Vero Cell Toxins in *Escherichia coli* and Related Bacteria: Transfer by Phage and Conjugation and Toxic Action in Laboratory Animals, Chickens and Pigs

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Sixty-eight of 519 strains of *Escherichia coli* and six of 10 strains of *Pseudomonas aeruginosa* produced toxins acting on Vero cells (VT⁺); all of 63 *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter* and *Proteus* strains were VT⁻. Most of the VT⁺ *E. coli* strains were from weaned pigs suffering from oedema disease and/or diarrhoea and belonged to serogroups O141:K85,88, O141:K85, O138:K81, and O139:K82; six VT⁺ *E. coli* strains were from diarrhoeic human babies, four of serogroup O26 and two of serogroup O128. The VT genes in two of the O26 strains and in the O128 strains were located in the genome of the phages with which they were lysogenized. One O141:K85,88 pig *E. coli* strain transferred its VT genes, probably by conjugation, to *E. coli* K12. The VTs of the human *E. coli* strains, the pig *E. coli* strains and the *P. aeruginosa* strains were antigenically different from each other; unlike the others, the *P. aeruginosa* VT was heat-resistant. Cell-free preparations of cultures of *E. coli* K12 to which the VT genes of the four human *E. coli* strains had been transferred caused fluid accumulation in ligated segments of rabbit intestine. Inoculated intravenously, they were lethal for mice and rabbits; similar preparations of *E. coli* K12 to which the VT genes of the pig *E. coli* strain had been transferred produced a disease in pigs that clinically and pathologically resembled oedema disease.

INTRODUCTION

Konowalchuk *et al.* (1977) found that some *Escherichia coli* strains synthesized a toxin (VT) that produced morphological changes in Vero cells different from those produced by the 'classical' heat-labile enterotoxin (LT); the cell response to VT was cytotoxic whereas that to LT was cytotoxic. Most of their VT⁺ strains were from human babies with diarrhoea and they belonged to the infantile enteropathogenic serogroups (EPEC) which do not produce LT or heat-stable enterotoxin (ST). These observations have been confirmed by other workers, although difference exists as to the serogroups involved and the incidence of VT⁺ strains within them (Scotland *et al.*, 1979, 1980a; Wade *et al.*, 1979; Wilson & Bettelheim, 1980). All the reports, however, indicate that VT production is most common in serogroup O26 and that O26 strains possessing the H11 antigen are the most likely to be VT⁺. An O26 strain shown to be VT⁺ by Konowalchuk *et al.* (1977), called H19, one of the several strains examined by these workers that belonged to the *E. coli* collection maintained in this institute, is of special interest in that Smith & Lingood (1971) had found that extracts of it dilated ligated rabbit intestine and that the enterotoxin (Ent) responsible for this reaction, although different from LT, could be transferred to *E. coli* K12. Scotland *et al.* (1979, 1980a) found that the K12 Ent⁺ derivative was VT⁺, suggesting that the enterotoxin and VT of H19 were identical. They also demonstrated that VT production was transferable from H19 to *E. coli* K12. Williams *et al.* (1978) and Scotland *et al.*

Abbreviations: VT, Vero cell toxin; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin.

(1980*b*) identified two large plasmids in H19, one coding for its antibiotic resistance and one for its colicin production. Scotland *et al.* (1980*b*) identified a third plasmid, but failed to show that this coded for VT production. Because there was, therefore, no definitive evidence that VT production in H19, although transferable, was plasmid-determined, it was decided to investigate the VT transfer mechanism in this strain and in any other strain from which VT could be transferred. A large number of strains were first examined, especially of *E. coli* from enteric diseases in domestic animals; Konowalchuk *et al.* (1977) and Kashiwazaki *et al.* (1980, 1981) had obtained a few VT⁺ strains from such sources. Strains found to be VT⁺ were examined for ability to transfer the characteristic to *E. coli* K12. Recipient organisms that had acquired the VT⁺ character from some strains, including H19, were noted to have become lysogenic in the process, an observation that led to the discovery that the VT genes in these strains were incorporated in the genome of the phages with which they were lysogenized. VT genes that were transferable were compared in their common *E. coli* K12 host, particularly in regard to the contribution they made to its enterotoxigenicity and to its lethality for laboratory animals and pigs; pigs were included because we found that many *E. coli* strains that had caused outbreaks of oedema disease in these animals were VT⁺.

METHODS

Bacterial strains. All the bacterial strains examined for VT production were believed to be epidemiologically unrelated. They had been maintained in this laboratory lyophilized and on Dorset egg medium at 5 °C for 3–15 years, during which time most of those that had been isolated from cases of enteric disease were examined by the ligated pig intestine methods of Smith & Gyles (1970) for heat-labile (LT) and heat-stable (ST) enterotoxin. Two *E. coli* K12 strains were used as potential recipients in most of the attempts to identify *E. coli* strains with transferable VT production. One, 711, was *lac pro his trp phe* λ⁺ and the other, C600, was *lac thr leu thi* λ⁻; the latter was usually employed in phage studies because it was not latently infected with phage λ. A *lac*⁺ λ⁺ prototrophic *E. coli* K12 strain called Proto was occasionally used instead of 711 and C600. The two derivatives of *E. coli* K12 designated 711(H19) Ent⁺ and Ent⁻ by Smith & Linggood (1971), according to whether or not they had, during mixed culture with *E. coli* H19, acquired the ability to dilate ligated rabbit intestine were also used in some of the studies.

Culture media and cultural conditions. Broth cultures consisted of organisms grown in 10 ml nutrient broth, Oxoid no. 2 (CM67). Evans medium (Evans *et al.*, 1973) was dispensed in 20 ml amounts in 250 ml Erlenmeyer flasks except when it was used for screening bacterial strains for VT production; then it was dispensed in 5 ml amounts in Universal bottles. The nutrient agar employed was Difco tryptose agar (B64) to which 0.02 M-CaCl₂ had been added. All cultures were incubated at 37 °C for 24 h; fluid cultures, except those in conjugation experiments, were shaken in a water bath (approx. 100 r.p.m.).

Experimental animals. White Swiss mice and New Zealand White rabbits, bred at this institute, were used as young adults. Large White pigs, aged 6–8 weeks, were obtained from a nearby farm. Chickens, aged 4–6 weeks, were from this institute's specified pathogen-free Light Sussex flock. The sex of the animals was not determined.

Preparation and testing of bacterial products for VT, enterotoxins and lethal toxin. Bacterial cultures in Evans medium were centrifuged twice at 2500 *g* for 30 min and the supernate passed through a Millipore membrane filter at 450 nm pore diameter. These preparations are referred to as extracellular fluids to distinguish them from extracts which were prepared in the same manner except that the cells in the culture were first disrupted by means of an ice-cold MSE Mullard 60 W ultrasonic disintegrator at maximum output employing a titanium probe of 19 mm diameter. Some extracts for enterotoxin studies were prepared from suspensions of bacterial cells that had been grown in Roux flasks of glucose agar (Smith & Linggood, 1971). Preparations that were only to be screened for VT production or to be tested for enterotoxin were not filtered; instead penicillin G (1000 units ml⁻¹) and neomycin (1000 µg ml⁻¹) were added to the former and neomycin and polymyxin (each at 100 µg ml⁻¹) to the latter.

Preparations were tested and assayed for VT by the method of Konowalchuk *et al.* (1977) except that the Vero cell cultures were contained in 10 × 1.25 cm glass tubes instead of in plastic dishes and were incubated for 3–4 d instead of 1–2 d before use. Enterotoxin tests were performed in rabbits by the method of Smith & Linggood (1971); test samples were inoculated in 1 or 4 ml amounts into ligated segments of small intestine approximately 10 cm long. The volume of fluid that had accumulated was measured immediately after the animals had been killed. To check for LT production in gene transfer experiments, VT⁻ samples were tested in Vero cells and VT⁺ samples were tested in Chinese hamster ovary cells (Konowalchuk *et al.*, 1977); ligated pig intestine tests were used for checking ST gene transfer. Tests for the lethal toxin were performed by the intravenous inoculation of mice, rabbits, chickens and pigs. Inoculated animals were inspected several times daily for clinical evidence of disease. Those that were near to death were killed, and they and any that had died were autopsied and their lesions

recorded. Because pigs are very susceptible to fatal endotoxic shock, some of these animals were inoculated with extracts whose endotoxin content had been reduced by the techniques of Clugston & Nielsen (1974); sodium deoxycholate was added to the inocula as recommended by these workers.

Antitoxic sera. Rabbits were given 0.1 ml of live broth culture in Freund's incomplete adjuvant (Difco) intramuscularly followed by multiple intravenous injections of 0.2–0.5 ml of extracellular fluid (5 injections), 0.1–0.2 ml of heat-killed broth culture (7) and 0.1–0.2 ml of live broth culture (4), in that order during a period of 7 weeks. Chickens were given, intramuscularly, eight weekly injections of 1.0 ml of extract that had been concentrated 10 times by freeze-drying, the first dose being in Freund's incomplete adjuvant, and then, intravenously, 1.0 ml of live broth culture.

Transfer of ability to produce VT. Broth cultures, in 0.02 ml amounts, of a VT⁺ strain of *E. coli* (the donor strain) and of a spontaneous nalidixic acid-resistant (Nal^r) mutant of one of the *E. coli* K12 strains (the recipient strain) were grown together in nutrient broth. The mixed culture was then diluted with an equal volume of broth and incubation continued for a further 24 h. Finally, it was cultured twice in broth containing sodium nalidixate (20 µg ml⁻¹) to eliminate donor organisms, and once in plain broth; the culture period was 24 h in each case. After confirmation that this culture, referred to as the 'final mated culture', consisted principally of organisms of the recipient strain, a 'VT-type' preparation was tested in Vero cells. If this preparation gave a positive reaction, other samples from cultures of individual colonies of the final mated culture were tested. Cultures yielding VT⁺ preparations were designated by the number of the K12 recipient strain followed by the symbol VT⁺ and the number of the donor strain in brackets. Negative cultures were designated in the same manner except that they were given the symbol VT⁻. Sometimes, Nal^r mutants of strains of the same serogroups as the donor strains were used as recipients instead of *E. coli* K12. In addition, the following modifications were employed in attempts to transmit VT. (1) The de-repressed plasmids F and I were implanted in donor strains in the hope of increasing their conjugative ability, a procedure which was facilitated by using plasmids that also possessed genes for tetracycline resistance. (2) If the donor strain possessed a self-transferable antibiotic resistance plasmid, one of the antibiotics against which it provided resistance, as well as sodium nalidixate, was added to the broth through which the mixed culture of donor and recipient was transferred. (3) Because their VT genes might be located in a self-transferable plasmid, donor strains were used to mobilize a non-conjugative streptomycin/sulphonamide plasmid from an *E. coli* K12 strain to a K12 recipient strain which was then tested for VT.

Identification of lysogenic strains. Supernates of centrifuged broth cultures were spot-inoculated on to plates of nutrient agar containing sodium nalidixate (20 µg ml⁻¹) and which had previously been inoculated with a lawn of a broth culture of a Nal^r mutant of either the λ⁻ *E. coli* K12 strain, C600, or of any other potentially suitable 'indicator' strain. The plates were incubated and examined for evidence of phage action. Sometimes, the supernates were either held at 58 °C for 40 min (referred to as heat treatment) or filtered through a Millipore filter of pore diameter 450 nm before they were examined for phage, on a lawn consisting of a Nal^s indicator strain growing on ordinary nutrient agar. Strains not found to be lysogenic by these methods were incubated in broth with organisms of a Nal^s indicator strain and the mixed culture was examined for phage in the manner previously described. In all studies in which it was essential that the phage preparations should contain no live bacteria, they were either heat-treated or filtered and then checked for bacteriological sterility.

Purification and propagation of phages. Single plaques were picked, mixed with a few drops of a broth culture of the propagating strain and plated on nutrient agar. A discrete plaque was picked as soon as plaques were distinctly visible and replated with the propagating strain. The process was repeated once. Finally, a discrete plaque with some of the surrounding bacterial growth was picked into broth and incubated until lysis occurred. The lysed culture was then heat-treated or filtered and tested for bacteriological sterility and for activity.

Phage-resistant bacterial mutants. The resistant growth from within a phage plaque or larger zone of phage lysis was picked into broth and incubated. The resulting culture was plated on nutrient agar, incubated, and a single colony picked and plated. This process was repeated once and the final culture was tested on nutrient agar to confirm its complete resistance to an undiluted preparation of the phage.

Cross-resistance tests. Mutants of the λ⁻ *E. coli* K12 strain, C600, resistant to each of the phages under investigation were isolated and the susceptibility of each mutant to each phage was determined by spot-inoculating the phages on lawns of broth cultures of the mutants.

Plasmid removal. This was attempted by serial transfer of strains in broth at 44 °C or in broth containing sodium lauryl sulphate (Tomoeda *et al.*, 1968) or acridine orange (Hirota, 1960).

RESULTS

Incidence of VT production in strains of E. coli and related bacteria

Of 519 strains of *E. coli* from diseased and healthy humans and domestic animals, 68 were VT⁺ (Table 1). VT production was commonest in strains from weaned pigs suffering from diarrhoea and/or oedema disease; about half of the O138:K81 and O139:K82 strains and all the

Table 1. *Production of VT by E. coli and other bacteria*

Bacterium	Source		Disease	Serogroup*	No. of strains	
	Animal				tested	VT+
<i>Escherichia coli</i>	Man	{	Neonatal diarrhoea	{ O26 O128 Others including O55 (8), O111 (3), O119 (6), O125 (3), O126 (6), O127 (2), O25, O124, O136, O143, O144, O152	8	4
			Dysentery-like infections	Unknown	12	2
			Cystitis	Unknown	56	0
			Ulcerative colitis	Unknown	6	0
			Generalized infections	Unknown	10	0
	Pig	{	None (from faeces)	Mostly unknown	9	0
				Unknown	46	0
			Post-weaning diarrhoea	Unknown	20	0
			and oedema disease	{ O138:K81 O139:K82 O141:K85 O141:K85,88 O141:K85,88	37	17
			Neonatal diarrhoea	{ Others including O8:K87,88 (13), O45:K?88 (5), O147:K89,88 (5), O149:K91,88 (6), O64:K?99 (1), O101:K30,99 (2), O9:K103,987P (4), O20:K101,987P (1)	24	11
Ox and sheep	{	{	'Undiagnosed' diarrhoea	{ O45:K?88 (5), O147:K89,88 (5), O149:K91,88 (6), O64:K?99 (1), O101:K30,99 (2), O9:K103,987P (4), O20:K101,987P (1)	25	25
			Mastitis	Unknown	4	4
			Neonatal diarrhoea	Mostly unknown	5	5
					37	0
					16	0
	{	{	Neonatal diarrhoea	{ O8:K85,99 (4), O9:K30,99 (1), O9:K32,99 (1), O9:K35,99 (1), O9:K-99 (1), O101:K30,99 (2)	11	0
			Neonatal generalized infections	Mainly O78	10	0
			'Undiagnosed' diarrhoea	Unknown	20	0
			Mastitis	Unknown	20	0
			Generalized infections	Mainly O78 (10) and O2:K1 (10)	30	0
<i>Salmonella</i> sp.	{	{	None (from faeces)	Unknown	20	0
			Generalized and enteric infections	18 serotypes	20	0
			Dysentery	<i>Sh. flexneri</i> (10) and <i>sonnei</i> (8)	18	0
			Mastitis	All different	10	0
			None (from faeces)	Unknown	5	0
	{	{	None (from faeces)	Unknown	10	0
			Unknown	Unknown	10	6
<i>Shigella</i> sp.	{	{	None (from faeces)	Unknown	10	0
			Unknown	Unknown	10	6
<i>Klebsiella pneumoniae</i>	{	{	None (from faeces)	Unknown	10	0
			Unknown	Unknown	10	6
<i>Enterobacter cloacae</i>	{	{	None (from faeces)	Unknown	10	0
			Unknown	Unknown	10	6
<i>Proteus mirabilis</i>	{	{	None (from faeces)	Unknown	10	0
			Unknown	Unknown	10	6
<i>Pseudomonas aeruginosa</i>	{	{	None (from faeces)	Unknown	10	0
			Unknown	Unknown	10	6

* The numbers in parentheses are the numbers of strains tested.

O141 :K85 and O141 :K85,88 strains in this category were VT⁺. All of five O141 :K85,88 strains that had caused diarrhoea in piglets were also VT⁺. None of the other 37 strains that had caused piglet diarrhoea was VT⁺; antigenically, these strains were different from those that had caused diarrhoea or oedema disease in weaned pigs. Eight of the 11 VT⁺ and 12 of the 13 VT⁻ O139 :K82 strains were non-enterotoxigenic. All the other strains that had caused diarrhoea or oedema disease in pigs or piglets were enterotoxigenic. The K88⁻ strains produced ST only and the K88⁺ strains produced ST and LT (LT/ST⁺); nearly all the enterotoxigenic strains and the O139 :K82 strains produced α -haemolysin (Hly⁺).

Six of 73 *E. coli* strains from infantile diarrhoea were VT⁺. These six comprised two of 12 O128 strains and four of eight O26 strains; none of them produced LT or ST. One VT⁺ O128 strain possessed the H2 antigen and the other possessed the H32 antigen. The four VT⁺ O26 strains possessed the H11 antigen and so did one of the VT⁻ O26 strains; one of the VT⁺ strains was H19, the strain referred to in the Introduction. VT production was not detected in six strains of *E. coli* that had caused dysentery-like infections in man or in 10 strains that had caused diarrhoea in calves or lambs. Neither was it detected in 137 strains that had caused generalized infections, cystitis or mastitis, or in 85 strains that had been isolated from the faeces of humans or animals that were healthy or were suffering from ulcerative colitis or diarrhoea of unknown aetiology ('undiagnosed' diarrhoea in Table 1). VT production was detected, however, in six of 10 *Pseudomonas aeruginosa* strains but not in 63 *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter* or *Proteus* strains.

VT transfer from E. coli strains

By growth in mixed culture, VT production was transferred from two VT⁺ *E. coli* O26 strains (H19 and H28), one VT⁺ *E. coli* O128 strain (H12), and one VT⁺ *E. coli* O141 :K85,88 strain (P101) to one or more of the *E. coli* K12 recipient strains. Strains H19, H28 and H12 were isolated from diarrhoea in infants and strain P101, an LT/ST⁺ strain, had caused diarrhoea in weaned pigs. Transfer was detected in all of five matings of H19 with three *E. coli* K12 strains, C600, 711 and Proto, 33 of 41 K12 isolates from these matings being VT⁺. Transfer was detected in eight of nine matings of H28 with the three K12 strains, 61 of 71 K12 isolates from the successful matings being VT⁺, and in three of four matings of H12 with 711 and Proto, 10 of 21 K12 isolates from the successful matings being VT⁺. Transfer of VT from P101 was more difficult to demonstrate. It was not detected in any of 27 matings with C600 or in any of 17 matings with Proto, but it was detected in four of 18 matings with 711. LT transfer occurred in 15 of the C600 matings, nine of the Proto matings and all of the unsuccessful 711 matings. Of 40 isolates from the four successful matings with 711, 20 were VT⁺. All of 11 VT⁺ isolates tested were LT⁺ as were eight of 10 VT⁻ isolates. Four VT⁺LT⁺ and four VT⁻LT⁺ isolates were ST⁺. None of the 711 VT⁺ isolates had acquired the Hly or the K88 plasmids of P101 but all of 10 VT⁺LT⁺, 10 VT⁻LT⁺ and two VT⁻LT⁻ isolates had acquired the colicin determinant.

Despite repeated attempts, which included the use of all the modifications of the mixed culture technique described in Methods, VT production was not transferred from any of the other 64 VT⁺ *E. coli* strains or the six VT⁺ *P. aeruginosa* strains to *E. coli* K12. Attempts to transfer VT production from the 17 VT⁺ O138 :K81 *E. coli* strains to two VT⁻ O138 :K81 strains and from 10 VT⁺ O139 :K82 strains to two VT⁻ O139 :K82 strains were also unsuccessful.

The examinations of laboratory cultures of K12 and other *E. coli* strains to which Hly, K88, LT, ST and Col plasmids had been transferred from VT⁺ pig *E. coli* did not reveal any accompanying transfer of VT. Also, loss of Hly and K88 plasmids was not accompanied by VT loss.

Attempts to remove the VT genes from *E. coli* 711 strains possessing the VT of *E. coli* H12, H19 or P101 by means of sodium lauryl sulphate or acridine orange treatment were unsuccessful; so were attempts to remove them from a VT⁺ O141 :K85,88, an O138 :K81 and an O139 :K82 strain by means of sodium lauryl sulphate.

Implication of phage in VT transfer

In two matings with H19, a mutant of C600 resistant to the colicin of H19 was used as recipient so that information could be obtained on the relationship between the transfer of VT,

colicin and antibiotic resistance (H19, unlike H28, the other O26 strain whose VT was transferable, possessed plasmids coding for colicin production and for resistance to tetracycline, streptomycin, spectinomycin and sulphonamides). Of 100 isolates of C600 from these matings, 56 had acquired antibiotic resistance and 13 had acquired colicin production; 50 had become lysogenic for C600. Eighteen selected from the 100 were examined for VT production. All seven that were VT⁺ were lysogenic and all 11 that were VT⁻ were non-lysogenic. The seven VT⁺ isolates and six of the VT⁻ isolates were antibiotic-resistant. Two of the VT⁺ isolates produced colicin, as did three VT⁻ isolates. Because of the correlation between the acquisition of VT production and lysogenicity, K12 isolates that had acquired VT during mixed culture with H19, H28, H12 and P101 were examined for lysogenicity for the K12 strains from which they were derived; those that had acquired VT from H19, H28 or H12, but not from P101, were lysogenic. Because this suggested that VT production in the three human strains was phage-mediated, further investigations were made with the two forms of *E. coli* K12 designated 711(H19) Ent⁺ and Ent⁻ by Smith & Linggood (1971) and which had been found by Scotland *et al.* (1980*a*) to be VT⁺ and VT⁻ respectively. Heat-treated extracellular fluids of broth cultures of the Ent⁺ and Ent⁻ forms contained approximately 10⁵ phage particles ml⁻¹ active on 711. Broth cultures of the resistant growth in 14 of 24 phage plaques present on the lawn of 711 used for the phage counts on the Ent⁺ culture were VT⁺. Cultures of bacterial growth picked from 16 inter-plaque regions of this lawn were VT⁻ and so were cultures of the resistant growth in all of 15 plaques present on the 711 lawn used for the phage counts on the Ent⁻ culture. Broth cultures of H19, unlike those of the Ent⁺ and Ent⁻ forms of 711, contained very few phage particles, usually fewer than 10 ml⁻¹; often none could be demonstrated, the probable reason for H19 being recorded by Smith & Linggood (1971) as non-lysogenic. Much higher numbers, in the region of 10⁷ ml⁻¹, were found in mixed broth cultures of H19 and 711. The results of testing cultures prepared from the resistant growth within phage plaques on a lawn of 711 inoculated with heat-treated extracellular fluids of broth cultures of H19 or H19 and 711 were similar to the results obtained with the 711 Ent⁺ culture, i.e. some were VT⁺ and some were VT⁻. For example, in one experiment all five phage-resistant cultures tested were VT⁺, in another all four tested were VT⁺, but in another all 14 were VT⁻. In one experiment in which the K12 strain Proto was used instead of 711, six phage-resistant cultures were VT⁺ and four were VT⁻. By contrast, when the λ^- strain C600 was used, VT production was always acquired together with phage resistance. For example, in five separate experiments broth cultures of the resistant growth of C600 within 5, 20, 14, 8 and 16 plaques were all VT⁺. Cultures prepared from each of 16 inter-plaque regions of the C600 lawn that had been spot-inoculated with extracellular fluid of the H19 culture in the last of these five experiments were VT⁻. None of the 16 VT⁺ cultures of C600 obtained in this experiment had acquired either the antibiotic resistance or the colicin plasmids of H19.

The phages carried by some of the VT⁺ phage-resistant mutants of 711 and C600 isolated in the previous experiments were purified and propagated on C600 and then tested against a selection of the mutants. This revealed the phages to be of two kinds: one, designated A, that lysed some of the mutants and one, designated B, that lysed none of them. Broth cultures of the phage-resistant mutants that grew within 12 plaques produced by each of the two phages on lawns of 711, C600 and a λ^+ form of C600, a total of 72, were tested for VT production. The 48 mutants of C600 and C600 λ^+ resistant to phages A or B were VT⁺ but only 11 of the 12 phage A-resistant mutants and seven of the 12 phage B-resistant mutants of 711 were VT⁺. The 72 cultures, like those of the phage-resistant mutants isolated in previous experiments, were, irrespective of VT-status, lysogenic for the K12 strain from which they were derived.

The phage infection state and the phage/VT relationship in *E. coli* H28 appeared identical with that in H19. Broth cultures of H28 contained very few phage particles but mixed cultures with K12 strains contained many. Cross-resistance tests indicated that the phage particles were of kinds A and B. Of the phage-resistant isolates obtained from 29 plaques present on lawns of strain 711 inoculated with heated extracellular fluids of three mixed broth cultures of H28 and 711, 16 were carrying phage A and were VT⁺ and 13 were carrying phage B, only two of which were VT⁺. In similar experiments employing C600 instead of 711, all of 17 phage-resistant isolates were VT⁺, 12 carrying phage A and five carrying phage B. As in the H19 phage studies,

Table 2. Activity of the VT-determining phages of *E. coli* H19, H28, H12 and H23 on strains of *E. coli* K12 C600 lysogenized with these phages

Strain*	Lysis by phage†:					
	H19A	H19B	H28A	H28B	H12	H23
C600(H19A)	-	-	-	-	+	+
C600(H19B)	+	-	+	-	+	+
C600(H28A)	-	-	-	-	+	+
C600(H28B)	+	-	+	-	+	+
C600(H12)	+	+	+	+	-	-
C600(H23)	+	+	+	+	-	-
C600	+	+	+	+	+	+
H19, H28, H12 and H23	-	-	-	-	-	-

* The numbers in parentheses indicate the phages with which C600 was lysogenized.

† All the phages had been propagated on C600, the λ^- *E. coli* K12 strain. +, lysis; -, no lysis.

broth cultures of 12 mutants of each of 711, C600 and C600 λ^+ resistant to each of the two H28 phages were VT-tested. Again, some of the mutants of strain 711, and especially the phage B-resistant ones (one of the 12 resistant to A and seven of the 12 resistant to B) were VT⁻ whereas all 24 of the C600 mutants were VT⁺. Three of the 12 C600 λ^+ mutants resistant to phage B, however, were VT⁻; all 12 of the phage A-resistant mutants were VT⁺. The 72 cultures were lysogenic for the K12 strain from which they were derived.

The phage-mediated nature of VT transfer from the O128 strain, H12, was established by examining broth cultures of the resistant growth from within 36 plaques on lawns of C600 that had been inoculated with the filtered extracellular fluid of broth cultures of H12; filtration was employed to render the fluid bacteria-free because the phage was heat-sensitive. All 36 cultures were VT⁺ whereas broth cultures of 16 non-plaquing regions on the inoculated lawns were VT⁻.

One of the two VT⁺ O26 strains, H30, and the VT⁺ O128 strain, H23, from which VT transfer had not been demonstrated, were lysogenic for C600, their phages being heat-sensitive. Cultures of the phage-resistant growth within all of 22 plaques of the H30 phage on a lawn of C600 were VT⁻ but those of the resistant growth within all of eight plaques of the H23 phage on a similar lawn were VT⁺, implying that VT production in H23, like that of H19, H28 and H12, was phage-determined. A comparison of the phages of these four strains by cross-resistance tests (Table 2) indicated that the H23 phage was identical with the H12 phage but different from the H19 and H28 phages; phages A of H19 and H28 were identical and so were phages B of these two strains. During the performance of these tests, double mutants of C600 were isolated that carried phage H19A or H19B in addition to phage of H12 or H23.

The VT-determining phages of H19, H28, H12 and H23 were not active on the four O26 strains or the 10 O128 strains classed in Table 1 as VT⁻. One of the O26 strains and four of the O128 strains were lysogenic but their phages were not responsible for their resistance to the VT-determining phages, because mutants of C600 resistant to the phages carried by these five strains were still susceptible to the VT-determining phages. The VT-determining phages were also inactive on eight human (not O26 or O128), 11 pig and seven calf and lamb enteropathogenic strains and on seven non-enteropathogenic *E. coli* strains, two VT⁻ *Pseudomonas aeruginosa* strains, three *Proteus mirabilis* strains, two *Klebsiella pneumoniae* strains, 11 *Salmonella* strains and 12 *Shigella flexneri* strains. The A and B phages of H19 and H28 were active on four of six rough strains but not on two smooth strains of *Shigella sonnei*; the H12 and H23 phages were not active on any of these strains. Resistant mutants obtained from within plaques of the A and B phages on lawns of the four rough strains were VT⁺.

The four VT⁻ O26 strains were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 $\mu\text{g ml}^{-1}$) by the method of Glover (1968) in the hope of obtaining mutants susceptible to phages H19 and H28. No mutants were detected initially; broth cultures were then left at room temperature for 3 weeks and re-examined. Eight of 10 colonies selected because of their differing colonial morphology on culture plates prepared from one of the four strains, strain H1A, were

Table 3. *Titres and heat stability of VT in extracellular fluids and extracts of different bacterial strains*

VT producer strain*	Antigenic structure	Reciprocal of VT titre† of		Inactivation temperature‡ (°C)
		Extracellular fluid	Extract	
<i>E. coli</i> H19	O26	2000	32000	80
<i>E. coli</i> 711 VT ⁺ (H19)	—	4000	32000	80
<i>E. coli</i> H28	O26	1000	32000	80
<i>E. coli</i> 711 VT ⁺ (H28)	—	1000	32000	80
<i>E. coli</i> H30 and H64	O26	1000	32000	80
<i>E. coli</i> H12	O128	512	16000	80
<i>E. coli</i> 711 VT ⁺ (H12)	—	512	16000	80
<i>E. coli</i> H23	O128	512	8000	80
<i>E. coli</i> C600 VT ⁺ (H23)	—	256	4000	80
<i>E. coli</i> P101	O141:K85,88	128	256	70
<i>E. coli</i> 711 VT ⁺ LT/ST ⁺ (P101)	—	128	256	70
<i>E. coli</i> P5 and P135	O141:K85,88	64–128	128–256	70
<i>E. coli</i> P61, P100 and P122	O141:K85	16–32	16–128	70
<i>E. coli</i> P8, P105 and P174	O138:K81	8–128	16–128	70
<i>E. coli</i> P3, P104 and P223	O139:K82	8–64	64–256	70
<i>P. aeruginosa</i> G1, G6, G8				
G15, G17, G19, G49, G69	—	1–8	2–16	> 100

—, No observation.

* The numbers in parentheses indicate the donor *E. coli* strains from which VT and/or LT/ST genes were transferred to the recipient *E. coli* K12 strains, 711 and C600.

† Highest dilution producing a cytotoxic effect in 25% of Vero cells after 4 d.

‡ Determined on samples of undiluted preparations held at 60, 65, 70, 75, 80 and 100 °C for 30 min; extracellular fluids and extracts yielded similar results.

susceptible to the A phages but not to the B phages of H19 and H28 and so were two of 10 colonies picked at random from these plates. Cultures of four of the phage-susceptible colonies and a non-susceptible colony were examined by the Division of Enteric Pathogens of the Central Public Health Laboratory, London, and reported to be rough. Resistant mutants obtained from within plaques of phage H19A on lawns prepared from cultures of each of six of the phage-susceptible colonies of H1A were VT⁺. Twelve isolates selected because of their differing colonial morphology from broth cultures of each of eight of the 10 VT[−] O128 strains that had been held at room temperature for 2 months were insusceptible to the O128 VT-determining phages.

In vitro characteristics of the VT of different strains of E. coli and P. aeruginosa

The human VT⁺ *E. coli* strains and the *E. coli* K12 strains possessing their VT genes were more potent producers of VT than pig VT⁺ *E. coli* strains or the K12 strain possessing the VT genes of the pig strain, P101; the VT titres of the extracellular fluids and extracts of these K12 strains closely resembled those of the strains from which their VT genes were derived (Table 3). The *P. aeruginosa* strains were the poorest toxin producers. Extracts usually contained more toxin than extracellular fluids; this was particularly so in the case of the extracts prepared from the human *E. coli* strains. The VT of the *P. aeruginosa* strains resisted heating to 100 °C for 30 min whereas the VT of the human and pig strains were destroyed at 80 °C and 70 °C respectively.

The appearance of Vero cells exposed to extracellular fluids or extracts of human and pig VT⁺ *E. coli* strains was similar and it closely resembled the description given by Konowalchuk *et al.* (1977); cytopathic effects were sometimes noted as early as 6 h in the case of the pig strains whereas in the case of the human strains they were not usually apparent until 12 h. Changes of a slightly different nature were noted by 6 h when the *P. aeruginosa* strains were tested, the cells being granular in appearance and often enlarged; by 24 h, most of the cell sheet was floating free in the medium.

VT-neutralizing antisera were obtained from rabbits inoculated with *E. coli* K12 strains 711 VT⁺ (H19), 711 VT⁺ (H28) or 711 VT⁺ (H12) (as defined in Table 3). They were not obtainable from the rabbits inoculated with *E. coli* 711 VT⁺ LT/ST⁺ (P101) or with *E. coli* P5, P122, P8, P215, P3 and P104, or *P. aeruginosa* G1. Chickens inoculated with *E. coli* 711 VT⁺ LT/ST⁺ (P101) and with *E. coli* P5, however, did yield low-titred VT-neutralizing antisera, but serum from chickens inoculated with *E. coli* strains P122, P8 and P3 had no neutralizing ability. The results of determining the effect of the antisera on the cytotoxicity of extracts of a variety of *E. coli* and *P. aeruginosa* strains are summarized in Table 4. The antisera against the VT of strains H19, H28 and H12 neutralized the VT of all three strains and the VT of strain H23. They also neutralized the non-transferable VT of strains H30 and H64. They did not neutralize the VT of any of 12 pig *E. coli* strains of four different serotypes or two *P. aeruginosa* strains. The antisera against the VT of *E. coli* 711 VT⁺ LT/ST⁺ (P101) and P5 neutralized the VT of all the pig *E. coli* strains but not the VT of the human *E. coli* strains or the *P. aeruginosa* strains. The antiserum against 711 VT⁻ LT/ST⁺ (P101) had no effect on the VT of any of the strains, but it did neutralize LT.

Dilatation of ligated segments of rabbit intestine by extracts of VT⁺ and LT/ST⁺ strains of E. coli K12

Table 5 summarizes the results of inoculating ligated segments of rabbit intestine with different amounts of extracts of Evans broth and glucose agar cultures of *E. coli* 711 VT⁺ (H19), 711 VT⁺ (H28), 711 VT⁺ (H12) and C600 VT⁺ (H23), and a K12 strain possessing the LT/ST genes of a pig enteropathogenic *E. coli* strain, P307A (antigenic formula O8:K87,88). With one exception, no response was elicited in the 48 segments of the six rabbits inoculated with 1 ml of extracts of Evans broth cultures of one or other of the four K12 VT⁺ strains. A moderate response usually occurred when the dose was increased to 4 ml and a much greater one when extracts of glucose agar cultures at this dosage were used. In general, the extracts of the K12 VT⁺ (O26) strains (711 VT⁺ (H19) and 711 VT⁺ (H28)) were more potent dilators than the extracts of the K12 VT⁺ (O128) strains (711 VT⁺ (H12) and C600 VT⁺ (H23)). Extracts of the K12 LT/ST⁺ (P307A) strain dilated all the segments into which they had been inoculated although stronger reactions were produced by the higher doses. Dilatation did not occur in any of the 72 segments inoculated with extracts of the VT⁻ LT/ST⁻ parent K12 strains. Extracts of strain 711 VT⁺ LT/ST⁺ (P101) strongly dilated ligated segments of rabbit intestine. The effect was completely neutralized by adding antisera prepared against strains 711 VT⁻ LT/ST⁺ (P101) or 711 LT/ST⁺ (P307A). Extracts of Evans broth cultures of the six VT⁺ *P. aeruginosa* strains, each tested on one occasion in 4 ml amounts, failed to dilate ligated rabbit intestine.

Intravenous inoculation of animals with extracts or extracellular fluids of VT⁺ cultures of E. coli K12

Mice. Extracts of VT⁺ *E. coli* K12 strains, administered intravenously, were more lethal for mice than extracts of the parent K12 strains (Table 6); extracts of strain 711 VT⁺ (H28) were the most lethal and those of C600 VT⁺ (H23) and 711 VT⁺ LT/ST⁺ (P101) the least. Extracts of 711 VT⁻ LT/ST⁺ (P101) were no more lethal than those of the parent 711 strain. Eighteen of another group of 26 mice inoculated with 0.5 ml of an extract of 711 VT⁺ LT/ST⁺ (P101) died; no deaths occurred in a comparable group of 21 mice inoculated with a similar dose of an extract of the 711 VT⁻ LT/ST⁺ (P101) strain. The mortality in the experiments with all the VT⁺ strains occurred in the second to the sixth day after inoculation, the survival times of the mice, in general, being dose-dependent. Paralysis of the hind limbs commonly occurred before death in the mice given the extracts of 711 VT⁺ LT/ST⁺ (P101); those given the extracts of K12 strains possessing the VT genes of the four human enteropathogenic strains showed no characteristic clinical sign of ill-health before death. Macroscopic pathological lesions were not a feature of the disease produced by any of the extracts.

Groups of 10 mice were inoculated intravenously with 0.5, 0.25 or 0.12 ml of extracellular fluid of strain 711 VT⁺ (H19). Apart from one mouse given 0.5 ml, none of the 30 mice died. When the experiment was repeated with an extract of the broth culture from which the extracellular fluid had been obtained, 29 of the 30 inoculated mice died; none of 10 mice given

Table 4. Neutralization of the VT of different bacterial strains by antisera

Mixtures of undiluted antiserum and four cytotoxic doses of extract of the bacterial strains were held at 37 °C for 1 h before being tested for activity against Vero cells. +, neutralization; —, no neutralization. For other details see Table 3.

VT producer strain	Antigenic structure*	Neutralization of VT by antisera prepared from <i>E. coli</i> strain:			
		711 VT ⁺ (H19) and 711 VT ⁺ (H28)	711 VT ⁺ (H12)	711 VT ⁺ LT/ST ⁺ (P101) and P5	711 VT ⁺ LT/ST ⁺ (P101)
<i>E. coli</i> H19, 711 VT ⁺ (H19), H28 and 711 VT ⁺ (H28)	O26	+	+	—	—
<i>E. coli</i> H30 and H64	O26	+	+	—	—
<i>E. coli</i> H12, 711 VT ⁺ (H12), H23 and C600 VT ⁺ (H23)	O128	+	+	—	—
<i>E. coli</i> P101, 711 VT ⁺ LT/ST ⁺ (P101), P5 and P135	O141:K85,88	—	—	+	—
<i>E. coli</i> P61, P100 and P122	O141:K85	—	—	+	—
<i>E. coli</i> P8, P105 and P174	O138:K81	—	—	+	—
<i>E. coli</i> P3, P104 and P223	O139:K82	—	—	+	—
<i>P. aeruginosa</i> G1 and G17	—	—	—	—	—

* Of the VT donor in the case of the *E. coli* K12 strains, 711 and C600.

Table 5. *Ability of extracts of different VT⁺ and LT/ST⁺ strains of E. coli K12 to dilate ligated 10 cm segments of rabbit intestine*

Employing the same dose regimen, extracts of each VT⁺ and LT/ST⁺ strain were tested once, and extracts of each K12 parent strain twice, in each of six rabbits, 18 rabbits being used in all. For other details see Table 3.

Strain from which extract prepared	Vol. of fluid (ml) accumulated in intestinal segments inoculated with extract of:		
	Evans broth culture		Glucose agar culture
	1 ml dose	4 ml dose	4 ml dose
711 VT ⁺ (H19)	0, 0, 0, 0, 0, 0	13, 10, 10, 9, 5, 4	34, 31, 23, 16, 12, 8
711 VT ⁺ (H28)	7, 0, 0, 0, 0, 0	15, 10, 10, 9, 8, 5	32, 29, 20, 17, 13, 12
711 VT ⁺ (H12)	0, 0, 0, 0, 0, 0	10, 9, 6, 6, 0, 0	20, 19, 19, 15, 12, 2
C600 VT ⁺ (H23)	0, 0, 0, 0, 0, 0	11, 4, 4, 2, 0, 0	27, 16, 15, 13, 10, 6
711 LT/ST ⁺ (P307A)	28, 22, 20, 18, 16, 13	32, 30, 20, 21*, 16*, 13*	45, 42, 27, 25, 23, 22
711	0, 0, 0, 0, 0, 0	2, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
C600	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0

* A 1 ml not a 4 ml dose.

Table 6. *Mortality in mice inoculated intravenously with extracts of VT⁺ strains of E. coli K12*

All doses of less than 0.25 ml were made up to that volume with Evans medium. For other details see Table 3.

Strain from which extract prepared	No. of 10 mice that died after receiving the following doses of extract (ml):							LD ₅₀ (ml)
	0.5	0.25	0.12	0.06	0.03	0.015	0.008	
711 VT ⁺ (H19)	—	10	10	9	3	—	—	0.03–0.06
711 VT ⁺ (H28)	—	10	10	10	9	4	1	0.016
711 VT ⁺ (H12)	—	10	10	3	1	—	—	0.06–0.12
C600 VT ⁺ (H23)	9	5	3	1	0	—	—	0.41
711 VT ⁺ LT/ST ⁺ (P101)	7	3	0	—	—	—	—	0.25–0.5
711 VT ⁺ LT/ST ⁺ (P101)	0	—	—	—	—	—	—	>0.5
711	0	0	—	—	—	—	—	>0.5
C600	0	1	—	—	—	—	—	>0.5

0.5 ml of an extract of the VT⁺ K12 parent strain died. In tests carried out on other occasions the extracellular fluids of 711 VT⁺(H19) were more lethal. Of a total of 27 mice given 0.5 ml, 22 died; only one of 17 given a 0.25 ml dose died. As in the case of the extracts, the extracellular fluids of 711 VT⁺(H28) were more lethal than a similar preparation of 711 VT⁺(H12); nine of 10 mice given 0.5 ml of the former died compared with none of 10 given 0.5 ml of the latter.

No deaths occurred in groups of six mice inoculated intravenously with 0.12 ml of extracts of *E. coli* 711 VT⁺(H19) or 711 VT⁺(H12) that had been incubated at 37 °C for 1 h with 0.12 ml of antiserum prepared against either of these strains; in similar experiments in which the antisera were replaced by the pre-vaccinal sera all the mice died. Neither did any deaths occur in groups of six mice inoculated with 0.12 ml of extracts of 711 VT⁺(H19) or 711 VT⁺(H12) that had been held at 80 °C for 30 min.

Rabbits. Given intravenously, extracellular fluids of strain 711 possessing the VT genes of the O26 strains, H19 and H28, were more lethal for rabbits than those of the parent 711 strain (Table 7); extracts were not compared because those of the K12 parent strains were lethal in low dosage. Most of the deaths in rabbits given the extracellular fluids of these two VT⁺ 711 strains occurred 30–48 h after inoculation; paralysis of the fore and/or hind limbs was usually present

Table 7. *Mortality in rabbits inoculated intravenously with extracellular fluids of VT⁺ E. coli K12 strains*

The numerator is the number of rabbits that died and the denominator is the number that were inoculated. For other details see Table 3.

Strain from which extracellular fluid obtained	No. of rabbits that died after receiving the following volumes of extracellular fluid (ml):						LD ₅₀ (ml)
	10	5	2.5	1.0	0.5	0.25	
711 VT ⁺ (H19)	—	1/1	4/4	3/4	3/4	0/4	0.51
711 VT ⁺ (H28)	—	4/4	3/4	3/4	0/4	—	1.06
711 VT ⁺ (H12)	—	4/22	2/8	0/4	—	—	>5
C600 VT ⁺ (H23)	—	1/8	—	—	—	—	>5
711 VT ⁺ LT/ST ⁺ (P101)	4/6	1/10	—	—	—	—	5-10
711 VT ⁻ LT/ST ⁺ (P101)	—	2/10	—	—	—	—	>5
711	3/8	1/12	—	—	—	—	>10

Fig. 1. Caecum of a normal rabbit (right) and of a rabbit inoculated with extracellular fluid of *E. coli* strain 711 VT⁺(H19) (left); the walls and spiral rings of the latter are oedematous and there is a haemorrhagic region (near the top). (70% of natural size.)

before death in the rabbits that survived for this time. A common lesion was gross enlargement of the caecum due to oedema of the walls of its pockets and spiral rings (Fig. 1). Large haemorrhagic areas were present in the mucous membrane of some of the oedematous caeca. There was little difference in lethality between extracellular fluids of K12 strains 711 VT⁺(H12), C600 VT⁺(H23) and 711 VT⁺LT/ST⁺(P101), and those of the parent K12 strains. Three of the rabbits that died following inoculation with extracellular fluids of 711 VT⁺(H12) and the one that died following inoculation with extracellular fluids of C600 VT⁺(H23) did, however, exhibit paralysis of the limbs before death, a clinical sign not observed in the rabbits that died following inoculation of extracellular fluids of 711 itself or 711 VT⁺LT/ST⁺(P101).

Table 8. *Effect of inoculating pigs intravenously with different preparations of E. coli K12 711 possessing or not possessing the VT genes of E. coli P101*

Pigs were inoculated intravenously with 2 ml extracellular fluids or extracts per kg body weight, or with an equivalent amount of extract treated by the method of Clugston & Nielsen (1974) (= 'Treated extract'). For other details see Table 3.

Preparation inoculated	Strain	No. of pigs	No. that survived endotoxic shock	No. of survivors with oedema disease	
				Clinical signs	Gastric oedema*
Extracellular fluid	711 VT ⁺ LT/ST ⁺ (P101)	3	2	1	1
	711 VT ⁻ LT/ST ⁺ (P101)	1	0	—	—
Extract	711 VT ⁺ LT/ST ⁺ (P101)	9	8	6	4
	711 VT ⁻ LT/ST ⁺ (P101)	6	3	0	0
Treated extract	711 VT ⁺ LT/ST ⁺ (P101)	5	4	3	1
	711 VT ⁻ LT/ST ⁺ (P101)	3	3	0	0

* All the pigs with gastric oedema had exhibited clinical signs of oedema disease.

Limb paralysis and caecal lesions were not noted in rabbits that died following inoculation with extracellular fluids of one of the rough mutants of the VT⁻ *E. coli* O26 strain, H1A, that was susceptible to the H19A and H28A phages. They were present, though, in most rabbits that died following inoculation with the more lethal extracellular fluids of the mutant rendered VT⁺ by lysogenization with phage H19A. After their contents had been removed, the weights of the caeca of six of these rabbits were 94, 82, 80, 67, 61 and 57 g; the weights of the caeca of four rabbits that survived inoculation of extracellular fluids of the VT⁻ parent strain were 37, 33, 30 and 25 g.

All of four rabbits inoculated intravenously with a mixture of 2.5 ml of extracellular fluids of 711 VT⁺(H19) and 2.5 ml of normal rabbit serum died; only one of four died after being given a similar mixture in which the normal serum was replaced with antiserum against 711 VT⁺(H19). Both of two rabbits remained well after being given 2.5 ml of 711 VT⁺(H19) extracellular fluids that had been held at 80 °C for 30 min.

Chickens. No deaths occurred in groups of five chickens inoculated intravenously with 0.5 ml of extracts of *E. coli* K12 strains 711 VT⁺(H19), 711 VT⁺(H28), 711 VT⁺(H12), C600 VT⁺(H23) and 711 VT⁺LT/ST⁺(P101).

Pigs. Of 14 pigs that survived endotoxic shock following intravenous inoculation with extracellular fluids, extracts or extracts treated by the method of Clugston & Nielsen (1974) of strain 711 VT⁺LT/ST⁺(P101), 10 subsequently developed clinical signs of oedema disease and of these six had gastric oedema (Table 8, Fig. 2). A higher proportion of the pigs inoculated with similar preparations of 711 VT⁻LT/ST⁺(P101) died from endotoxic shock; none of the six that survived subsequently exhibited any clinical or pathological signs of oedema disease. These pigs had originated from the same six litters as those given the VT⁺ preparations. Endotoxic shock, when present, became evident immediately after inoculation and had disappeared completely by 24 h. The signs of oedema disease, principally ataxia and mental confusion, commenced at this time and usually became progressively worse, some of the affected pigs being unable to rise at 48 h, the time when most pigs were killed for pathological examination.

DISCUSSION

The results of the survey imply that VT production is not a common feature of *Escherichia coli* in the U.K. and that it is mainly confined to a few serogroups that cause enteric disease in human beings or pigs. Amongst the 'classical' human enteropathogenic serogroups (EPEC), its incidence and its apparent confinement to O26 and O128 strains is in agreement with the findings of Scotland *et al.* (1980a). Konowalchuk *et al.* (1977), working in Canada, reported a

Fig. 2. Stomach of a pig inoculated with an extract of *E. coli* strain 711 VT⁺LT/ST⁺(P101). Extensive submucosal oedema can be seen in the cut surface of the wall. ($\times 3$).

higher incidence in human strains, with VT production being found in serogroups additional to O26 and O128. Amongst pig enteropathogenic strains, no correlation could be found between VT status and production of α -haemolysin, K88 antigen or LT and ST enterotoxins; some VT⁺ and some VT⁻ strains possessed these characteristics, others did not. Furthermore, the removal of plasmids determining these characteristics or their transfer to other bacterial hosts was not accompanied by a corresponding loss or acquisition of VT production. The one characteristic that the VT⁺ pig strains had in common was their aetiological role in enteric infections, especially oedema disease, of weaned animals. Of the four main serogroups that are implicated in these infections in the U.K., all the O141:K85 and O141:K85,88 strains and about half the O138:K81 and O139:K82 strains were VT⁺; Konowalchuk *et al.* (1977) and Kashiwazaki *et al.* (1980, 1981) also found two O138:K81 strains, an O139:K82 strain and an untypable oedema disease-producing strain to be VT⁺. No reason could be found for the VT⁻ status of some of our O138:K81 and O139:K82 strains. They may have lost their VT genes during their long stay in this laboratory. Most of the strains, though, were maintained both on egg media at 5 °C and lyophilized, and no difference was detected in VT reaction between representatives of the same strain kept under each condition.

The results demonstrated that the transferable nature of VT production in the two *E. coli* O26 strains, H19 and H28, and the two *E. coli* O128 strains, H12 and H23, was due to the genes responsible for VT synthesis being incorporated in the genome of the phages with which these strains were lysogenized. Experiments with the λ^- K12 strain, C600, showed that lysogenization with phage of each of two O26 strains rendered C600 VT⁺. This did not always happen when the λ^+ K12 strain, 711, was used instead of C600; some organisms remained VT⁻ after lysogenization. Since this incomplete correlation between the acquisition of the phages and the VT genes of the O26 strains was also observed, albeit to a lesser extent, in the case of the λ^+ form of C600, it is possible that the λ phage itself may be implicated in some way. That two kinds of phage particles shown to be dissimilar by cross-resistance tests mediated VT transfer in both H19 and H28 strongly points to the common identity of these two strains despite the fact that H19, but not H28, possessed conjugative plasmids determining antibiotic resistance and colicin production. At what period in their evolutionary development they and the VT⁺ O128 strains became lysogenized is of interest bearing in mind that their phages were not active on any of the VT⁻ O26 or O128 strains and that these strains were not found to be carrying phages that made them resistant to the VT-determining phages. The fact that rough forms of one of the VT⁻ O26

strains, H1A, were susceptible to the H19A and H28A phages suggested that they became lysogenized at a period when they did not possess their present O antigen. Whether the VT genes were already incorporated in the phage genome or became so later is conjecturable. No conclusive information was obtained, either, on the location of the phages, and hence of the VT genes, in the O26 and O128 strains. The studies of Scotland *et al.* (1980*b*) produced no positive evidence for an extrachromosomal location of the VT genes in H19. The failure of VT genes to become established in some λ^+ K12 organisms, but not in λ^- K12 organisms, that had been lysogenized with the O26 VT-determining phages favours a chromosomal location. If this is indeed so, then the fact that C600 organisms were obtained that were lysogenized with both the O26 and the O128 VT-determining phages suggests that the VT genes can occupy at least two sites in the C600 chromosome.

In contrast to the VT of the four human *E. coli* strains, the VT of the pig strain, P101, was not phage-mediated. Plasmids may well have been implicated. If so, then the VT genes were not located on the same plasmid as the Hly, K88, LT or ST genes of P101, because none of the VT⁺ *E. coli* K12 organisms isolated from mixed cultures of P101 and K12 were Hly⁺ or K88⁺ and, although they were LT⁺ and ST⁺, so were some of the VT⁻ isolates. It is possible, though, that the enterotoxin plasmids might have been involved in their transfer.

Cross-neutralization tests revealed that there were at least three antigenically distinct VTs, those of the human *E. coli* strains, the pig *E. coli* strains and the *P. aeruginosa* strains. The latter were grouped together because they were not neutralized by any of the *E. coli* antisera; they were also heat resistant, unlike the VTs of the human and pig *E. coli* strains. By contrast, Konowalchuk *et al.* (1977) found that an antiserum against an O26 VT⁺ strain did not neutralize the VT of an O128 strain. However, it did neutralize the VT of an O18, an O111 and an O126 human *E. coli* strain. It failed to neutralize the VT of an O138:K81 pig strain, an observation that was confirmed by Kashiwazaki *et al.* (1981) using antisera against two O26 strains. These workers failed to produce an antiserum against the VT of the O138:K81 strain. It is noteworthy that a long course of vaccination was required to produce antiserum against the VT of our pig strain, P101, and then its titre was low, an observation that provides further evidence of the poor antigenicity of the VT of pig strains.

That genetic material additional to that coding for antibiotic resistance and colicin production is transferred from the O26 strain H19 to *E. coli* K12 during mixed culture was first demonstrated by Smith & Linggood (1971) by means of the ligated rabbit intestine test. Organisms that had received this material, designated Ent⁺, were later shown by Scotland *et al.* (1980*a*) and in the present work to be VT⁺. In their rabbit studies, Smith & Linggood inoculated ligated intestinal segments with 4 ml of extracts of glucose agar cultures, a test dose greater than the 1 ml of extracellular fluids of Evans broth cultures that most investigators use in this particular model. In the present work, this lower dose of extracts of *E. coli* K12 possessing the VT genes of H19 or of the other three VT⁺ human strains failed to dilate ligated intestinal segments whereas similar doses of extracts of K12 possessing the 'classical' LT/ST genes of *E. coli* P307A did cause dilatation. To produce a positive response with the VT⁺ K12 strains a dose of 4 ml, preferably prepared from glucose agar cultures, was necessary. Since this dose of extracts of the VT⁻ parent K12 strains did not provoke an accumulation of fluid in ligated segments it can be concluded that the four human strains possess enterotoxigenic activity, albeit of a lower potency than P307A and, probably, of 'classical' LT⁺ strains in general. It must be appreciated, though, that these observations apply to ligated rabbit intestine inoculated with the products of bacterial growth in artificial culture media. Whether they accurately reflect the response of the intact human intestine to the products of VT⁺ and LT⁺ organisms proliferating at its epithelial surface is another matter. Efforts to demonstrate an enterotoxigenic role for the VT genes of the pig enteropathogenic strain, P101, were complicated by the fact that our isolates of *E. coli* K12 that had received its VT genes had also received its 'classical' LT/ST genes. However, the failure of extracts of these isolates to dilate ligated rabbit intestinal segments after treatment with antisera prepared against the LT of P101 and P307A suggested that the VT genes of P101 have no such role. This, and the failure of VT⁺ LT⁻ ST⁻ O138:K82 strains to dilate ligated segments of pig intestine, suggest that the pig VT does not play an important part in the

aetiology of diarrhoea. The finding that several of the pigs inoculated with extracts of the K12 VT⁺(P101) strain developed the clinical signs and pathological lesions of oedema disease, together with the finding in our survey that a high proportion of *E. coli* strains that cause this disease are VT⁺, implies that it may instead be more importantly involved in this aspect of *E. coli* infection. However, because the number of pigs used in these toxicity tests was small and because several of those inoculated with extracts of the K12 VT⁻(P101) strain died from endotoxic shock before they could have developed signs of oedema disease, further work is necessary to definitely establish VT as an aetiological factor in oedema disease.

The fact that lysogenization of *E. coli* K12 with the phages of the four human enteropathogenic strains resulted in the acquisition of the ability to destroy Vero cells, dilate rabbit intestine and kill mice and rabbits strongly suggests that one toxin is responsible for all three phenomena. Supporting evidence for this was provided by the findings that extracts of the lysogenized K12 strains that had the highest VT titres were also the most enterotoxic and the most lethal for mice and rabbits and that all three properties of the extracts had the same degree of heat stability and were neutralized by the same antisera. Final proof of their common identity, however, must await more precise examination of the genome of the phages. Should it be confirmed, then it may be better for the toxin to be named in a manner that emphasizes its pathogenic effects rather than its effect on Vero cells, especially as the latter effect, although diagnostically valuable, is not specific to this particular VT; to continue with the name 'Vero cell toxin' would seem as inappropriate as referring to the 'classical' LT as Chinese hamster ovary cell toxin. The actual naming of the toxin, though, should take into consideration recent studies on its common identity with *Shigella dysenteriae* toxin (O'Brien *et al.*, 1982, 1983); the similarity between the clinical signs and pathological lesions in our rabbits inoculated with this toxin and those well documented for *Sh. dysenteriae* toxin accord with this observation. It is further suggested that the naming of the VT produced by the pig enteropathogenic strains be deferred until additional information is available on its association with oedema disease.

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REFERENCES

- CLUGSTON, R. E. & NIELSEN, N. O. (1974). Experimental oedema disease of swine (*E. coli* enterotoxemia). I. Detection and preparation of an active principle. *Canadian Journal of Comparative Medicine* **38**, 22-28.
- EVANS, D. G., EVANS, D. J. & PIERCE, N. F. (1973). Differences in the response of rabbit small intestine to heat-labile and heat-stable enterotoxins of *Escherichia coli*. *Infection and Immunity* **7**, 873-880.
- GLOVER, S. W. (1968). In *Experiments in Microbial Genetics*, p. 17. Edited by R. C. Clowes & W. Hayes, Oxford & Edinburgh: Blackwell Scientific Publications.
- HIROTA, Y. (1960). The effect of acridine dyes on mating type factors in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **46**, 57-64.
- KASHIWAZAKI, M., OGAWA, T., ISAYAMA, Y., AKAIKE, Y., TAMURA, K. & SAKAZAKI, R. (1980). Detection of Vero cytotoxic strains of *Escherichia coli* isolated from diseased animals. *National Institute of Animal Health Quarterly (Japan)* **20**, 116-117.
- KASHIWAZAKI, M., OGAWA, T., NAKAMURA, K., ISAYAMA, T., TAMURA, K. & SAKAZAKI, R. (1981). Vero cytotoxin produced by *Escherichia coli* strains of animal origin. *National Institute of Animal Health Quarterly (Japan)* **21**, 69-72.
- KONOWALCHUK, J., SPEIRS, J. I. & STAVRIC, S. (1977). Vero response to a cytotoxin of *Escherichia coli*. *Infection and Immunity* **18**, 775-779.
- O'BRIEN, A. D., LAVECK, G. D., THOMPSON, M. R. & FORMAL, S. B. (1982). Production of *Shigella dysenteriae* Type 1-like cytotoxin by *Escherichia coli*. *Journal of Infectious Diseases* **146**, 763-769.
- O'BRIEN, A. D., LIVELY, T. A., CHEN, M. E., ROTHMAN, S. W. & FORMAL, S. B. (1983). *Escherichia coli* O157:H7. Strains associated with haemorrhagic colitis in the United States produce a *Shigella dysenteriae* 1 (Shiga)-like cytotoxin. *Lancet* **i**, 702.
- SCOTLAND, S. M., DAY, N. P. & ROWE, B. (1979). Production by strains of *Escherichia coli* of a cytotoxin (VT) affecting Vero cells. *Society for General Microbiology Quarterly* **6**, 156-157.
- SCOTLAND, S. M., DAY, N. P. & ROWE, B. (1980a). Production of a cytotoxin affecting Vero cells by strains of *Escherichia coli* belonging to traditional enteropathogenic serogroups. *FEMS Microbiology Letters* **7**, 15-17.
- SCOTLAND, S. M., DAY, N. P., WILLSHAW, G. A. & ROWE, B. (1980b). Cytotoxic enteropathogenic *Escherichia coli*. *Lancet* **i**, 90.
- SMITH, H. WILLIAMS & GYLES, C. L. (1970). The relationship between two apparently different enterotoxins produced by enteropathogenic strains of

- Escherichia coli* of porcine origin. *Journal of Medical Microbiology* **3**, 387–401.
- SMITH, H. WILLIAMS & LINGGOOD, M. A. (1971). The transmissible nature of enterotoxin production in a human enteropathogenic strain of *Escherichia coli*. *Journal of Medical Microbiology* **4**, 301–305.
- TOMOEDA, M., INUZUKA, M., KUBO, N. & NAKAMA, S. (1968). Effective elimination of drug resistance and sex factors in *Escherichia coli* by sodium dodecylsulphate. *Journal of Bacteriology* **95**, 1078–1089.
- WADE, W. G., THOM, B. T. & EVANS, N. (1979). Cytotoxic enteropathogenic *Escherichia coli*. *Lancet* **ii**, 1235–1236.
- WILLIAMS, P. H., SEDGEWICK, M. I., EVANS, N., TURNER, P. J., GEORGE, R. H. & MCNEISH, A. S. (1978). Adherence of enteropathogenic strains of *Escherichia coli* to human intestinal mucosa is mediated by a colicinogenic conjugative plasmid. *Infection and Immunity* **22**, 393–402.
- WILSON, M. W. & BETTELHEIM, K. A. (1980). Cytotoxic *Escherichia coli* serotypes. *Lancet* **i**, 201.