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The survival of multi-antibacterial drug-resistant *Escherichia coli* and *Salmonella typhimurium* in stored static slurry from a veal calf unit

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SUMMARY

Salmonella typhimurium phage type DT 193 survived in small numbers, in stored static slurry derived from veal calves, for the 7-week period of observation. The viable coliform count fell by 1½ logs during the first 2 weeks of storage, thereafter there were only relatively small fluctuations in the coliform population. In all 735 of 752 *Escherichia coli* isolates examined from eight samples of slurry were resistant to 3–6 antibacterial drugs. There was no dramatic change in the overall level of drug resistance amongst the *E. coli* with time. Chloramphenicol resistance was recorded in 400 (55%) of the *E. coli*. It was always associated with multiple resistance, with 96% of the strains being resistant to 5 or 6 drugs, although the proportion of isolates of each of the ten most prevalent O-serotypes resistant to chloramphenicol was variable and ranged between none and 97·5%. The use of biotyping together with O-serotyping indicated that the *E. coli* population was extremely complex, although certain components of the population remained relatively stable within the dominant flora with time since several of the more common O-serotype/biotype combinations were isolated from more than half of the eight slurry samples examined.

INTRODUCTION

In the last decade there has been considerable interest in the survival of pathogenic micro-organisms in slurry, a mixture of faeces, urine and water, and their potential danger as a source of infection to farm livestock (Jones, 1980). On the other hand there is little published information on the survival of micro-organisms containing R plasmids in the slurry and in the environment following the spreading of slurry on farm land (Kelly & Collins, 1978). The purpose of this investigation was to study the survival of naturally occurring *Salmonella*

typhimurium and *Escherichia coli*, which were resistant to several antibacterial drugs, in stored static slurry and also to assess the influence of storage on the *E. coli* population with regard to O-serotype, biotype and the R determinants that they contained.

MATERIAL AND METHODS

The slurry

The slurry was obtained from a veal-calf-rearing farm in the county of Somerset. Details of the management of the farm, including the use of antibacterial drugs, have been summarized by Linton, Timoney & Hinton (1981).

The concrete floors of the calf house were hosed down daily and the resultant mixture of faecal material, urine and washing water was collected as a slurry in an underground tank. This was emptied once or twice a week and the slurry spread onto fields adjacent to the buildings. A bulk sample of slurry was obtained on 19 February 1979 and was subsequently stored out of doors in a galvanized dustbin lined with a polyethylene sack and covered with a rubber lid. Sequential samples for bacteriological examination were taken from the bin on days 1, 6, 13, 20, 27, 34, 41 and 48 after the contents had been thoroughly stirred. The weather during the seven weeks of the survey was extremely cold and, on some occasions, it was necessary to break the ice which had formed on the surface before the sample could be collected. The slurry appeared to contain little solid material. The pH of the last five samples was measured and was found to vary between 7·7 and 8·0.

Isolation and identification of salmonella

The sample (1 ml) of neat slurry was incubated at 37 °C in 15 ml selenite broth (Hobbs & Allison, 1945). The broth was subcultured on brilliant green phenol red agar (Edel & Kampelmacher, 1969) at 24 and 48 h and the plates were incubated at 37 °C in air. After 24 h suspicious colonies were investigated by standard biochemical and serological techniques (Edwards & Ewing, 1972). The serological identification of all salmonella isolates was confirmed, and representative strains were phage typed by the Salmonella and Shigella Reference Laboratory, Colindale Avenue, London. In order to obtain the most probable number (MPN) of salmonella in each sample, 1 ml volumes of decimal dilutions (10^{-1} and 10^{-2}) of the slurry were similarly processed (Linton, Jeanett & Heard, 1970).

Studies on Escherichia coli

The number of viable coliforms in the sample of slurry was determined using a technique described previously (Linton, Howe & Osborne, 1975). Counts were made on bile lactose agar without salt (BLA) (Oxoid Ltd. CM7b) and on BLA incorporating either 25 μg/ml chloramphenicol, 10 μg/ml streptomycin or 25 μg/ml tetracycline. All plates were incubated for 24 h at 37 °C in air.

One hundred colonies typical of *E. coli* were picked from the unsupplemented BLA plates from each sample. A total of 752 colonies were identified as *E. coli* on the basis of positive indole and Eijkman tests. These strains were O-serotyped by a microtitre agglutination technique using 157 typing sera (Hartley *et al.* 1975;

Table 1. *The coliform count* on stored static slurry*

Plating media†	Day of sampling							
	1	6	13	20	27	34	41	48
BLA	5·60	5·44	4·00	3·90	3·95	4·10	4·16	4·88
BLA + C	5·00	4·30	3·65	3·54	3·54	4·08	4·11	4·18
BLA + S	5·48	4·40	4·04	3·88	4·02	4·13	4·11	4·54
BLA + T	5·51	4·48	3·85	3·74	3·88	4·16	4·18	4·40

* Counts expressed logarithmically.

† BLA, bile lactose agar; C, chloramphenicol; S. streptomycin; T, tetracycline.

Howe & Linton, 1976) and biotyped on the basis of their ability to ferment five carbohydrates (Hinton, Allen & Linton, 1982).

Determination of in vitro *resistance to antibacterial drugs*

The antibiotic resistance pattern of all isolates of *Salm. typhimurium* and *E. coli* was determined by the disk diffusion method (Howe & Linton, 1976). The antibacterial sensitivity disks used included ampicillin (A) (25 μg), chloramphenicol (C) (50 μg), kanamycin (K) (30 μg), streptomycin (S) (25 μg), sulphafurazole (Su) (500 μg) and tetracycline (T) (50 μg).

RESULTS

Survival of Salm. typhimurium *and* E. coli *in static slurry*

Salm. typhimurium phage type DT193, with the resistant pattern A C K S Su T, was isolated from all eight weekly samples. The most probable number of viable *Salm. typhimurium* in each sample varied between > 10 and > 100 organisms/ml of slurry.

The presumptive coliform counts on BLA and BLA containing either chloramphenicol, streptomycin or tetracyline are shown in Table 1. All counts fell by approximately 1½ logs during the first two weeks of storage; thereafter only relatively small fluctuations occurred in the coliform population.

Studies on E. coli *in slurry*

Only one of the 752 *E. coli* strains isolated was sensitive to all six antibacterial drugs, while 29 resistance patterns were recorded in the remainder. In all 735 (98%) of the *E. coli* were resistant to three or more drugs. Ten patterns, all of which involved at least 1% of the isolates and contained R determinants for S and Su, accounted for 713 (95%) of the strains (Table 2). The proportion of isolates exhibiting these patterns fluctuated between samplings, although five of the ten most common patterns were identified in all eight samples and accounted for 620 (82%) of the *E. coli* examined. There was no dramatic change in the overall level of resistance amongst the *E. coli* during the period of observation, the average number of R determinants carried by each isolate fluctuating between 4·4 and 5·0 (Table 2).

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Table 2. *Distribution and the number of* E. coli *isolated on each sampling occasion from stored static calf slurry according to the pattern of resistance to six antibacterial drugs*

Resistance pattern*	Day of sampling 1	6	13	20	27	34	41	48	Total
K S Su	1	—	—	—	—	13	—	—	14
S Su T	8	7	6	7	2	6	15	6	57
A C S Su	6	—	—	1	—	2	—	—	9
A S Su T	1	5	5	4	8	—	1	1	25
K S Su T	4	12	26	24	22	22	17	33	160
A C K S Su	8	1	—	—	—	3	—	—	12
A C S Su T	7	13	6	3	3	—	1	—	33
A K Su T	1	6	12	10	3	11	15	9	67
C K S Su T	19	16	6	12	5	9	14	15	96
A C K S Su T	34	36	33	36	26	20	27	28	240
Other patterns	8	2	4	1	4	8	7	5	39
Average no. R determinants/ isolate	4·9	5·0	4·8	4·9	4·7	4·4	4·5	4·7	4·7
No. *E. coli* examined	97	98	98	98	73	94	97	97	752

* A = ampicillin, C = chloramphenicol, K = kanamycin, S = streptomycin, Su = sulphafurazole and T = tetracycline. (Each of the patterns listed was recorded in > 1 % of the *E. coli* isolates.)

Chloramphenicol resistance was recorded in 400 (53 %) of the 752 strains of *E. coli*. It was always associated with muliple resistance, with 96 % of the strains being resistant to five or six drugs.

It was possible to O-serotype 519 (69 %) of the *E. coli*. A total of 48 O-serotypes were identified although 18 were present in only a single sample and a further six in two. The number of the dominant O-serotypes, each of which was represented by at least 1 % of the total isolates, fluctuated with time, although nine of the 10 most frequently isolated O-serotypes were present in six or more of the eight samples of slurry (Table 3).

The patterns of antibacterial drug resistance recorded in the ten O-serotypes identified 17 times or more are listed in Table 4. There were obvious differences in the distribution of the various R determinants between some of these O-serotypes, the most notable being that 37 of the 38 strains of *E. coli* O 154 had the resistance pattern A C K S Su T while all O 13 strains were resistant to four drugs or less and none contained R determinants to A or C. On the other hand 13 of the 14 *E. coli* with the resistance pattern KSSu were isolated from one of the eight samples (Table 2), and this was due to the simultaneous appearance in that sample of several O-serotypes and non-typable strains carrying that combination of R determinants (Table 4).

Chloramphenicol resistance was recorded in 31 (65 %) of the 48 O-serotypes. The proportion of chloramphenicol-resistance strains in the O-typable *E. coli* was 71·5 %

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Table 3. *The distribution of* E. coli *isolated from stored static slurry according to O-serotype*

O-serotypes	Day of sampling* 1	6	13	20	27	34	41	48	Total
9	2 (2)	19 (12)	11 (6)	12 (6)	15 (6)	14 (5)	12 (7)	12 (6)	97 (50)
8	14 (12)	4 (3)	6 (3)	4 (3)	4 (3)	2 (1)	8 (3)	6 (3)	48 (31)
19	12 (12)	12 (12)	6 (4)	7 (5)	2 (2)	4 (1)	1 (1)	—	44 (37)
40	1	4	5 (2)	3 (3)	3	8 (4)	10 (9)	7 (4)	41 (22)
154	—	—	4 (3)	9 (9)	9 (9)	4 (4)	5 (5)	7 (7)	38 (37)
21	—	8 (7)	2 (2)	4 (3)	5 (4)	3 (1)	3 (1)	1 (1)	26 (19)
23	5 (3)	6 (2)	4	2	—	3	4 (1)	2	26 (6)
13	—	—	—	9	1	5	—	6	21
25	2	2 .	3 (1)	6	—	4	1	—	18 (1)
86	3 (3)	2 (2)	2 (1)	3 (3)	1 (1)	2 (1)	2 (1)	2 (2)	17 (14)
101	8 (5)	3 (1)	—	1	1	—	1	—	14 (6)
15	1 (1)	1	—	3 (1)	3 (1)	1 (1)	2 (1)	1 (1)	12 (6)
1	4 (4)	1 (1)	1 (1)	3 (3)	—	1 (1)	—	1 (1)	11 (11)
2	—	1 (1)	2 (1)	1 (1)	1 (1)	2 (2)	2 (2)	—	9 (8)
3	3 (2)	2 (2)	—	3 (3)	—	1	—	—	9 (7)
54	1 (1)	1 (1)	2 (2)	2 (2)	—	—	2 (2)	1 (1)	9 (9)
16	—	1	2	—	1	1	3	—	8
155	—	4 (3)	1 (1)	—	—	3 (1)	—	—	8 (5)
Others†	13 (10)	6 (4)	13 (5)	9 (3)	9 (2)	4 (2)	6 (2)	3 (1)	63 (29)
NT	28 (24)	21 (15)	34 (14)	17 (7)	18 (5)	32 (10)	35 (9)	48 (18)	233 (102)
Totals	97 (79)	98 (66)	98 (46)	98 (52)	73 (34)	94 (34)	97 (44)	97 (45)	752 (400)

* Figures in parentheses are the numbers of chloramphenicol-resistant *E. coli* isolates of each serotype.
† Thirty O-serotypes isolated 1 to 6 times in total.

Table 4. *The number of* E. coli *amongst the dominant O-serotypes isolated from stored static slurry according to the pattern of resistance to six antibacterial drugs*

Resistance pattern*	No. of *E. coli* amongst: O-serotype 9	8	19	40	154	21	23	13	25	86	Other O-serotypes	NT† strains	Total isolates	No. of O-serotypes represented
K S Su	—	—	—	—	—	—	—	2	1	—	4	7	14	6
S Su T	5	9	3	—	—	—	7	2	—	2	12	17	57	13
A C S Su	—	—	6	—	—	—	—	—	—	—	2	1	9	2
A S Su T	16	—	1	—	—	1	1	—	—	—	2	4	25	5
K S Su T	7	3	3	12	—	3	5	16	16	—	24	71	160	21
A C K S Su	1	2	—	—	—	1	—	—	—	2	—	5	12	4
A C S Su T	2	—	23	—	—	—	—	—	—	—	7	1	33	5
A K S Su T	14	3	—	5	1	2	7	—	—	—	17	18	67	14
C K S Su T	23	12	4	4	—	—	—	—	1	—	21	31	96	16
A C K S Su T	23	14	4	16	37	18	6	—	—	12	48	62	240	23
Other patterns	6	4	—	4	—	1	—	1	—	1	6	16	39	12
No. of *E. coli*	97	48	44	41	38	26	26	21	18	17	143	233	752	48
Proportion (%) resistant to chloramphenicol	57	65	32	54	97	73	23	—	6	82	57	44	53	

* A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, sulphafurazole; T, tetracycline.
† NT, Non-typable.

Table 5. *The number of* E. coli *serotypes* O9/*biotype* 30, O19/*biotype* 19, O4/*biotype* 22, O154/*biotype* 22 *isolated from stored static slurry according to the pattern of resistance to six antibacterial drugs*

O-serotype	Biotype*	Resistance pattern†	No. *E. coli* isolated at each sampling								
			1	2	3	4	5	6	7	8	Total
9	30	A K Su	—	—	—	—	1	—	—	—	1
		A S Su	—	—	—	—	—	1	—	—	1
		S Su T	—	2	—	—	—	—	—	—	2
		A S Su T	—	2	2	2	8	—	—	—	14
		K S Su T	—	—	—	—	—	1	—	—	1
		A K S Su T	—	—	—	—	—	1	4	2	7
		C K S Su T	1	6	—	3	1	2	—	2	15
		A C K S Su T	—	1	—	1	1	—	-	—	3
		Total	1	11	2	6	11	5	4	4	44
19	19	S Su T	—	—	—	—	—	1	—	—	1
		A C S Su	5	—	—	—	—	—	—	—	5
		A S Su T	—	—	1	—	—	—	—	—	1
		K S Su T	—	—	—	1	—	—	—	—	1
		A C S Su T	5	7	3	2	1	—	—	—	18
		C K S Su T	—	—	—	1	—	—	—	—	1
		A C K S Su T	—	—	—	1	—	1	1	—	3
		Total	10	7	4	5	1	2	1	—	30
40	22	A K T	—	—	1	—	—	1	—	—	2
		K S Su T	1	3	—	—	3	2	1	1	11
		A C K S T	—	—	—	—	—	—	1	—	—
		A C K Su T	—	—	—	—	—	—	—	1	1
		A C K S Su T	—	—	1	2	—	2	4	3	12
		Total	1	3	2	2	3	5	6	5	27
154	22	A K S Su T	—	—	1	—	—	—	—	—	1
		A C K S Su T	—	—	2	8	9	2	3	4	28
		Total	—	—	3	8	9	2	3	4	29

* Hinton, Allen & Linton (1982).

† A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, sulphafurazole; T, tetracycline.

as compared to 43·5% in the non-typable strains. This difference is statistically significant ($P = < 0{\cdot}001$). The proportion of isolates of each of the most prevalent O-serotypes resistant to chloramphenicol was variable and ranged between none and 97·5% (Table 4). The total number of O-serotypes represented in each of the different drug resistance patterns is also listed in Table 4.

Four hundred of the 519 O-typable *E. coli* were biotyped on the basis of their ability to ferment five carbohydrates. Nearly a third of these (130) were accounted for by four O-serotype/biotype combinations. The distribution of these *E. coli* at each sampling, and according to their drug resistance pattern, is set out in Table 5. Five or more resistance patterns were recorded for three of the four O-serotype/biotype combinations while all six of the O-serotype/biotype/resistogram combinations identified 11 times or more were present in four to six of the eight

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slurry samples examined. All *E. coli* serotype O154 examined were of biotype 22, and the fact that all but one had the resistance pattern A C K S Su T suggests the possibility that these isolates were all derived from a single clone of cells (Table 5).

DISCUSSION

Viable *Salm. typhimurium* persisted in small numbers in stored static slurry throughout the seven-week period of observation. This was not unexpected, since salmonellae may persist for nearly 300 days in slurries of farm animal origin (Jones, 1980). The small numbers of *Salm. typhimurium* recorded in this investigation were unlikely to pose a significant threat to farm stock grazing fields dressed with this slurry, since it has been shown that calves only become infected with *Salm. dublin* following the grazing of pasture 18 h after the application of a slurry containing 10^6 *Salm. dublin*/ml but not one containing only 10^3/ml (Taylor & Burrows, 1971).

The number of viable coliforms in the slurry fell in the early period of storage although the population remained reasonably stable thereafter. This suggests that the majority of *E. coli* were either preserved by the cold (the experiment was undertaken during the winter) or the population was maintained by the *E. coli* multiplying at a rate sufficient to replace those that were dying.

Analysis of the structure of the dominant *E. coli* flora, using O-serotyping and biotyping, indicated that certain components of the population remained relatively stable during the seven-week period since the most common O-serotype/biotype combinations were isolated from more than half of the samples examined. Nevertheless, the composition of the total *E. coli* population was extremely complex since not only were a relatively large number of O-serotypes identified (48) but the distribution of R determinants was such as to give a wide range of different patterns to antibacterial drugs amongst many of those O-serotypes (Table 4).

A disturbing feature arising from this investigation was the high level of chloramphenicol resistance amongst the *E. coli*, although the incidence was far from uniform amongst the dominant *E. coli* O-serotypes and was not detected in several of them (Table 4). Interestingly, the proportion of the *E. coli* exhibiting chloramphenicol resistance (55 %) was very similar to that observed in *E. coli* faeces isolated from the faeces of 10 calves reared for five weeks on the farm some six months earlier (49 %) (Linton *et al.* 1981).

Chloramphenicol resistance was always associated with several other R determinants. Its maintenance in the farm environment (the slurry was derived from over 200 calves) was probably due in part to the therapeutic use of drugs other than chloramphenicol, since we were informed that chloramphenicol was not used orally for therapeutic purposes. It is possible that multiply resistant strains of *E. coli* persisted in the calf house between batches, despite disinfection, although these would have been present in small numbers since it has proved difficult to isolate *E. coli* from the wooden crates in which the calves are reared after they have been cleaned (unpublished observations). On the other hand chloramphenicol resistance can be regularly demonstrated amongst the minority *E. coli* in the faeces on the day calves arrive on the farm by the use of BLA supplemented with chloramphenicol

as the medium for primary isolation of the *E. coli* (Linton *et al.* 1981 and unpublished observations). This suggests that the source of many of the chloramphenicol-resistant *E. coli* was probably from outside the farm. However, the intensive nature of veal production, coupled with the use of antibacterial drugs for therapeutic purposes, favours the maintenance of a high level of chloramphenicol resistance amongst the *E. coli* flora of the calves on this farm, since chloramphenicol is always associated with multiple drug resistance. This fact, coupled with protracted survival of the *E. coli* in the slurry, suggests that farms of this type present a potential reservoir of plasmids coding for chloramphenicol resistance in the environment even when this drug is never prescribed for either prophylaxis or the treatment of diseased calves.

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