A Search for Transmissible Pathogenic Characters in Invasive Strains of *Escherichia coli*: the Discovery of a Plasmid-controlled Toxin and a Plasmid-controlled Lethal Character Closely Associated, or Identical, with Colicine V

By H. WILLIAMS SMITH

Houghton Poultry Research Station, Houghton, Huntingdon PE17 2DA

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SUMMARY

Most invasive strains of *Escherichia coli* from man and domestic animals were lethal for chickens and mice. The lethal characteristic was not, in general, transferred when invasive strains were grown in mixed culture with non-pathogenic strains of *E. coli*, although two transmissible plasmids coding for pathogenic properties were discovered.

One plasmid, designated Vir, was found in an *E. coli* strain causing bacteraemia in a lamb. It transferred at high rate to several strains of *E. coli*, including a *rec A*-K12 strain, to *Salmonella typhi*, *Salm. typhimurium* and *Shigella sonnei*. Culture filtrates and, especially, bacterial ultrasonicates of Vir⁺ strains were toxic for chickens, mice and rabbits. The toxin was heat-sensitive, acid-sensitive and non-diffusible. Organisms producing it were agglutinated by specific Vir⁺ antisera; their toxic activity was not neutralized. The transfer factor of the Vir plasmid was *fi*⁺ and could transfer antibiotic-resistance determinants in addition to the Vir determinant.

The other plasmid was first discovered in an *E. coli* strain F120, isolated from an outbreak of bacteraemia in chickens. Organisms of *E. coli* K12 and of other *E. coli* strains acquiring this plasmid during mixed culture with F120 were increased in lethality for chickens and mice; this was associated not with toxic activity but with greater ability to survive in blood and peritoneal fluids. Strain F120 possessed transmissible ColV and ColIb plasmids; increased lethality was closely associated with the ColV plasmid. When the ColV plasmids of another six wild strains of *E. coli* of varied origin were transferred to organisms of *E. coli* K12, the lethality increase was similar to that for ColV transfer from F120. No lethality change accompanied transfer of other Col plasmids. It was concluded that colicine V itself might be responsible for the increased lethality.

Strains of *E. coli* associated with bacteraemia in man and animals commonly produced colicine V.

INTRODUCTION

Several characters possessed by enteropathogenic *E. coli* are controlled by transmissible plasmids. These include the production of enterotoxin (Smith & Halls, 1968; Smith & Linggood, 1971, 1972; Skerman, Formal & Falkow, 1972), K88 antigen (Ørskov & Ørskov, 1966), K99 antigen (Smith & Linggood, 1972) and α -haemolysin (Smith & Halls, 1967). Whereas enteropathogenic strains of *E. coli* are usually confined to the alimentary tract, other pathogenic strains are invasive and produce their harmful effects in other tissues. It seemed worth determining whether any of the properties peculiar to invasive strains were also plasmid-controlled.

The main approach to the problem was to grow invasive strains of *E. coli* in mixed culture

with non-pathogenic strains under conditions providing maximum opportunity for conjugation, and then to determine whether the non-pathogenic strains had become lethal for chickens and mice.

Most of the invasive strains produced a fatal bacteraemia when administered parenterally. No transmissible genetic element was demonstrated for the bacteraemic character of the invasive strains, but a toxin in one of two strains that produced it was controlled by a transmissible plasmid. Also, the production of colicine V, a common feature of invasive strains, was associated with increased lethality. Although, perhaps, not strictly invasive, some *E. coli* strains isolated from cases of cystitis and ulcerative colitis were also examined.

METHODS

Experimental animals. Specified pathogen-free Light Sussex chickens were employed. Unless otherwise stated, they were $3\frac{1}{2}$ weeks old and weighed approximately 300 g when put under experiment. The mice were young adults of the White Swiss breed (Tuck TO) and the rabbits were New Zealand Whites. All the animals were fed *ad libitum* on commercial diets not containing antibiotics.

E. coli strains. The invasive strains had been isolated from man and animals under conditions strongly suggesting a pathogenic role. The majority of the animal strains had been isolated in this laboratory; the others were received from Drs Mary Cooke, F. and I. Ørskov, B. Rowe, R. Sakarzaki and W. J. Sojka. Apart from *E. coli* K12, all the strains classed as non-pathogenic had been isolated from the faeces of healthy men (prefix H), pigs (prefix P) or sheep (prefix S); the antigenic formula of strain H209 was O9; K31; H14 and that of S1 was O78; K80. All strains were maintained on Dorset egg medium at 5 °C.

Culture media and cultural conditions. Nutrient broth contained (g/l): Oxoid 'Lab. Lemco' 10; Oxoid peptone, L37, 10; glucose, 1; NaCl, 5. Its pH was adjusted to 7.5 before autoclaving at 105 °C for 20 min. Except where stated, broth cultures consisted of organisms grown in 10 ml broth for 24 h in a shaking water bath at 37 °C; they contained approximately 1.5×10^9 viable organisms/ml. The addition of 0.36 % agar to nutrient broth constituted soft agar. All cultures in this medium were incubated at 37 °C for 24 h, after which the bacteria and fluids were expressed through muslin by gentle squeezing; the fluids contained approximately 6.0×10^9 viable organisms/ml.

Determination of lethality of strains of E. coli for experimental animals. Unless otherwise stated, broth cultures were injected intravenously (i.v.) into chickens in 0.3 ml amounts (5×10^8 viable organisms) and intraperitoneally (i.p.) into mice in 0.05 ml amounts (7.5×10^7 viable organisms). The animals were observed daily for 4 days and the livers of those that died were cultured on MacConkey agar to confirm recovery of inoculum organisms.

Transfer of lethal characters. Nutrient broth was seeded with 0.02 ml volumes of broth cultures of a lethal strain of *E. coli* and of a streptomycin-resistant mutant of a non-lethal strain – the prospective recipient. The mixed culture was incubated at 37 °C for 48 h without shaking and 0.2 ml inoculated into broth containing 30 µg streptomycin sulphate/ml. After incubation at 37 °C for 24 h, 0.2 ml of the culture with streptomycin was inoculated into more streptomycin-containing broth, the latter incubated as before and then sub-cultured into plain broth. This culture, referred to as the passaged mating culture, was then examined to confirm, as far as possible, that it did not contain donor organisms. For this purpose, and whenever it was necessary to distinguish donor from recipient strains, differences in their growth characteristics, antibiotic sensitivity or antigenic structure were exploited; some of the recipients were *lac*⁻ and others were mutants resistant to nalidixic

acid, spectinomycin or ampicillin in addition to streptomycin. If no donor organisms were found in the passaged mating culture, 0.5 ml was injected i.v. into a chicken and, if the chicken died, its liver was cultured to test that only organisms of the recipient strain were present. If so, the same lethality test was repeated in at least three animals and, if the result was still positive, the passaged mating culture was inoculated onto MacConkey agar to give well-separated colonies on incubation. A number of these colonies was purified by replating and then tested for lethality.

Occasionally, the above procedure was varied. For example, if the donor strain was itself streptomycin-resistant, an ampicillin-resistant or nalidixic acid-resistant mutant of the recipient strain was employed; the mating culture was then passaged through broth containing 30 µg ampicillin or sodium nalidixate/ml. Also, if the donor strain possessed an R factor, the mating culture was sometimes passaged through broth containing one of the antibiotics to which the donor strain was resistant in addition to the antibiotic to which the recipient was resistant.

Colicines. These were identified by the method of Lewis (1968) using the indicator and colicine-producing strains of Professor P. Fredericq of the University of Liege; final identification of the colicines produced by all the bacterial strains employed in comparative lethality tests was achieved by Professor Fredericq himself.

The identification of bacterial strains possessing transfer factors. This was performed by the antibiotic resistance determinant-mobilization test (Anderson, 1965) as described by Smith & Linggood (1970), the presence of a transfer factor being identified by its ability to mobilize a streptomycin/sulphonamide resistance determinant in an *E. coli* K12 strain.

Fertility-inhibiting (fi) character of transfer factors. This was determined by transmitting an F-linked tetracycline-resistance determinant from an *E. coli* K12 strain to another K12 strain possessing the transfer factor under test. The latter strain was then examined for susceptibility to visible lysis by the F-specific phage MS2 (Davis, Strauss & Sinsheimer, 1961).

The estimation of ColV⁺ and ColV⁻ organisms in the blood and liver of chickens and in the blood and peritoneal washings of mice. Chickens were injected i.v. with equal numbers of washed ColV⁺ or ColV⁻ organisms of the same strain of *E. coli*. At intervals viable numbers were estimated in blood removed from wing veins. Viable numbers in the liver were estimated on 1 g organ samples removed at the end of the experiment and ground with sterile sand and 9 ml volumes of phosphate buffer (pH 7.0) in a pestle and mortar. Some chickens were infected with equal numbers of both ColV⁺ and ColV⁻ organisms. Colonies from blood and liver samples of these chickens were spot-inoculated directly onto Petri plates of nutrient agar over which had been spread a diluted culture of *E. coli* K12 to differentiate the ColV⁺ from the ColV⁻ colonies. When colicine V-resistant (*colV^r*) organisms were included in the mixed inoculum given to chickens, their colonies were differentiated from those of ColV⁺ and ColV⁻ ones by their insusceptibility to colicine V and inability to produce it. Mice were also injected i.p. with equal numbers of washed ColV⁺ or ColV⁻ organisms of the same strain, killed 18 h later, and viable numbers estimated in heart blood and washings rinsed from the peritoneal cavity with 1 ml saline. Colonies obtained from mice infected with equal numbers of both ColV⁺ and ColV⁻ organisms were differentiated as those from infected chickens.

The survival of ColV⁺ and ColV⁻ organisms in chicken serum. Broth cultures of ColV⁺ and ColV⁻ organisms of the same strain of *E. coli* were diluted 1 in 20000 in phosphate buffer, and 0.08 ml volumes added to 0.5 ml volumes of chicken serum and incubated in a shaking water bath at 37 °C. At intervals, viable numbers were estimated. Sometimes both kinds of organisms were added to the same sample of chicken serum. In this case,

Table 1. *The lethality for chickens and mice of strains of E. coli from different sources*

Chickens received 0.3 ml volumes of a broth culture intravenously, and mice 0.05 ml intraperitoneally.

Source of <i>E. coli</i>		No. strains tested	No. lethal for:	
Host	Clinical condition		Chickens	Mice
Man	Bacteraemia or meningitis	45	27	36
	Cystitis	9	5	7
	Ulcerative colitis	19	8	9
	Neonatal diarrhoea	19	2	—
Chickens	Bacteraemia	40	39	—
Calves and lambs	Bacteraemia	14	13	—
	Neonatal diarrhoea	7	0	—
Pigs	Neonatal and post-weaning diarrhoea	12	2	—
Man, chickens and pigs	None*	14	2	—

* Isolated from the faeces of healthy individuals.

50 colonies from each of two sets of viable-count plates were examined for colicine production as previously described. The first set were those of organisms present in the serum sample at the commencement of the experiment, and the second set were those prepared when the number of viable organisms in the sample had been reduced by 90 to 95 %. In control tests, samples of sera were heated at 65 °C for 30 min before inoculation to confirm that the serum could support the multiplication of ColV⁺ and ColV⁻ organisms.

Bacterial counts. Viable organisms in broth cultures and tissue fluids were estimated by the method of Miles & Misra (1938) using MacConkey's agar.

Antisera. These were prepared by the multiple i.v. injection of rabbits with live broth cultures.

Statistical analysis. The results of lethality tests were analysed in 2 × 2 contingency tables and examined for significance by the χ^2 test by Mr J. G. Rowell of the A.R.C. Statistics Group at Cambridge University.

RESULTS

The lethality for chickens and mice of strains of E. coli from different sources

Chickens were inoculated intravenously and mice intraperitoneally with strains of *E. coli* associated with different disease conditions in man and domestic animals (Table 1). All except two of the 54 strains isolated from animals with bacteraemia were lethal for chickens and so were over half of the strains from human beings with bacteraemia, meningitis, ulcerative colitis or cystitis; a slightly higher proportion of the human strains were lethal for mice. By contrast, of the strains isolated from the faeces of human beings or animals – whether healthy or suffering from *E. coli* diarrhoea – only a small proportion were lethal for chickens. In the chickens and mice that died in these experiments, death usually occurred within 1 to 2 days of infection and, at autopsy, large numbers of organisms of the infecting strain were cultured from their livers. Supernatant fluids of centrifuged broth cultures of the lethal human strains were injected i.v. into chickens in 1.0 ml amounts and into mice in 0.2 ml amounts; none of the animals died.

Table 2. *The incidence of transfer factors in invasive strains of E. coli*

Source of strains	No. examined	Percentage with transfer factors
Calves and lambs	18	78
Chickens	65	43
Human beings	45	9

Table 3. *The rate of transfer of the Vir plasmid from E. coli s5 to different recipient strains*

Recipient strain	No. examined	Colonies of recipient strains from a mating with s5
		Percentage Vir ⁺ *
<i>E. coli</i> K12	32	37
<i>E. coli</i> K12recA ⁻	30	30
<i>E. coli</i> H209	102	14
<i>E. coli</i> H209K ⁻ †	13	100
<i>E. coli</i> H103	20	25
<i>Shigella sonnei</i>	20	60
<i>Salmonella typhimurium</i>	24	17

* Assessed by lethality for chickens (1/colony tested) of approx. 5×10^8 viable organisms i.v. from broth cultures grown from single colonies of recipients. Only those broth cultures of *Salm. typhimurium* that produced lesions characteristic of Vir activity were recorded as Vir⁺.

† K antigen lost.

The incidence of transfer factors in invasive strains of E. coli

E. coli strains associated with bacteraemia, cystitis and ulcerative colitis in man and bacteraemia in calves, lambs and chickens were examined for transfer factors (Table 2); none of these strains possessed characters known to be transferable, such as antibiotic resistance and colicine and haemolysin production. Transfer factors were common in the animal strains, being five times more frequent in chicken and nine times more frequent in calf and lamb strains than in the human strains.

The transfer of lethality from invasive strains of E. coli to non-lethal strains

In all, 550 passaged mating cultures were tested for lethality in chickens. These were derived from one or other of five recipient strains of *E. coli* that had been grown in mixed culture with one or other of 200 potential donor strains that were lethal for chickens and that had originated from human beings (40), calves and lambs (20), and chickens (140). The five recipient strains, K12, P13, H209, H103 and S1, were chosen for use principally because they were good recipients of R factors, did not possess transfer factors and were always non-lethal under the conditions of test; if employed in increased dosage and/or in 2-week-old (instead of 3½-week-old) chickens, some strains exhibited a low degree of lethality (see Table 6 for example). During culture in broth, strain H209 usually gave rise to K⁻ variants so that passaged mating cultures of this strain consisted of mixtures of K⁺ and K⁻ organisms. The only chickens that died were those given passaged mating cultures grown with either of two of the 200 potential donor strains. Subsequent investigations, described below, indicated that these two strains possessed transmissible plasmids coding for lethal

characters. One of the plasmids was provisionally designated Vir, while the other, on account of its close association with colicine V production, was not named.

The Vir plasmid

Strain s5 (antigenic formula O15:K?:H21), the *E. coli* strain possessing the Vir plasmid, had been isolated from the blood of a lamb that had died from an *E. coli* bacteraemia. The presence of the plasmid was first suspected when the passaged mating culture of the recipient strain H209 grown with s5 proved lethal for chickens. A further eight matings between these two strains all gave rise to passaged mating cultures of H209 that were chicken-lethal. The examination of single colonies from one passaged mating culture and from matings of s5 with other recipient strains, including *E. coli* K12recA⁻, revealed a high rate of transfer of lethal activity, the highest rate being to K⁻ organisms of H209 (Table 3). Unlike other recipients, the *Salmonella typhimurium* strain was lethal for chickens. However, its Vir⁺ forms could be distinguished from Vir⁻ forms by the characteristic lesions Vir⁺ organisms produced in chickens (see below).

Vir transfer was not detected during culture of a H209K⁻ strain in broth containing 5 ml of a membrane filtrate (Millipore; 450 nm) of a broth culture of strain s5. The latter was not latently infected with bacterial viruses active on H209, *E. coli* K12 or any of the other strains tested. It possessed an fi⁺ transfer factor (Tra) detected by antibiotic resistance determinant mobilization tests in all of five Vir⁺ strains of H209 obtained from a mating with s5 but not in five Vir⁻ strains obtained from the same mating; four Vir⁺ and four Vir⁻ strains of *Salm. typhimurium* obtained in a similar manner were Tra⁺ and Tra⁻ respectively, as were 12 Vir⁺ and 20 Vir⁻ strains of *E. coli* K12. Vir⁻ forms were not detected in a H209 Vir⁺ strain during acridine orange treatment. Vir⁻ forms of *Salm. typhimurium*, however, were found in the tissues of chickens inoculated with Vir⁺ organisms of this strain.

Serological studies on Vir⁺ bacteria

Antisera were prepared against *E. coli* s5 and against Vir⁺ and Vir⁻ forms of *E. coli* H209 and H209K⁻, those prepared against the Vir⁺ forms of H209 and H209K⁻ being subsequently absorbed with live organisms of the corresponding Vir⁻ forms. Slide tests with these antisera revealed that s5, and the Vir⁺ but not the Vir⁻ forms of H209 and H209K⁻, possessed a common antigen. In these tests, bacterial suspensions that had been held at 60 °C for 30 min agglutinated more slowly than live suspensions; suspensions held at 100 °C for 30 min did not agglutinate. The antigen was also demonstrated in all of 14 Vir⁺ strains of H209 or H209K⁻ obtained from several matings with s5 but not in any of eight Vir⁻ strains obtained from these matings. From other matings, all of four strains of *Salmonella typhimurium*, 12 strains of *Shigella sonnei* and five strains of *E. coli* H103, all classed as Vir⁺ by lethality tests, possessed the antigen whereas all of four strains of *Salm. typhimurium*, eight strains of *Sh. sonnei* and 15 strains of H103 classed as Vir⁻ did not.

Because of the complete correlation found between lethality and agglutination tests, subsequent attempts to transfer Vir were monitored by the slide agglutination test. Vir was transferred to a chicken bacteraemia strain of *E. coli* (O2:K1) and to Vi⁺ and Vi⁻ forms of *Salmonella typhi*, Vir⁺ colonies derived from the Vi⁺ form agglutinating poorly with Vi⁺ antiserum. Transfer to the *E. coli* strain was confirmed by lethality tests; these tests were not performed for the *Salm. typhi* strain. Transfer was not detected to 12 strains of *E. coli* enteropathogenic for man, calves or pigs, to three chicken bacteraemia strains, to three strains of *Shigella flexneri* or to one strain each of *Salm. dublin*, *Salm. cholerae-suis*, *Salm. pullorum*, *Proteus rettgeri*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.

Table 4. *The lethality for chickens, rabbits and mice of filtrates and ultrasonicates of broth cultures of Vir⁺ and Vir⁻ forms of E. coli H209K⁻*

Forms of H209K ⁻	Material injected i.v.	Dose (ml)	No. died/no. injected		
			Chickens	Rabbits	Mice
Vir ⁺	Culture filtrate	5.0	—	0/2	—
		0.4	3/3	—	0/8
		0.2	1/3	—	—
		0.1	0/3	—	—
	Ultrasonicate	0.8	—	2/2	—
		0.4	—	1/3	1/1
		0.1	—	—	3/3
		0.05	3/3	—	0/3
		0.025	3/3	—	—
		0.012	3/3	—	—
		0.006	5/6	—	—
		0.003	1/3	—	—
		0.002	0/3	—	—
		—	—	—	—
		—	—	—	—
		—	—	—	—
		—	—	—	—
		—	—	—	—
Vir ⁻	Culture filtrate	5.0	0/2	—	—
	Ultrasonicate	5.0	0/3	0/2	—
		1.0	0/3	—	—
		0.4	—	—	0/3

Culture filtrates were membrane-sterilized supernatants from centrifuged broth cultures; ultrasonicates were membrane-sterilized supernatants from broth cultures centrifuged after ultrasonic treatment (M.S.E., 100 W, 3/4 in probe for 5 min with cooling).

The incidence of Vir activity in strains of E. coli

By slide agglutination tests, the Vir antigen was detected in only one of 190 epidemiologically-unrelated human (50), bovine (74), ovine (38) and porcine (28) strains of *E. coli*; these strains had been isolated from the faeces of healthy individuals or from cases of diarrhoea or bacteraemia. The one Vir⁺ strain, like s5, had been isolated from the blood of a lamb suffering from *E. coli* bacteraemia. It had the same antigenic formula as *E. coli* s5 and produced lesions typical of Vir activity when inoculated into chickens. Although it possessed a transfer factor, all attempts to transfer its Vir activity failed.

The lethality for chickens, rabbits and mice of filtrates and ultrasonicates of broth cultures of Vir⁺ and Vir⁻ organisms

Different amounts of sterile membrane filtrates and ultrasonicates of broth cultures of *E. coli* H209K-Vir⁺ and Vir⁻ were injected i.v. into chickens, rabbits and mice (Table 4). The filtrates and ultrasonicates of the Vir⁻ culture produced no ill effect. By contrast, both preparations from the Vir⁺ culture were lethal – especially the ultrasonicates; chickens were more susceptible than rabbits and mice. Two groups of three chickens given 0.4 ml of the Vir⁺ ultrasonicate i.p. or i.m. died; a group given 5.0 ml orally remained well.

Results similar to those above were obtained with Vir⁺ and Vir⁻ forms of other strains, provided similar cultural conditions were employed; in conditions of partial or strict anaerobiosis, no toxic effect was detected. Live cultures, culture filtrates and ultrasonicates of all the Vir⁺ strains produced the same syndrome when injected i.v. into chickens, rabbits and mice. Depending on the dose, death occurred within 6 to 24 h. Always present in dead chickens were large amounts of clear yellow serous fluid in the abdominal cavity and pericardial sac, and characteristically pale-coloured livers. Pale livers were common, too, in the dead rabbits and mice, but not the excess fluid. The livers of four chickens, one

Table 5. *The effect of temperature and pH on lethality for chickens of filtrates of a broth culture of E. coli H209K⁻Vir⁺*

Filtrate temperature* (°C)	Lethality of filtrate*	Filtrate pH†	Lethality of filtrate‡
100.0	0/3	10.0	3/3
80.0	0/3	9.0	5/5
60.0	0/9	8.0	2/2
57.5	0/8	7.0	10/10
55.0	0/8	6.0	2/2
52.5	0/11	5.0	0/9
50.0	0/13	4.0	0/3
47.5	2/5	—	—
45.0	12/13	—	—
37.0	8/8	—	—

* Temperature to which the filtrate, at pH 7.4, was exposed for 30 min.

† Adjusted to pH 7.0 before injection.

‡ No. of chickens that died/no. inoculated i.v. with 1.0 ml of filtrate.

mouse and one rabbit were examined histologically by Dr J. E. T. Jones, who reported that the main lesion present was focal necrosis of the liver cells with, in the case of the chicken livers, a pronounced and widespread distension of the sinusoids with erythrocytes.

The effect of heat, pH and dialysis on the lethality of filtrates and ultrasonicates of Vir⁺ cultures

Broth culture filtrates of *E. coli* H209K⁻Vir⁺ were held at different temperatures and pH values and then injected i.v. into chickens (Table 5). Holding at 50 °C or more for 30 min, or at pH 5.0 or lower for 1 h, rendered the filtrate innocuous. Similar results were obtained with sterile ultrasonicates of broth cultures of this strain.

Using Visking seamless cellulose tubing, 20 ml of a broth culture filtrate of H209K⁻Vir⁺ were dialysed against 20 ml of broth for 2 days. Two chickens were then injected i.v. with 2.0 ml volumes of the diffusate; both remained well. Dialysis was continued for a further day against 1 l broth, and 0.2 ml volumes of the dialysate were then injected i.v. into two chickens. Both died, indicating that little or none of the filtrate toxicity had been lost during dialysis.

The effect of antiserum on Vir activity

Broth culture filtrates of *E. coli* H209K⁻Vir⁺ in 0.4 ml amounts were incubated at 37 °C for 1 to 3 h with 0.4 to 2.0 ml volumes of an antiserum against live broth cultures of this strain, and the mixtures then injected i.v. into four chickens; all four died, exhibiting lesions characteristic of Vir activity. Similarly, failure of antiserum protection was noted with sterile ultrasonicates in chickens (0.02 ml ultrasonicate + 0.4 to 2.0 ml antiserum) and in mice (0.2 ml ultrasonicate + 0.2 to 0.4 ml antiserum).

The influence of the Vir plasmid on the pathogenicity of Salm. typhimurium for chickens

Two groups of 25 one-day-old chickens were given orally 0.5 ml of a broth culture of either a Vir⁺ or a Vir⁻ form of *Salm. typhimurium*. Very little difference was noted in the course of the disease between the two groups: by the sixth day after infection, 22 chickens in the Vir⁺ group and 18 in the Vir⁻ group had died; the results of i.p. administration of

Table 6. *The lethality for chickens of strains of E. coli possessing different combinations of the ColV and Ib plasmids of E. coli F120*

Strain tested	Origin of its plasmids	No. of chickens injected*	Percentage of chicken deaths
H209 Col V ⁺ Ib ⁺	2nd mating with F120	70	61
H209 Col V ⁺	1st mating with F120	118	59
H209 Col V ⁺	3rd mating with F120	37	51
H209 Col V ⁺	Mating with <i>E. coli</i> K12 Col V ⁺ whose Col V plasmid had been acquired from F120	25	52
H209 Col Ib	1st mating with F120	30	13
H209 Col Ib	3rd mating with F120	37	16
H209 Col V ⁻ (Col V ⁺)	1st mating with F120, Col V plasmid acquired then lost	70	10
H209 Col ⁻	1st mating with F120, no Col plasmids acquired	92	10
H209 Col ⁻	2nd mating with F120, no Col plasmids acquired	30	7
P10 Col V ⁺ Ib ⁺	Mating with F120	30	100
P10 Col Ib ⁺	Mating with F120	30	3
P10 Col ⁻	Mating with F120, no Col plasmids acquired	30	3
K12 Col V ⁺ Ib ⁺	Mating with P10 Col V ⁺ Ib ⁺ whose Col plasmids had been acquired from F120	30	80
K12 Col V ⁺	Mating with F120	22	86
K12 Col V ⁺	Mating with H209 Col V ⁺ whose Col V plasmid had been acquired from F120	110	87
K12 Col V ⁺	6th successive transfer of Col V plasmid between strains of K12	30	73
K12 Col Ib	Mating with H209 Col Ib ⁺ whose Ib plasmid had been acquired from F120	30	7
K12 Tra ⁺	Mating with H209 Col V ⁺ (transfer factor acquired)	30	15
K12 Col ⁻	Unmated recipient strain	148	7

* Two-week-old chickens received i.v. 7.5×10^8 viable organisms from broth cultures of the H209 strains or 3.0×10^9 viable organisms from soft agar cultures of the P10 and K12 strains.

Vir⁺ and Vir⁻ cultures to three-day-old chickens were also similar. About half of the *Salm. typhimurium* organisms isolated from dead chickens given the Vir⁺ form had lost the Vir plasmid.

The ColV plasmid

The discovery that a plasmid coding for colicine V production was associated with lethality stemmed from an observation that all of four different passaged mating cultures of *E. coli* H209 that had been grown with an O78:K80 strain of *E. coli*, F120, were lethal for chickens on i.v. injection. *E. coli* F120, which had been associated with an outbreak of bacteraemia in broiler chickens, produced two colicines, Ib and V. Both colicines were controlled by transmissible plasmids which transferred at a high rate from F120 to *E. coli* H209 and K12; the exconjugants had acquired one or other, or both, of the plasmids. Of 37 colonies of H209 cultured from one of these matings, 16 had acquired the ColIb plasmid of F120, three the ColV plasmid and two had both plasmids. When the K⁻ form of H209 was used as recipient, 13 of 34 colonies cultured had acquired ColIb only and the remainder had either ColV or both plasmids. The Col plasmid status of the colonies could easily be ascertained by spot-inoculating them on to 'lawns' of *E. coli* K12, K12 ColIb⁺ and K12ColV⁺ on nutrient agar. Neither plasmid was completely stable in H209, and Col⁻ forms could be obtained without difficulty from Col⁺ forms; these Col⁻ forms were devoid of transfer factors. The transfer factors of the ColV plasmid were classed as fi⁺, although one of

Table 7. *The lethality for mice of forms of E. coli H209 that had acquired the ColV or Ib plasmids of E. coli F120*

Form of H209 administered	No. of mouse deaths
ColV ⁺	68
ColIb ⁺	47
Col ⁻ (ColV ⁺)*	33

* ColV plasmid and transfer factor acquired during mating with F120, then lost. Each of a group of 100 mice was given i.p. 5×10^7 viable organisms.

50 ColV⁻ colonies of *E. coli* K12 obtained from one mixed culture of this strain and H209 ColV⁺ was fully susceptible to the MS2 phage; the other 49 were Tra⁻.

The transfer of ColIb and ColV from E. coli F120 to other strains of E. coli: its effect on the lethality of these strains for chickens and mice

Chickens, 2 weeks old, were injected i.v. with Col⁺ and Col⁻ forms of *E. coli* H209, P10 and K12, obtained from matings of these strains with *E. coli* F120 or with strains to which the ColIb and ColV plasmids of F120 had been transferred. In these (Table 6) and subsequent infection experiments a suitably high mortality rate was achieved by using 2-week-old in place of 3½-week-old chickens, and by substituting soft agar cultures, with their higher bacterial content, for broth cultures of the *E. coli* K12 and P10 strains. Most deaths occurred 1 to 3 days after inoculation, no characteristic lesions being noted at autopsy. All the strains acquiring the Col V plasmid of F120, but not its Ib plasmid, were more lethal than the Col⁻ strains from which they were derived. Also, loss of Col V from the H209 strain was accompanied by loss in lethality, while the acquisition of only the transfer factor of Col V by *E. coli* K12 was not accompanied by a significant increase in lethality. The increased lethality associated with possession of the ColV plasmid was still exhibited by the last recipient after six successive transfers between K12 strains. Several other Col⁻, ColIb⁺ and ColV⁺ forms of *E. coli* H209 and K12 obtained from other matings of these strains with F120 were also tested in smaller groups of chickens; the results were similar to those in Table 6.

Mice were inoculated i.p. with forms of *E. coli* H209 that had or had not acquired the ColV or Ib plasmids of *E. coli* F120 (Table 7). The mortality rate in the group given the ColV⁺ form was much higher than in that given the Col⁻ form ($P < 0.001$); mortality in the group given the ColIb⁺ form was only slightly higher ($0.05 < P < 0.1$). In all, of 130 mice given the ColV⁺ form, 92 died; of 130 mice given the ColV⁻ form, 38 died.

The transfer of ColV and other Col plasmids from enterobacterial strains to E. coli K12: its effect on the lethality of the K12 strain for chickens

Groups of 30 two-week-old chickens were injected i.v. with a strain of *E. coli* K12 to which had been transferred ColV and other Col plasmids from enterobacterial strains (Table 8). Apart from differing in other respects, all seven ColV donor strains were epidemiologically unrelated; six had been isolated from cases of bacteraemia (two in humans and four in chickens), and one had been isolated from the faeces of a healthy pig. Four of these donor strains possessed transmissible Col plasmids in addition to ColV, and these co-transferred with ColV to the K12 strain. The K12 strains possessing plasmids similar, in the main, to the additional plasmids possessed by the four donor strains were included in the tests principally to determine whether the presence of the additional plasmids

Table 8. *The lethality for chickens of an E. coli K12 strain that had acquired ColV and other plasmids from different enterobacterial strains*

Plasmids acquired by the K12 strain under test	Plasmid donor strains	No. of deaths following infection with the K12 strain†
ColV, ColE1, ColIa	<i>E. coli</i> H261	27
ColV, ColIa	<i>E. coli</i> H247	27
ColV, ColIa	<i>E. coli</i> F157	26
ColV, ColIb	<i>E. coli</i> F107	21
ColV	<i>E. coli</i> F120	26
ColV	<i>E. coli</i> P72	27
ColV	<i>E. coli</i> F70	25
ColE1, ColIa	Paracolon	5
ColIa	Paracolon	2
ColIb	<i>E. coli</i> F120	2
ColI	<i>Salm. typhimurium</i> (Meynell, 1968)	4
ColE1	<i>E. coli</i> F105	1
ColE1 (F ⁺)*	<i>E. coli</i> B165 and K12F ⁺	4
ColE1 (F ⁻)*	<i>E. coli</i> B165	4
F	<i>E. coli</i> K12F ⁺	1
None	—	3

* The non-transferring ColE1 plasmid of *E. coli* B165 had been transferred to the K12 strain by transfer factor F; the E1 (F⁺) strain had also acquired F but the E1 (F⁻) strain had not.

† Two-week-old chickens, 30/group, each received i.v. 3.0×10^9 viable organisms from soft agar cultures. Strains isolated from human beings have the prefix H; from chickens, F; from cattle, B; and from pigs, P.

in the four strains influenced their pathogenicity. The K12F⁺ strain was tested mainly because the two forms of the K12 strain that had received ColV plasmids from *E. coli* F70 and P72 had become, as a result, fully susceptible to the MS2 phage, indicating that their transfer factor was probably F. The seven forms of the K12 strain acquiring ColV plasmids from the different *E. coli* strains had similar virulence for chickens and this was significantly higher than that of the K12 strain itself or its forms with other Col plasmids or F.

Mice in groups of 60 were each inoculated i.p. with 5×10^7 viable organisms of *E. coli* H209 or with forms of this strain that had acquired the ColI, Ia, Ib (from *E. coli* F107, not F120) and the ColE1 plasmids listed in Table 8; there was no significant difference between the mortality rate in the groups given the Col⁺ forms of H209 and the group given the H209 strain itself.

During the search for the wild ColV⁺ *E. coli* strains used in the above experiments, 179 bacteraemia strains of *E. coli* were examined for colicine production. Of 38 human strains, seven produced colicine V and eight produced other kinds of colicine. The corresponding proportions were: for 12 bovine strains, five with ColV and three with other colicines; for 12 sheep strains, three with ColV and three with other colicines; and for 117 chicken strains, 78 with ColV and 18 with other colicines.

*The lethality for chickens of sterile preparations of
cultures of ColV⁺ E. coli*

Ultrasonicates of bacterial suspensions expressed from soft agar cultures of ColV⁺ forms of *E. coli* K12 and H209 and rendered organism-free by membrane filtration were injected i.v. in 5 ml amounts, into eight 2-week-old chickens; none died. No deaths occurred in

Table 9. *Recovery of viable organisms from the blood and livers of chickens at intervals after inoculation of ColV⁺ or ColV⁻ forms of E. coli K12*

Form inoculated	Chicken no.	10 ⁻⁵ × No. of organisms/ml, in:			
		Blood at			Liver at 24 h
		3 h	6 h	24 h	
ColV ⁺	1	170	200	1500	5200
	2	580	480	1200	1000
	3	300	500	600	2200
	4	300	450	280	6500
	5	300	400	200	570
ColV ⁻	6	300	100	27	1200
	7	400	300	18	1500
	8	450	300	15	170
	9	150	150	4	450
	10	170	80	2	400

For each form, doses of 3.0×10^9 viable organisms were inoculated i.v. into groups of 5 two-week-old chickens which were killed 24 h later.

any of 14 chickens injected i.v. with 5 ml volumes of broth cultures of these strains killed either by chloroform or by incubation at 37 °C for 4 h after addition of 100 µg neomycin/ml culture. The ColV⁺ organisms employed in these and in subsequent experiments had acquired their ColV plasmid from *E. coli* F120.

The concentration of ColV⁺ and ColV⁻ organisms in the tissues of infected chickens and mice

The blood and liver concentrations of ColV⁺ and ColV⁻ organisms at intervals after i.v. injection of chickens were estimated (Table 9). So was the ratio of ColV⁺ and ColV⁻ organisms, and sometimes *colV^r* organisms (colicine V-resistant mutants), determined in chickens receiving mixtures of equal numbers of organisms (Table 10). In both experiments, higher concentrations of ColV⁺ than of ColV⁻ organisms or, where applicable, of *colV^r* organisms, were usually found in the blood – especially 24 h after inoculation. Slightly higher concentrations of ColV⁺ than of the other organisms were sometimes found in the liver. In similar experiments in mice, higher concentrations of ColV⁺ than of ColV⁻ organisms of *E. coli* H209 were found in the peritoneal fluid and blood 18 h after i.p. injection (Tables 11 and 12).

The results of giving mixtures of organisms to chickens and mice might have been influenced by colicine V production and/or by ColV transfer occurring *in vivo*. The possibility in broth suspensions of colicine production by ColV⁺ organisms suppressing ColV⁻ organisms and/or of ColV transfer was assessed; 10 ml volumes of broth were inoculated with 0.1 ml amounts of broth cultures of ColV⁺ and ColV⁻ organisms of *E. coli* H209 and incubated at 37 °C for 24 h. Of 100 colonies sampled from this mixture, 58 were ColV⁺ at 0 h and 50 were ColV⁺ at 24 h. In a similar experiment with ColV⁺ (nalidixic acid-resistant mutants) and ColV⁻ (ampicillin resistant) organisms of *E. coli* K12, 39 of the 100 colonies obtained from the mixed culture at 0 h were ColV⁺; at 24 h 49 ColV⁺ were obtained, the slight increase probably being accounted for by the fact that eight of the 49 colonies consisted of *amp^r* organisms that had acquired the ColV plasmid.

Table 10. The ratio of *ColV*⁺, *ColV*⁻ and *colV*^r* organisms of *E. coli* strains H209 or K12 in the blood and livers of chickens at intervals after inoculation of equal numbers of these organisms

Organisms inoculated	Time after inoculation (h)	Tissue examined	No. of colonies† that were:		
			<i>ColV</i> ⁺	<i>ColV</i> ⁻	<i>colV</i> ^r
<i>ColV</i> ⁺ and <i>ColV</i> ⁻ forms of H209	0.05	Blood	90	60	—
	3	Blood	113	37	—
	6	Blood	117	33	—
	24	Blood	150	0	—
	24	Liver	104	46	—
<i>ColV</i> ⁺ and <i>ColV</i> ⁻ forms of K12	0.05	Blood	78	72	—
	3	Blood	114	36	—
	6	Blood	118	32	—
	24	Blood	141	9	—
	24	Liver	92	58	—
<i>ColV</i> ⁺ , <i>ColV</i> ⁻ and <i>colV</i> ^r forms of K12	0.05	Blood	39	55	56
	3	Blood	90	23	37
	6	Blood	97	23	30
	24	Blood	134	1	15
	24	Liver	60	36	54

* *colV*^r = colicine V-resistant mutants of *E. coli* K12.

† Three chickens were inoculated i.v. with mixtures of 2×10^8 washed viable organisms of each pair or trio of strains; 50 colonies from each chicken sample were tested (i.e. 150 in all).

Table 11. Viable numbers of organisms in the peritoneal washings and blood of mice 18 h after inoculation with *ColV*⁺ or *ColV*⁻ forms of *E. coli* H209

Form inoculated	Mouse no.	$10^{-3} \times$ no. of organisms/ml of:	
		Peritoneal washings*	Blood
<i>ColV</i> ⁺	1	2 000 000	15 000
	2	8 000 000	10 000
	3	2 500 000	10 000
	4	2 500 000	6 000
	5	2 000 000	1 000
	6	3 000 000	600
<i>ColV</i> ⁻	7	700 000	1 250
	8	7 500	100
	9	2 000	2
	10	1 750 000	1
	11	15	< 1
	12	5	< 1

For each form, doses of 5×10^7 viable organisms were inoculated i.p. into groups of 6 mice.

* The peritoneal washings were obtained by rinsing out the abdomen with 1 ml saline.

The survival of ColV⁺ and *ColV*⁻ organisms in chicken serum

Fresh samples of serum, each taken from a different healthy 4-week-old chicken, were inoculated with mixtures of diluted broth cultures of *ColV*⁺ and *ColV*⁻ or *colV*^r organisms of *E. coli* K12 and H209 and incubated at 37 °C. The ratio of the two kinds of organism in each sample was determined at the start and at the time when the bacterial concentration had decreased by more than 90 % (Table 13). The *ColV*⁺ organisms of *E. coli* K12 survived much better in all six samples than did the *ColV*⁻ or *colV*^r organisms. A similar trend was

Table 12. *The ratio of ColV⁺ and ColV⁻ organisms of E. coli H209 in the peritoneal washings and blood of mice 18 h after inoculation of equal numbers of both organisms*

Mouse no.	No. of ColV ⁺ colonies amongst 100 colonies cultured from:	
	Peritoneal washings	Blood
1	100	100
2	100	100
3	100	100
4	100	99
5	100	98
6	100	98
7	100	97
8	92	100

Doses of 2.5×10^7 viable organisms of both strains were inoculated i.v. into each of 8 mice which were killed 18 h later.

Table 13. *The comparative survival of ColV⁺, ColV⁻ and colV^r organisms of E. coli K12 or H209 in samples of chicken serum inoculated with mixtures of these organisms*

Serum samples inoculated with organisms of	No. of serum sample	No. of ColV ⁺ colonies among 50 colonies cultured at:	
		0 h	1-2 h*
ColV ⁺ and ColV ⁻ K12	1	21	35
	2	21	44
	3	21	48
	4	21	46
	5	21	41
	6	21	43
ColV ⁺ and colV ^r K12	1	14	45
	2	14	42
	3	14	40
	4	14	34
	5	14	39
	6	14	32
ColV ⁺ and ColV ⁻ H209	1	23	34
	2	23	45
	3	23	46
	4	23	39
	5	23	39
	6	23	45

* At this time the concentration of viable organisms was < 10 % of the approx. 10^4 /ml* present at 0 h.

noted for the ColV⁺ and ColV⁻ organisms of *E. coli* H209 (ColV⁺ and ColV⁻ organisms of K12 and H209 survived equally well as mixtures in one of the serum samples that had been heated to 65 °C for 30 min, the concentration of both kinds of organisms increasing about tenfold during a 3 h incubation period).

Next, serum samples were inoculated with either ColV⁺ or ColV⁻ organisms of *E. coli* K12. The concentration of viable ColV⁻ organisms in the six samples decreased by 99 to 100 % (median 99.5 %) during 1 h incubation, whereas the concentration of viable ColV⁺

organisms decreased by only 94.5 to 99 % (median 97.5 %); for any particular sample of serum, the concentration of surviving ColV⁻ organisms at this time was 3 to 12 times less than that of ColV⁺ organisms.

Agglutination tests between ColV⁺ and ColV⁻ organisms and antisera

Antisera were prepared against *E. coli* F120 and against forms of *E. coli* H209 and K12 with and without the ColV plasmid of F120. These five antisera were then employed in tube O-agglutination tests against F120 and forms of *E. coli* H209, *E. coli* P13 and *Shigella sonnei* both with and without the ColV plasmid of F120; *E. coli* K12 organisms were not employed because of their rough nature. The tests were repeated by the slide method using live bacterial suspensions. None of the strains that had acquired the ColV plasmid of F120 had acquired an antigen by these tests.

DISCUSSION

The results indicate that the specific toxic activity of *E. coli* s5 is controlled by a transmissible plasmid designated, provisionally, Vir. Because of its high susceptibility to heat and low pH and its failure to dialyse, the toxin is probably protein in nature. Furthermore, the agglutination of Vir⁺ organisms by specific antiserum suggests that Vir may be a surface antigen. If so, it is not an internationally recognized one (I. Ørskov, W.H.O. *Escherichia coli* Centre, Copenhagen, personal communication). Although the organisms were agglutinated, their toxic activity was not neutralized by antiserum because pretreated culture filtrates and ultrasonicates were fully lethal for experimental animals. This, of course, is not an unusual phenomenon – some endotoxins, for example, resemble Vir in this respect. Even so, the possibility that the apparent identity of the agglutinogen and the toxin of Vir⁺ organisms is merely a reflexion of two different genes being located on the same plasmid cannot be entirely overlooked.

Vir⁺ strains of *E. coli* appear to be uncommon in nature, only two strains being found amongst the 190 examined from man and domestic animals; both were isolated from sheep, which are rarely given antibiotics orally. Had the Vir plasmid originated in the *E. coli* flora of a species of domestic animal frequently given antibiotics orally, e.g. pigs, the plasmid might well have become more common because its transfer factor can transfer antibiotic resistance determinants in addition to the Vir determinant.

Although ultrasonicates and culture filtrates of Vir⁺ organisms were lethal for experimental animals, especially chickens, toxin production was not detected, for example, under anaerobic or partially anaerobic conditions. The question arises whether toxin is produced in significant amounts *in vivo*. Certainly the acquisition of the Vir plasmid by the *Salm. typhimurium* strain did not lower the survival rate of infected chicks, possibly because of the comparative instability of the plasmid in this particular strain.

In all the transfer experiments involving the ColV plasmid of *E. coli* F120, including that of six consecutive transfers from one strain of *E. coli* K12 to another, the subsequent animal inoculation studies revealed that the acquisition of ColV was always accompanied by an increase in lethality. This was not so with strains that had apparently only acquired the transfer factor of the ColV plasmid or that had only acquired the ColIb plasmid of F120. Also, spontaneous loss of the ColV plasmid by a recipient strain was accompanied by a return to normal lethality. Thus it seems likely that the genetic elements responsible for this increased lethality and for colicine V production in F120 are located on the same plasmid. The subsequent observation of lethality increase on transfer of ColV plasmids to *E. coli* K12 from another six wild ColV⁺ strains of *E. coli*, that differed widely in their origins, indicates

that there is a general association between the genes for increased lethality and those for colicine V production. In fact, colicine V itself may well be responsible for the increased lethality of the organisms that produce it.

Heatley & Florey (1946) and Braude & Sieminski (1964, 1965) found purified preparations of colicine V to be non-toxic for mice. Preparations used by Hutton and Goebel (1961), however, were toxic, but it is possible that the O antigen which their preparations still contained might have accounted for this. The results of the agglutination tests in the present studies indicate that genes controlling O antigen production in F120 had not been transferred with those controlling colicine V production. Also, the large doses of ultrasonicates, culture filtrates and dead bacteria of ColV⁺ recipient strains given to chickens were harmless. It therefore seems improbable that the increased lethality of the ColV⁺ strains was due to a direct toxic effect.

The recovery pattern of ColV⁺ and ColV⁻ organisms in chickens or mice revealed that the ColV⁺ organisms survived better in, or were removed less rapidly from, the blood and peritoneal fluids than the ColV⁻ organisms. This may well be the reason for their greater lethality. The bactericidal activity of serum is probably involved to some degree because ColV⁺ organisms survived better than the corresponding ColV⁻ organisms in chicken serum in the *in vitro* tests.

Whether or not the increased lethality possessed by ColV⁺ organisms is indeed due to the colicine they produce cannot be decided. However, from the practical point of view, colicine V production, at least when plasmid-controlled, should be regarded as a pathogenic characteristic. It is noteworthy that production of this colicine was a common feature of strains of *E. coli* associated with bacteraemia in man and domestic animals.

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Note added in proof. Four of 30 antibiotic sensitive O78:K80 strains of *E. coli* isolated from calves with bacteraemia have been found to produce Vir. Although the four strains possessed transfer factors, it was only possible to transmit Vir from two of them.

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