Furazolidone resistance in *Salmonella gallinarum*: the relationship between *in vitro* and *in vivo* determinations of resistance

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SUMMARY

Of 22 strains of *Salmonella gallinarum* isolated from recent outbreaks of infection in poultry in Greece (15), Amman (3), Kenya (2), Lebanon (1) and Yemen (1), 20 were more resistant to furazolidone *in vitro* than 6 strains that had been isolated in the U.K. in the 1950s; the minimum inhibitory concentration of furazolidone was approximately 0.3 µg/ml for the sensitive strains and 1.3 or 2.5 µg/ml for the more resistant strains.

Furazolidone given continuously in the food did not control experimental infections in chickens caused by most of the strains that had been classed as more resistant by the *in vitro* tests. Chloramphenicol, trimethoprim and sulphadiazine or mixtures of the latter two were the best antibiotics for treating these infections, but they were less satisfactory than furazolidone for treating infections caused by the furazolidone-sensitive strains.

As a group, the furazolidone-resistant strains and furazolidone-resistant mutants of one of the sensitive strains were less virulent for chickens than the sensitive strains.

INTRODUCTION

Because its superiority was convincingly demonstrated in experimental and field studies (Pomeroy, 1972), furazolidone, administered in the feed on a flock basis, became, some 25 years ago, the most common antibiotic to be used in controlling *Salmonella gallinarum* infection in poultry. It has also been widely used in some countries to control other diseases of poultry and as a growth promoter. Despite this extensive usage, there have been surprisingly few reports of strains of *S. gallinarum* emerging that are furazolidone-resistant. Hall & Cartrite (1961) examined four strains from outbreaks in Texas that had not responded to furazolidone therapy. Although all four were considered to be furazolidone-sensitive one developed resistance when serially passaged in furazolidone-containing culture medium. It also appeared to do so in experimentally infected turkeys because they did not respond to treatment with food containing 0.011% furazolidone, a concentration that is usually adequate for this purpose. Stuart, Keenum & Bruins (1962, 1967) isolated two strains that experimentally produced a disease not controlled by feeding diets containing 0.022% furazolidone; the premises from which one of the strains had been isolated had a 4-year history of *S. gallinarum* infection being treated with furazolidone.

In recent years *S. gallinarum* infection has become more prevalent, especially in the Eastern Mediterranean and Middle Eastern areas. Because of reports that the feeding of furazolidone-containing diets was failing to prevent outbreaks occurring, *S. gallinarum* strains were obtained from these areas so that their *in vitro* and *in vivo* resistance to furazolidone could be determined. Because some were resistant, other antibiotics were studied to determine whether or not they provided a suitable alternative to furazolidone in the treatment of disease caused by these strains. The results of these investigations are reported here.

MATERIALS AND METHODS

Chickens

Salmonella-free Light Sussex chickens aged 7 days were used in all experiments. They were kept under good hygienic conditions in groups of about 30 on wire-mesh 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, antibiotics as pre-mixes were incorporated in the food by means of a mechanical mixer.

S. gallinarum strains

Of the strains studied, 22 were from recent outbreaks of clinical disease in chickens in Amman (nos. 6–8), Greece (nos. 10–24), Yemen (no. 25), Lebanon (no. 26) and Kenya (nos. 27 and 35). Another six (nos. 1–5 and 9), preserved in the lyophilized state, had caused outbreaks in the U.K. in the 1950s; strain 9 had been used in previous chemotherapeutic studies (Smith, 1955). All 26 were judged to be smooth because they were not agglutinated in slide tests by 0.2 % acriflavine solution. For use, they were grown in 10 ml of nutrient broth (Oxoid CM 67) in a shaking water bath at 37 °C for 24 h. These cultures contained approximately 10⁹ viable organisms per ml.

Antibiotic sensitivity tests

These were performed on Sensitest Agar (Oxoid) by the disk method (Smith, 1977) using disks containing (µg): ampicillin (25), chloramphenicol (50), neomycin (30), spectinomycin (25), streptomycin (25), oxytetracycline (50), furazolidone (15), sodium nalidixate (30), trimethoprim (1.25) and sulphonamides (300). Furazolidone sensitivity tests were also performed by the strip method using paper strips that had been soaked in solutions containing 100 and 500 µg/ml of furazolidone and then placed at right angles to streaks of broth cultures, diluted 10 times, on plates of Sensitest agar; the plates were read after incubation at 37 °C for 24 h.

Minimum inhibitory concentrations (MIC) were determined by spot-inoculating a series of plates of Sensitest Agar, containing twofold differences in concentration of the antibiotic under test, with broth cultures of different strains suitably diluted so that each spot contained approximately 200 viable organisms. The plates were incubated at 37 °C for 24 h and the lowest concentration that prevented visible growth was recorded as the MIC.

Isolation of furazolidone-resistant mutants of S. gallinarum and E. coli K 12

Nutrient broth in 500 ml amounts and containing 4 µg/ml of furazolidone were inoculated with all the organisms of the furazolidone-sensitive strain 9 or of *E. coli* K 12 that grew on a Roux flask of nutrient agar. The broth was incubated at 37 °C and at 24, 48 and 72 h afterwards 0.3 ml was removed and inoculated over the whole surface of a plate of nutrient agar containing 4 µg/ml of furazolidone. The plates were incubated and colonies that grew upon them were picked, purified and checked for furazolidone resistance.

Attempted transfer of furazolidone resistance

Nutrient broth in 10 ml amounts was inoculated with 0.1 ml each of a broth culture of a furazolidone-resistant strain of *S. gallinarum* and of a spontaneous nalidixic acid-resistant mutant of the furazolidone-sensitive *S. gallinarum* strain 9 or of *E. coli* K 12. After incubation at 37 °C for 24 h, 0.3 ml of the mixed culture was inoculated onto plates of nutrient agar containing 20 µg/ml of sodium nalidixate and 0.6 µg/ml of furazolidone. The plates were incubated at 37 °C for 24 h and examined for colonies of the recipient strains.

The effect of antibiotic treatment on the course of S. gallinarum infection

Groups of chickens were given 0.3 ml of a shaken broth culture of *S. gallinarum* directly into the crop by means of a Pasteur pipette passed down the oesophagus. All groups, except control groups, were then fed continuously on antibiotic-containing diets. To confirm that death was not due to emergent antibiotic-resistant mutants of the infecting strain, the livers of all chickens that died were cultured on plates of MacConkey's agar and a disk containing the antibiotic with which they had been treated placed in the middle of the inoculated area of each plate. The organs of all surviving chickens were examined for macroscopic lesions characteristic of the chronic form of *S. gallinarum* infection.

RESULTS

The in vitro sensitivity of Salmonella gallinarum strains to furazolidone and other antibiotics

The 28 strains of *S. gallinarum* were classified into a resistant and a sensitive group by the furazolidone disk tests. The zones of inhibition that surrounded 15 µg disks in tests with the sensitive group comprising the six strains isolated in the U.K. in the 1950s and the two Kenyan strains were 13–14 (median 14) mm in diameter. The zone diameters in tests with the other 20 strains – the resistant group, comprising the 15 from Greece, the three from Amman and the one each from Lebanon and Yemen – were 8–10 (median 9) mm. No zones were discernible in tests with four mutants of the sensitive *S. gallinarum* strain 9 or with one mutant of *E. coli* K 12. Testing one strain from each group with disks containing 3, 9 and 30 µg of furazolidone (Plate 1) revealed differences in zone size between the two strains at all three concentrations of antibiotic tested.

Differences were also noted between the two groups of strains when two strains of the sensitive group and 12 of the resistant group were tested for furazolidone

sensitivity by the strip method (Plate 2). In these tests, the strains that had been classified as resistant grew right up to the edge of the strip that had been impregnated with the more dilute solution of furazolidone (100 µg/ml).

The minimum inhibitory concentration (MIC) of furazolidone for the resistant strains was either 1.3 µg/ml (5 strains) or 2.5 µg/ml (15 strains); that for the mutants of strain 9 and the *E. coli* K 12 strain was 5.0 µg/ml. The MIC for the 8 sensitive strains was 0.3 µg/ml; that for *E. coli* K 12 was 0.15 µg/ml. The MIC of ampicillin, chloramphenicol, oxytetracycline, sodium nalidixate, trimethoprim and sodium sulphadiazine for all the *S. gallinarum* strains was approximately 1.5, 3.0, 3.0, 12.0, 0.75 and 50 µg/ml respectively.

Attempts to transfer the furazolidone resistance from 7 resistant strains were unsuccessful.

To obtain information on the nature of furazolidone resistance, organisms of the sensitive *S. gallinarum* strain 9 that had been grown on a Roux flask of nutrient agar that had been incubated at 37 °C for 24 h were removed into 50 ml of an aqueous solution of 30 µg/ml of furazolidone. The suspension was incubated at 37 °C for 2 h in a shaking water bath, centrifuged at 2500 *g* for 30 min and the supernatant fluid filtered through a membrane filter of pore diameter 450 µm. Culture plates were then made with 7 ml of molten triple-strength nutrient agar at 60 °C to which had been added 14 ml of the filtrate. Similar plates were made with furazolidone solution that had been exposed to organisms of the *E. coli* K 12 strain and to furazolidone-resistant mutants of this strain and those of the *S. gallinarum* strain 9. Each of the 4 plates was then inoculated with the 4 strains and incubated at 37 °C for 24 h. All 4 strains grew on the plates containing furazolidone that had been exposed to organisms of the furazolidone-sensitive strain 9 or *E. coli* K 12. Strain 9 and *E. coli* K 12 failed to grow on the plates containing furazolidone that had been exposed to the furazolidone-resistant mutants of these strains; the mutants either failed to grow on these plates or grew only very slightly. The set of plates containing furazolidone that had been exposed to furazolidone-sensitive or resistant organisms of strain 9 or neither are illustrated in Plate 3. Similar results were obtained when the experiments were repeated with some of the resistant and sensitive wild-type *S. gallinarum* strains.

*Furazolidone treatment of chickens infected with S. gallinarum strains
of differing in vitro sensitivity to this antibiotic*

Furazolidone was highly effective in treating groups of chickens that had been infected with the eight *S. gallinarum* strains that had been classified as furazolidone-sensitive; none of the chickens died and very few were found at the end of the experiment to have lesions characteristic of the chronic form of *S. gallinarum* infection (Table 1). By contrast, furazolidone had no effect in controlling the disease caused by most of the strains that had been classified as resistant or by the furazolidone-resistant mutant of strain 9; even in the case of the few strains against which furazolidone had an *in vivo* effect, the disease was only incompletely controlled in that either some of the chickens in the treated groups died and/or there was a high incidence of lesions in the survivors.

The results of infecting groups of chickens with either the sensitive strain 9 or the resistant strain 6 and then treating them with food containing different

Table 1. *The effect of treatment with furazolidone on the mortality rate in groups of 15 chickens inoculated with S. gallinarum strains of differing in vitro sensitivity to that antibiotic*

Infecting strain	Minimum inhibitory concentrations of furazolidone ($\mu\text{g/ml}$)	Percentage of chickens that died in group	
		Treated with furazolidone*	Not treated
5	0.3	0 (0)†	100
2	0.3	0 (7)	100
1	0.3	0 (13)	100
3	0.3	0 (0)	93 (0)
35	0.3	0 (0)	93 (0)
9	0.3	0 (0)	93 (0)
4	0.3	0 (0)	87 (0)
27	0.3	0 (0)	87 (0)
24	2.5	80 (33)	87 (50)
17	2.5	93 (100)	67 (80)
6	2.5	67 (80)	53 (100)
13	1.3	60 (83)	67 (80)
12	2.5	60 (100)	60 (100)
21	2.5	40 (11)	53 (29)
7	2.5	40 (100)	40 (33)
14	1.3	33 (20)	47 (75)
22	2.5	47 (100)	27 (100)
16	2.5	0 (67)	7 (86)
20	2.5	0 (93)	0 (93)
26	1.3	27 (64)	53 (57)
25	2.5	33 (80)	87 (100)
18	2.5	0 (73)	53 (71)
Furazolidone-resistant mutant of 9	5.0	80 (33)	87 (0)

* 0.04 % in the food commencing one day after infection.

† The figures in parentheses are the percentages of survivors with lesions characteristic of chronic *S. gallinarum* infection when killed 16 days after inoculation.

concentrations of furazolidone are summarized in Table 2. Food containing concentrations as low as 0.0025 % was highly effective in treating chickens infected with strain 9 whereas food containing concentrations as high as 0.16 % was completely ineffective in treating chickens infected with strain 6; concentrations higher than 0.16 % were not tested because they were known to be toxic. In another experiment, groups of 33 chickens were given a 9-day course of food containing 0.04 % furazolidone commencing one day before infection with strain 6, at infection time or 1, 2 or 3 days afterwards. The mortality rate in each of these groups was 73, 79, 85, 82 or 79 % respectively; the mortality rate in an untreated group was 79 %. By contrast, the mortality rate in a similar experiment in which administration of the furazolidone-containing food was commenced 1, 2, 3, 4 or 5 days after infection with strain 9 was 6, 0, 0, 6 and 36 % respectively; the mortality rate in an untreated group was 94 %.

Table 2. *The mortality rate in groups of 25 chickens given diets containing different concentrations of furazolidone following inoculation with the furazolidone-sensitive S. gallinarum strain 9 or the resistant strain 6*

Percentage of furazolidone in the diet of chickens*	Percentage that died following inoculation with strain	
	9	6
0.16	—	84 (0)†
0.08	—	76 (33)
0.04	0 (0)	68 (75)
0.02	4 (0)	—
0.01	0 (0)	—
0.005	4 (0)	—
0.0025	8 (0)	—
0.0012	40 (40)	—
0.0006	80 (40)	—
nil	88 (33)	72 (57)

* The feeding of the furazolidone-containing diets was commenced one day before inoculation and was continued for 16 days.

† The figures in parentheses are the percentages of survivors with lesions characteristic of chronic *S. gallinarum* infection when killed 16 days after infection.

Table 3. *The mortality rate in groups of 35 chickens given diets containing different antibiotics for 9 days commencing one day after they were inoculated with the furazolidone-resistant strain 6*

Antibiotic in the diet of chickens	Percentage that had died by the following days after inoculation	
	10	30
Furazolidone	57	83 (17)*
Nalidixic acid	26	60 (71)
Ampicillin	14	43 (60)
Oxytetracycline	20	43 (45)
Chloramphenicol	0	17 (59)
Trimethoprim and sulphadiazine	0	17 (62)
Trimethoprim	6	29 (52)
Sulphadiazine	3	26 (73)
None	46	80 (43)

* The figures in parentheses are the percentages of survivors with lesions characteristic of chronic *S. gallinarum* infection.

All the antibiotics except trimethoprim and sulphadiazine were given at a concentration of 0.04 % in the food. The concentrations of trimethoprim and sulphadiazine employed were 0.04 % and 0.20 % respectively.

The effect of antibiotics other than furazolidone on the treatment of chickens infected with a furazolidone-sensitive or a furazolidone-resistant strain of S. gallinarum

The results of infecting chickens with the furazolidone-resistant strain 6 and 1 or 3 days later giving them diets containing different antibiotics for 9 or 16 days are summarized in Tables 3, 4 and 5; varying the time interval between infection

Table 4. *The mortality rate in groups of 30 chickens given diets containing different antibiotics for 16 days commencing one day after they were inoculated with the furazolidone-resistant strain 6*

Antibiotic in the diet of chickens	Percentage that had died by the following days after inoculation	
	17	30
Furazolidone	80	80 (100)
Nalidixic acid	57	57 (60)
Ampicillin	53	53 (71)
Oxytetracycline	23	27 (86)
Chloramphenicol	0	0 (60)
Trimethoprim and sulphadiazine	10	17 (28)
Trimethoprim	17	20 (46)
Sulphadiazine	3	2 (48)
None	67	70 (78)

For other details see legend to Table 3.

Table 5. *The mortality rate in groups of 33 chickens given diets containing different antibiotics for 9 days commencing 3 days after they were infected with the furazolidone-resistant strain 6*

Antibiotic in the diet of chickens	Percentage that had died by the following days after inoculation	
	12	30
Furazolidone	58	67 (64)
Nalidixic acid	58	64 (50)
Ampicillin	64	70 (60)
Oxytetracycline	24	36 (57)
Chloramphenicol	12	12 (31)
Trimethoprim and sulphadiazine	9	12 (83)
Trimethoprim	12	15 (61)
Sulphadiazine	9	12 (83)
None	64	67 (45)

For other details see legend to Table 3.

and the commencement of treatment and varying the duration of treatment had only a slight influence. Nalidixic acid and ampicillin had no or only a slight effect on the mortality rate and oxytetracycline had only a slight effect. Chloramphenicol, trimethoprim, sulphadiazine and a mixture of the last two antibiotics had a marked effect, but a high proportion of the chickens that had been treated with these agents had lesions characteristic of chronic *S. gallinarum* infection; there was no evidence of a synergistic effect when the trimethoprim and sulphathiazole were given in combination.

Furazolidone given for 9 days commencing one day (Table 6) or 3 days (Table 7) after inoculation with the more virulent furazolidone-sensitive strain 9 had a pronounced curative effect. The mortality rate was markedly reduced and none

Table 6. *The mortality rate in groups of 28 chickens given diets containing different antibiotics for 9 days commencing one day after they were inoculated with the furazolidone-sensitive strain 9*

Antibiotic in the diet of chickens	Percentage that had died by the following days after inoculation	
	10	30
Furazolidone	3	3 (0)
Nalidixic acid	61	64 (10)
Ampicillin	79	79 (0)
Oxytetracycline	54	71 (25)
Chloramphenicol	4	57 (50)
Trimethoprim and sulphadiazine	14	54 (62)
Trimethoprim	64	86 (0)
Sulphadiazine	39	64 (70)
None	79	79 (17)

For other details see legend to Table 3.

Table 7. *The mortality rate in groups of 32 chickens given diets containing different antibiotics for 9 days commencing 3 days after they were inoculated with the furazolidone-sensitive strain 9*

Antibiotic in the diet of chickens	Percentage that had died by the following days after inoculation	
	12	30
Furazolidone	6	6 (0)
Nalidixic acid	97	97 (0)
Ampicillin	100	100
Oxytetracycline	72	78 (57)
Chloramphenicol	94	100
Trimethoprim and sulphadiazine	47	53 (53)
None	91	94 (50)

For other details see legend to Table 3.

of the surviving chickens in the two groups that had been treated with this antibiotic had lesions in its organs. By contrast, the other antibiotics had very little effect on the course of the disease. This was particularly noticeable in the experiment in which the onset of treatment was delayed until 3 days after inoculation (Table 7).

The virulence of furazolidone-resistant strains of S. gallinarum

In the experiments involving the treatment of infections caused by different *S. gallinarum* strains with furazolidone (Table 1) the mortality rates in the untreated groups of chickens infected with the 14 furazolidone-resistant strains, 0–87% (median 53%) were, in general, lower than those in the groups infected with the 8 sensitive strains, 87–100% (93%). Decreased virulence associated with the furazolidone resistance was also noted when groups of chickens were inoculated

Table 8. *The survival times of groups of 33 chickens that were inoculated with the furazolidone-sensitive strain of S. gallinarum, strain 9 and four furazolidone-resistant mutants of it*

Strain inoculated		Percentage of chickens that were dead by the following days after inoculation												
		5	6	7	8	9	10	11	12	13	14	15	16	21
Furazolidone-sensitive parent	{	6	27	79	82	88	88	88	91	91	91	91	91	91
		3	30	73	85	91	91	94	94	97	97	97	97	97
Furazolidone-resistant mutant	{	1	0	9	30	42	45	55	58	61	64	67	70	70
		2	0	9	21	42	45	48	58	61	61	67	67	70
		3	6	12	33	45	58	76	82	85	85	88	88	88
		4	0	0	0	0	0	0	0	3	3	3	6	6

with the sensitive strain 9 and four furazolidone-resistant mutants of this strain (furazolidone MIC for all four = 5.0 µg/ml). In three of the mutants the decreased virulence was mainly manifested by prolonged survival time of infected chickens; in the fourth mutant it was manifested by a very low mortality rate (Table 8).

DISCUSSION

All except two of the 22 recently isolated strains of *Salmonella gallinarum* differed from the six isolated in the 1950s in being about 4–8 times less sensitive to furazolidone. Although the actual degree of their resistance (MIC approximately 1.3 or 2.5 µg/ml) was, by ordinary standards, of a low order, the *in vivo* studies revealed that it was usually sufficiently high to negate the therapeutic effect of furazolidone, even when it was used at much higher dose rates than those normally employed. Furthermore, the experiments in which the potency of a furazolidone solution was assessed after exposure to fully sensitive and resistant organisms revealed that the resistance was probably associated with reduced uptake of the antibiotic. Our failure to transmit this resistance confirmed earlier observations that furazolidone resistance was not transmissible (Smith, 1966).

If the recently isolated strains and those isolated in the 1950s are typical of the *S. gallinarum* that are causing disease in poultry during these two periods then it is apparent that a remarkable ecological change has taken place in the intervening years in that a predominantly furazolidone-sensitive *S. gallinarum* population has been replaced by a predominantly furazolidone-resistant one. Should this be true, then furazolidone from being the antibiotic of choice in controlling *S. gallinarum* infection may now become an antibiotic of very limited usefulness in this respect. Further investigations are definitely needed before this can be claimed. Whatever the outcome, it is obvious that, in the future, isolates from outbreaks of *S. gallinarum* infection should always be checked for sensitivity to furazolidone before resorting to treatment with this antibiotic. Also, the tests must be carefully conducted and interpreted because if, for example, disk methods are employed, the difference between a strain which is sensitive *in vivo* and one which is not is relatively small. It is probable, too, that the need for great care in assessing furazolidone sensitivity also applies when the treatment of diseases

caused by bacteria other than *S. gallinarum* is under consideration. The use of furazolidone in the actual treatment of outbreaks of *S. gallinarum* infection is unlikely to be the main cause of the high incidence of furazolidone-resistant strains found in the present work. The routine and widespread use of this antibiotic as a feed additive for poultry flocks in the hope of preventing outbreaks of disease caused by *S. gallinarum* and other infectious agents is much more likely to be the cause. If this is so, then it is yet another example of the consequence of the indiscriminate use of antibiotics.

The furazolidone-resistant strains, as a group, were less virulent than the sensitive ones. That this difference in virulence was associated with furazolidone resistance was strengthened by the observation that the furazolidone-resistant mutants of the sensitive strain 9 were less virulent than their parent strain. Although one could argue from this that the widespread use of furazolidone has had an overall beneficial effect in that it has tended to select *S. gallinarum* organisms that are less virulent, it must be borne in mind that all the strains in this investigation were from flocks in which they were posing a disease problem. Also, we did not find any antibiotics that were a suitable therapeutic alternative to furazolidone, an observation that is in keeping with those of other investigations (Pomeroy, 1972).

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Plate 2

Plate 3

EXPLANATION OF PLATES

PLATE 1

The top half of the plate has been inoculated with the furazolidone-sensitive *S. gallinarum* strain 9 and the bottom half with the furazolidone-resistant strain 6. The disks from left to right of each culture contain respectively 30, 9, and 3 μ g of furazolidone.

PLATE 2

Each plate has been inoculated with two furazolidone-sensitive and 12 furazolidone-resistant strains of *S. gallinarum*. The paper strip on the left plate has been soaked in 200 μ g of furazolidone/ml in di-methyl-formamide and the strip on the right plate has been soaked in 100 μ g/ml.

PLATE 3

The top left nutrient agar plate contains furazolidone that had been exposed to the furazolidone-sensitive strain 9, the top right plate contains furazolidone that had been exposed to a furazolidone-resistant mutant of strain 9 and the bottom plate contains unexposed furazolidone.

The top left, top right, bottom left and bottom right quadrant of each plate had been inoculated respectively with a furazolidone-resistant mutant of strain 9, strain 9, a furazolidone-resistant mutant of *E. coli* K 12 and *E. coli* K 12.