with 10 LD50; it was completely ineffective in a group inoculated with 100 LD50. By contrast, the furazolidone-containing diet failed completely to control the mortality in groups of chickens inoculated with 10 LD50 of strains whose furazolidone MIC was 1·25–20 μg/ml (numbers 6–12 in Table 4); it was usually ineffective when the inocula were reduced to one or three LD50. This was so with wild strains and with laboratory-isolated mutants of the very sensitive strains, numbers 2 and 4. Furazolidone at lower dietary concentrations than 400 mg/kg controlled the mortality caused by the very furazolidone-sensitive strain 2; increasing it had no effect on the mortality caused by the less sensitive strain 7 (Table 5).

Neither tetracycline, trimethoprim, chloramphenicol nor ampicillin, given in the food, was as effective in controlling the disease produced in chickens by three O2:K1 *E. coli* strains as was furazolidone in controlling the disease produced by the one fully furazolidone-sensitive strain of the three (Table 6).

Table 6. *The effect of feeding diets containing* 400 *mg/kg of different antibiotics to chickens inoculated intramuscularly with O2:K1* Escherichia coli *strains of differing furazolidone sensitivity (MIC)*

Furazolidone MIC for strain (μg/ml)	No. of strain	No. of LD50 inoculated	No. of 30 chickens that died during treatment with					
			Fur	Tc	Tp	Cm	Ap	Neither
0·16	2	10	0	6	16	18	24	26
1·25	7	10	29	10	27	26	28	28
		1	14	0	0	2	11	10
20·0	12	10	27	6	20	26	24	29

Fur, furazolidone; Tc, tetracycline; Tp, trimethoprim; Cm, chloramphenicol; Ap, ampicillin. The MIC of tetracycline, trimethoprim, chloramphenicol and ampicillin for the three strains was 0·75, 0·75, 1·5 and 1·5 respectively.

DISCUSSION

Although the available evidence (Smith, 1980; Smith & Lovell, 1981) indicated that nine years after the banning of the use of therapeutic antibiotics as feed additives, pigs and broiler chickens in the UK were still a large reservoir of tetracycline-resistant and furazolidone-resistant faecal *E. coli*, a comparison of the results of the present survey with those of the similar one performed in 1972 (Heller & Smith, 1973) suggests that there has been a reduction in the incidence of tetracycline and furazolidone-resistant *E. coli* strains that cause generalized infections in broiler chickens; the reduction, by almost one half, was striking in the case of tetracycline resistance. Although there may be other reasons for this reduction, decreased exposure to these antibiotics as a direct result of the banning of their use as feed additives is a likely one. The sulphonamides were not included in the ban as far as chickens were concerned because it was thought that they would still be needed as feed additives to control coccidiosis in these animals. However, in recent years the anti-coccidial preparations that have come into general use have not contained sulphonamides – this may be the reason for the decline in sulphonamide resistance brought to light by the surveys. It is noteworthy when speculating on the cause of the decreased incidence of resistance to the antibiotics formerly used as feed additives that the trend, although only slight, in regard to antibiotics that had not been used in this manner, was to increase between the years 1972 and 1982.

As was the case with *S. gallinarum* (Smith, Tucker & Lovell, 1981), furazolidone was very effective in controlling the experimental infections produced by the O2:K1 strains of *E. coli* whose furazolidone MICs were 0·32 μg/ml or lower but it was largely ineffective in controlling infections caused by the strains whose MICs were 1·25 μg/ml or higher. If these results reliably reflect the situation in the field, it appears that furazolidone is now of limited value in controlling outbreaks of generalized *E. coli* infections in chickens in the UK because the furazolidone MIC of most of the strains examined in the 1982 survey was 1·25 μg/ml or higher. This incidence of furazolidone resistance is particularly unfortunate because our results revealed furazolidone to be much more effective than the other antibiotics tested in controlling infections produced by fully furazolidone-sensitive *E. coli* strains.

It is apparent, too, that great care is required in the conduct of *in vitro* tests to detect this resistance.

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