Identification of *slt-II* gene homologue in a selection of pathogec bacterial species

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Abstract

Identification and characterization of bacterial genes that are induced during the disease process are important in understanding the molecular mechanism of disease and can be useful in designing antimicrobial drugs to control the disease. A molecular genetic approach was taken using Southern blot hybridization to gain clearer picture of the distribution of nucleotide sequences homology of the Shiga-like toxins gene (*slt-II gene*) in different strains of gram-negative bacteria. To identify and distinguish those strains of gram-negative bacteria carrying a sequence of gene encode VT2-A and B subunits toxin, cloned DNA fragment and Polymerase chain reaction (PCR) amplification fragment were used as specific probes, large scale isolation of chromosomal DNA from different bacterial species was performed, digested with restricted enzyme (EcoR1) and subjected to Gel Electrophoresis through 1% (w/v) agarose gels. Initially, an 850-base-pair Sma1-Pst1 (Ava1-Pst1) cloned DNA specific gene probe, present in the A-subunit of the Shiga-like toxin II (*slt-II*) gene, was provided and used to hybridized the chromosomal DNA of the bacterial strains. This fragment obtained from the recombinant plasmid NTP707, which carries a 4.7 Kb *Eco*RI fragment encoding *slt-II* operon. The plasmid (pNTP707) was digested with *Eco*RI, and the isolated 4.7-kb fragment was digested with enzymes Sma1 and Pst1, yielding fragment of approximately 850 bp containing sequences of VT2 (*SLTII*) gene A-subunit. These fragments were chemically labelled by DIG-labelling. A *slt*-II specific probe was generated by PCR, positive amplification was obtained, resulting in a band of the expecting size on an agarose gel and was used as probe. Two sets of oligonucleotides primers were used, with the first pair homologous to VT2-A subunit gene and the second pair homologous to the VT2-A and part of B-subunit genes. About 90% Nucleotide sequences homologue of *Eco*RI fragment was identified in *Klebsiella luteus* strain have been detected by Southern hybridization and direct sequence analysis of the PCR product generated from these bacteria. VT production in several *E. coli* strains of human origin is due to the presence of lysogenic phages. No signal was visible on the DNA from any other strain even at low stringency (2x SSC, 64°C). This was the first step to proving that part of the *slt* gene sequence is present in *K. luteus*. *K. luteus* chromosomal DNA was cut using *Eco*RI and *Xba*I, and by both. A significant result was obtained by hybridizing an EcoR1 fragment digest from *K. luteus* chromosomal DNA, which gave two bands in different size ranges, approximately 4.7 and 5.5 kb. This also suggested that this strain might have two copies of nucleotide sequence homologous to the *slt-II* gene. The results in this study suggest that the oligonucleotide probes can provide important information about the diversity of the *slt-I* and *slt-II* genes

1. Introduction

Pathogenesis is the process by which pathogens cause disease or disorder, often by expressing virulence-factor-encoding genes [Pakbin el al., 2020]. Virulence factors are specific molecules, primarily proteins produced and released by bacteria, fungi, protozoa, and viruses. These factors are encoded by specific genes located on the chromosome or mobile genetic elements (e.g., plasmids or transposons) in bacterial pathogens [Alegbeleye el al., 2018]. *Escherichia coli* is a genetically diverse bacterium commonly present in a wide range of environments (Kim el al., 2020). There are six recognized enteric pathotypes of *E. coli*, including Shiga toxin-producing *E. coli* (STEC) (Denamur el al., 2021). About nine diseases have been described for *E. coli* strains isolated from humans causing diarrheagenic and extraintestinal diseases [Nash el al., 2010]. All enteric *E. coli* pathogens are a significant cause of illness and mortality, but the risk of potentially life-threatening illness with long-term sequela, and limited treatment options (Agge el al., 2015). Many of these pathotypes constitute public health concerns as foodborne pathogens and caused several fatal outbreaks in developing and developed countries (Alegbeleye el al., 2020). Children, the elderly, and immuno-compromised people are the most susceptible to this type of infection (Doyle el al., 1991, Padhye el al., 1992). STEC infections can result in a range of patient outcomes, including asymptomatic infection, uncomplicated diarrhea, bloody diarrhea (BD), and life-threatening hemolytic uremic syndrome (HUS) (Karpman el al., 2013). Enterohemorrhagic *E. coli* (EHEC) cause diarrhea, hemorrhagic colitis (HC) with bloody diarrhea, and hemolytic uremic syndrome (HUS) in humans and are implicated in several foodborne outbreaks in developed countries. EHEC as foodborne A/E pathogens are mainly transmitted to humans through contaminated food and water [van Hoek el al., 2019]. The most important serotype playing a role in EHEC outbreaks is *E.coli* O157: H7, which is still considered a serious health concern in Japan, Europe, and North America. The Shiga-like toxin (SLT), also termed Verotoxin and encoded by *stx* genes, is the main virulence factor in EHEC serotypes which belongs to the Shiga-toxin producing *E. coli* group and is responsible for pathological manifestations leading to specific disease symptoms caused during EHEC infections, such as HUS and renal failure [Joseph el al., 2020]. SLT includes two subgroups, stx1 and stx2, and different subtypes. Stx2a, stx2c, and stx2d positive EHEC isolates are strongly associated with HC and HUS compared to other stx-subgroups and subtypes [Ferdous el al., 2016], both toxins are made up of A and B subunits (Yutsudoet al., 1987; reviewed by O'Brien and Holmes, 1987). SLT production in several *E. coli* strains of human origin is due to the presence of lysogenic phages. SLT -encoding phages were first isolated from *E. coli* H19, serotype 026 : H11 (Scotland et al., 1983; Smith et al:, 1983). Bacteriophage 933W, a well-studied Stx-converting phage, can self-induce from *E.coli* O157:H7 EDL 933 strain and subsequently infect other susceptible *E.coli* strains to form a new lysogen (Bullwinkle el al., 2012). Several methods have been employed for the detection of VTEC infection, however, testing of individual isolates may result in poor sensitivity of detection, unless very large numbers of isolates are analyzed. Furthermore, Karch et al. 1989b have recently reported a high frequency of loss of SLT genes from clinical isolates of *E. coli* during subculture. Specific DNA probes for *slt-I* and *slt-II* genes that do not cross-hybridize with each other have been developed from cloned genes (Willshaw et al., 1985, 1987). Strains could also be tested for the presence of the various SLT genes by hybridization to DNA or oligonucleotide probes (Thomas el al., 1991) or by polymerase chain reaction (PCR) amplification (Brian el al., 1992). SLT genes have been cloned in *E. coli* strain K12 from phages originating in strains of serogroups 026 and 0157 (Newland **et** al., 1985; Willshaw **et** al., 1985,1987; Huang **et** al., 1986). The aim of this study to suggest that

the oligonucleotide probes can provide important information about the diversity of the *slt-I* and *slt-II* genes.

Material and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in material and methods and cultures were routinely grown in LB, NB and Penassay broth or on LB agar plates at 37°C. VTEC and non-VTEC reference culture isolates, included *E. coli* O157:H7 (stx1 & stx2 genes) and an *E.coli* O157:H7 strain carrying only the stx2 gene were from Biological Science Department, University of Warwick. The *E. coli* K12 strain 36R363 which carries a recombinant plasmid (NTP707) with a 4.7 kb fragment of the *slt*-II operon was kindly provided by Dr. B. Rowe, Central Public Health Laboratory, London. Large scale extraction of chromosomal DNA was performed by modification of the method of Maniatis *et al*. (1982).

Large scale preparation of plasmid DNA using a Cesium Chloride Gradient

An overnight culture (35 ml) of bacteria in LB medium with the appropriate antibiotics was pelleted using an MSE centrifuge at 4000g, for 20 min at 4°C. The cell pellet was washed in TES buffer, centrifuged and resuspended in STE buffer by vortexing. 1ml of lysozyme solution (10 mg ml^{-1}) dissolved in 0.25M Tris buffer, pH8.0, was added and the solution incubated at 40°C for 5 min with occasional inversion. 2.5 ml EDTA (0.5 M, pH 8.0) was added and the suspension was left for a further 10 min. 16 ml of Triton lysis buffer was added rapidly, the solution shaken vigorously, and incubated for 20 min on ice. The suspension was centrifuged at 14000g for 30 min at 4°C. The supernatant was decanted through muslin into a 200 ml measuring cylinder containing 28.5 g cesium chloride. The volume was made up to 38 ml with TES buffer and cesium chloride was dissolved before adding ethidium bromide. The mixed solution was transferred to an Oakridge centrifuge tube, 2ml of 5 mg ml^{-1} ethidium bromide solution (EtBr) was added and the tube was centrifuged at 18000g for 20 min at 4°C. The supernatant was decanted into Beckman heat-seal Vti50 centrifuge tubes, through a 20 ml syringe containing siliconised glass wool and a 16 gauge needle. The tubes was topped up to the neck with cesium chloride (71.25% w/v aqueous solution) and heat sealed. The balanced tubes were centrifuged (Beckman ultracentrifuge, 45,000g) for 14 to 24h at 20°C. The lower band, which was usually the plasmid band, was identified using long wave UV. The top of the tube was pierced by a needle and the plasmid band carefully removed in a maximum volume of 5ml, using a 10 ml syringe with a 16 gauge needle inserted just below the band. EtBr was removed by extracting the sample 5 times with an equal volume of sodium chloride-saturated isopropanol. The plasmid DNA was precipitated by adding 2 volumes distilled water and 2 volumes of ethanol. After 2 to 24 h at -20°C, the sample was centrifuge at 14000g for 30 min at 4°C. The pellet was washed with 70% (v/v) ethanol to remove residual cesium chloride. The DNA was air dried and re-dissolved in 2ml TE buffer and stored at -20°C until required.

Preparation of bacterial chromosomal DNA

Overnight cultures (25 ml) were pelleted and the pellet frozen at -70°C for 30 min and thawed on ice. The pellet was re-suspended in 10 ml Tris-EDTA solution. 1ml lysozyme solution was added with 100 µl of proteinase K solution (20 mg ml^{-1}) and the tube was placed on ice for 10 min. 250 µl of 10% (w/v) SDS was added and the tube was placed in a 65°C water bath for 30 min until the solution had cleared. 1.5 ml of sodium perchlorate was added and the tube was again incubated at

65°C for 20 min with occasional mixing. The volume was made up to 30 ml with Tris-EDTA, split into three universals and extracted three times with a phenol/chloroform (1:1) mixture and once with chloroform alone. The extracts were collected in a clean beaker and ethanol (two volumes) was added gently. The genomic DNA was precipitated and collected using a glass rod in a gentle swirling/spooling action. The DNA was air dried and re-suspended in Tris-EDTA.

Restriction endonuclease treatment of DNA

Restriction endonuclease digests were usually performed in a final volume of 20 µl. The digestion of chromosomal and plasmid DNA was performed according to the manufacturer's recommendation (BRL, New England Bio labs or Amersham) most usually for an hour at 37°C. "Double enzyme" digests were performed in the tube if the restriction buffers were similar. A two step reaction using the lower salt concentration buffer first and then altering the salt concentration by the addition of 1M NaCl (heat treatment was used to denature the first enzyme) was used. The sample was then re-incubated with the second enzyme for another hour. If the buffers were incompatible the DNA was cut with one enzyme for an hour then extracted with phenol chloroform, recovered by ethanol precipitation and re-suspended in 15µl of TE before the second restriction enzyme digest. All restriction digests were analysed by agarose gel electrophoresis and if a band was required it was purified by Gene clean.

Oligonucleotides used as primers

The sequence of the two pairs of oligonucleotide primers (forward and reverse) which were used in this study for the amplification of the *slt*-II genes and for the synthesis of the DIG-labelled hybridisation probes used in PCR were designed from published nucleotide sequences of Shiga-like toxin type II (*slt*-II) structure genes cloned from 933W bacteriophage of the enterohaemorrhagic *E.coli* O157:H7 strain 933 (Jackson *et al.*, 1987a). The nucleotide sequence of each primer and the corresponding locations within the VT2 gene are given in Table 1.

Table 1: Base sequences, locations, and predicted size of amplified products for the VT2-specific oligonucleotide primers

Primer Designation	Oligonucleotide sequence (5′-3′)	Location within gene	Size of Amplified product (bp)	Length of primer (bp)
VT2-a	5′ GCATAGCTCATCG GAACAAG	4-23	737	20
VT2-b	5′ - CTGAACTCCATTAA CGCCAG	722-741		20
VT2-c	5′ GGTTCGAATCCAG TACAACG	124-143	1240	20
VT2-d	5′ - CAGCGACTGGTCC AGTATTC	1345-1364		20

The Polymerase Chain Reaction (PCR)

The PCR technique was used two pairs of primers. In this technique a DNA Thermal Cycler (Perkin-Elmer, Norwalk, USA) was used. A Taq polymerase enzyme with buffer (GIBCO) was

used as per the manufacturer's instructions. The PCR composition was as follows: 50 mM $MgCl_2$ (5 µl), 10x Taq polymerase buffer (5µl), 100 ng μl^{-1} Primer I (forward primer) (1µl), 100 ng μl^{-1} Primer II (reverse primer) (1µl), 100 ng μl^{-1} Genomic DNA (1µ), 10 mM. DNTP (1µl), Sterlised H_2 O 35.5µl PCR consisted of 94°C for 45 sec, 50°C, 55°C, 65°C or 70°C for 45 sec or 1min and 72°C for 45sec. PCR was repeated for 35 cycles.

Southern blot protocol

The Southern blot transfer method was carried out following the procedure of Maniatis *et al*. (1982).

Incorporation of Digoxigenin-11-dUTP during PCR

Digoxigenin-11-dUTP (DIG-dUTP) was incorporated by Taq DNA polymerase during the polymerase chain reaction by using a PCR DIG Probe according to the manufacturer's instruction. Synthesis kit (Boehringer Mannheim DIG system). Standard conditions were used. To a 500 µl tube, was added, 1µl DNA (100 ng µl-1 chromosomal DNA), 5 µl reaction buffer (5x), 2 µl dNTP labelling mixture (Boehriger Mannheim), 10 µl of each primer (~1.5 ng final concentration; 1 µM stock) and 4 5 µl mM MgCl2, 18 µl H2O. DIG labelled oligonucleotide was purified by agarose gel 1.0% (w/v) electrophoresis (the presence of the DIG label in the oligonucleotide causes a slower rate of migration during gel electrophoresis). Labelled PCR product was cut from the gel and individual fragments of DNA were isolated after recovery from the gel using the 'Geneclean II' kit (Bio 101 Inc.) following the manufacture's instruction, before being resuspended in 15 µl of TE buffer. The labelled oligonucleotide was stored at -20°C until required. Prior to hybridisation, the labelled probe was heated to 100°C for 10 minuts and then placed on ice for 5 minutes, before being added to the hybridisation solution. The amplified DNA products were labelled chemically by including 17.5 µM DIG in a 20 µl PCR mixture during the polymerase chain reaction (PCR DIG Probe Synthesis kit section Boehringer Mannheim Biochemical, Inianapolis, Ind.).

Probe synthesis

Probes used in the Southern blot hybridization were labelled using the non-radioactive digoxgenin-11-dUTP (DIG) kit (Bohehringer Mannheim) by either Random Primed DNA labelling or Incorporation of Digoxigenin-11-dUTP during PCR; according to the manufacturer's instruction. The labelled DNA to be used as a probe, generated by PCR, was always purified from an agarose gel using 'gene clean' before use. Non-radioactive probes were generally made using PCR because of the more efficient labelling of the probe. Hybridization of DIG probes to Southern filters. Hybridizations of Southern blot filters with a digoxygenin -11-dUTP labelled (DIG) DNA probes were carried out essentially according to the manufacturer's instructions. exposed to X-ray film (Fuji medial) at room temperature, the exposure time varied between 1-16h.

DIG detection

Several alternative methods were used for the detection of the DIG-labelled probes. The Boehringer Mannheim DIG system was used for the detection of DNA probes in Southern hybridisation**.** The membrane was blocked for 30 min. using 1% (w/v) non-fat dry milk in buffer1 (100 mM Tris pH 7.5, 150 mM NaCl). Following a brief wash in buffer, the membrane was incubated for 30 min with a 1:5,000 dilution of AP-conjugated anti-Digoxygenin Fab fragment in buffer 1. The membrane was washed for 30 min. in buffer 1, equilibrated for 2 min. in buffer2 (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and developed using 40 µl of the AP substrates nitro blue tetrazolium (75 mg/ml in dimethyl formamide) and 5-bromo-4-chloro-3-

indolyl phosphate (50 mg/ml in dimethyl formamide) per 10ml of buffer2. Colour development was terminated in 2 to 3min by briefly washing the membranes in 10mM Tris (pH 8.0), 1mM EDTA and air drying the colorimetric substrates (NBT) and (BCIP) to generate a purple/brown precipitate directly on the membrane. DNA templates were phenol: $CHCI_3$ extracts prior to incorporation of Digoxigenin-11-dUTP.

Nucleotide Sequencing of PCR product

PCR products to be sequenced using the 373A DNA Sequencer (Applied Biosystems) were extracted from agarose gels using the Gene clean Kit. A 0.03 μg - 0.09 μg of DNA (PCR) and 1.6 pmol primer were made up to a final volume of 6 μl with H_2O in 0.5 ml tube mixed and sent for automated sequence analysis. Sequencing was carried out using the Taq Dye Deoxy ™ Terminator Cycle Sequencing (Applied bio system) Kit. The cycles used in this reaction were 96 °C for 30 sec.; 50°C for 15 sec.; 60°C for 4 min. for a total of 30 cycles After reading the sequence, the data were further analysed using the GCG data base and the results displayed via the Gene-Doc programme.

Result

To gain a clearer picture of the distribution of the *slt*-II gene in a range of different bacteria, a molecular genetics approach was taken using hybridization to detect homologues of this gene. Initially, Initially, the recombinant plasmid pNTP707 digested with *Eco*RI, was constructed from insertion of a 4.7 kb *Eco*RI fragment encoding the entire *slt*-II operon (*slt*-II A and *slt*-II B genes (fig. 1A), into a chloramphenicol (Cm^r) resistance gene of vector plasmid pACYC184 (4244 bp) (Willshaw et al., 1987) (fig.1b**)**. The construction of generated recombinant plasmid pNTP707 shown (fig.2) The pNTP707 was prepared from *E. coli* K12 (60R363) by the caesium chloride density gradient method (as described in methods), and introduced into *E. coli* CC118 by electroporation and the transformants were suspended in LB medium. Several dilutions were plated on agar containing tetracycline (Tc) to select for pNTP707.

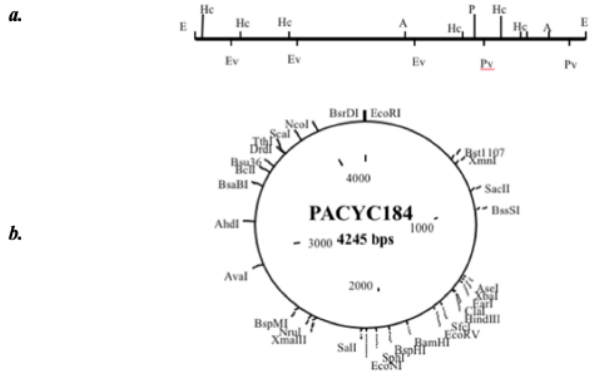

Fig 1 : (a) 4.7 kb fragment containing the *slt* 2 gene b) vector plasmid pACYC184. Restriction enzyme maps

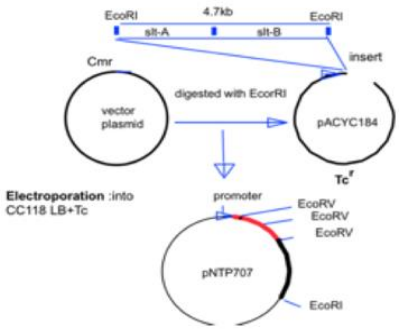

Fig 2*.The recombinant plasmid pNTP707 construction*

In fig. (2) a. Restriction sites are given within the 3250 bp *Sph*I to *Eco*RI fragment isolate from the hybrid plasmid pNN76. b. The location and orientation of the structural genes, *slt*-IIA and *slt*-IIB, are shown above the restriction map.

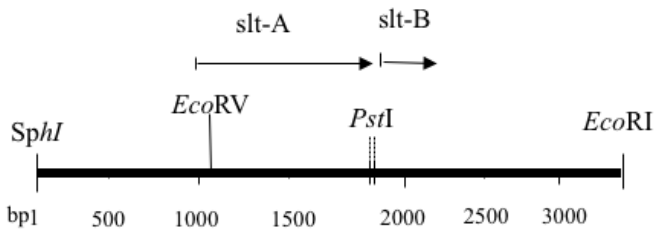

Fig. 3. Restriction enzyme map of the *slt* 2 operon (Jackson et al., 1987b) Restriction sites are given within the 3250 bp *Sph*I to *Eco*RI fragment isolated from the hybrid plasmid pNN76. The location and orientation of the structural genes, *slt*-IIA and *slt*-IIB, are shown above the restriction map.

To confirm the presence of pNTP707 within *E.coli* CC118, plasmid DNA was prepared from trans formants displaying tetracycline resistance (Tc^r), restricted with *Eco*RI and electrophoresed on 1% (w/v) agarose gels. Two fragments were produced, the first fragment was 4.7 kb, which is the insert containing the *slt*-II gene and the second fragment was 4.2 kb, which is the vector plasmid pACYC184 (Fig.4: lane 3 and 4). To ensure maintenance of plasmid during preparation, bacteria were cultured on LB containing kanamycin (50 microg m^{-1}) and tetramycin (15 mig m^{-1}).

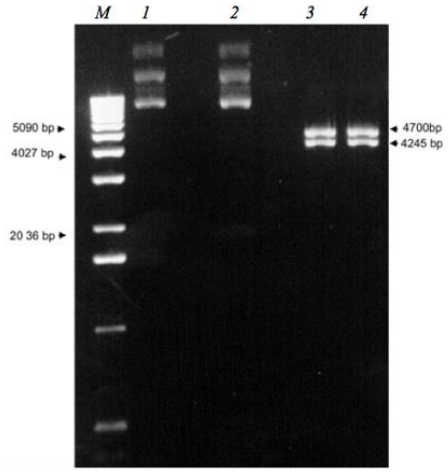

Fig 4. Key to tracks : Lane: M. l ladder 1kb DNA as a molecular size marke. Lane: 1- 2 is plasmid DNA (NTP707)-unrestricted. Lane: 3- 4 is plasmid DNA (NTP707) - restricted with *Eco*RI.

DNA probe for *slt* II gene

Using the whole plasmid pNTP707 as DNA probe for *slt* II gene. The plasmid was linearized and purified by phenol/chloroform extraction and ethanol precipitation prior to labelling with Digoxigenin-11-dUTP, and used as template DNA. Analyses using the whole plasmid contains 4.7 kb, which is the insert of the *slt*-II gene as a probe were unsuccessful may be because during the labelling the digoxigenin-11-dUTP (DIG) diffused in the entire plasmid and become less concentration and sensitivity. No cross-hybridization between the probe, pNTP707, and the DNA from the bacterial strains was observed after stringent washes. No positive signal was observed by Southern blot hybridization, may be because the 4.7 kb *Eco*RI cloned DNA fragment encoding *slt*-II operon was not separated from the vector sequences before labelling.

Using *Slt* II cloned DNA specific gene probe

An 850-base-pair Sma1-Pst1 (Ava1-Pst1) cloned DNA specific gene probe, present in the A-subunit of the Shiga-like toxin II *(slt*-II) gene, was used to hybridize the chromosomal DNA of the different bacterial strains. This fragment obtained from the recombinant plasmid NTP707. The plasmid pNTP707 (~1μg) was digested with *Eco*RI enzyme yielding isolated 4.7-kb fragment

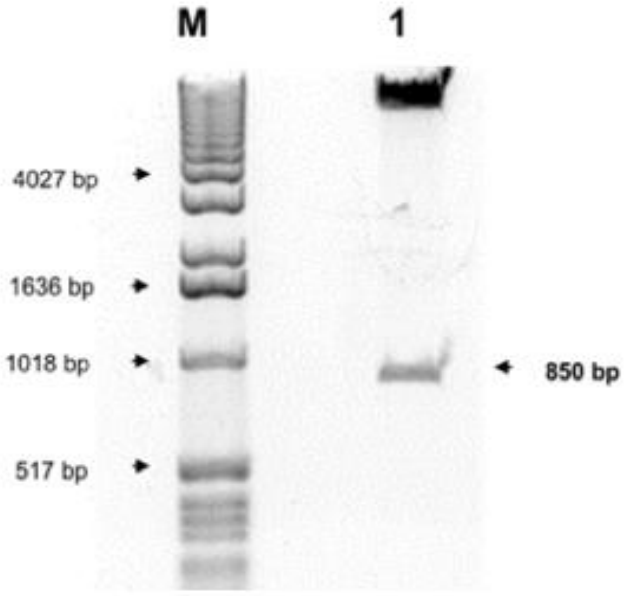

Fig 5 Lane: M. λ ladder 1kb DNA as a molecular size marker. Lane 1 *slt* II fragment (0.85 kb)

The 4.7-kb fragment further digested with the Sma1 and Pst1 enzymes, resulting in a segment of approximately 850 bp (as shown). Overall, it appears that our experiment involved a series of digestions of a plasmid with different restriction enzymes to isolate a specific fragment of DNA (the 850 bp fragment containing the *slt*-II A-subunit gene), which was subsequently used as a probe to hybridize the chromosomal DNA of the different bacterial strains, likely for detecting the presence of this gene in other DNA samples. The DNA fragment were separated by agarose gel and the band was excised from the gel and purified by Gene- Cleaning procedure, the DNA was then labelled with the digoxigenin-11-dUTP (DIG using the non-radioactive digoxgenin-11-dUTP (DIG) kit (Bohehringer Mannheim) by Random Primed DNA labelling
own i n Fig. 5, Lane 1)

Table 2 DNA probes for VT1 and VT2 gene sequences

Strains	slt production	Plasmid carried	Vector plasmid	Inserted DNA Fragment	Probe fragment	Reference
60R363	Yes	NTP707	pACYC184	4.7Kb *Eco*RI	VT2 0.85*Sma*I & *P*stI	Willshaw *et al*., 1987

Southern blot hybridization with a DNA probe

Southern blot hybridisation to detect the presence of homologues of the *slt* II gene in various bacterial species. Large scale isolation of chromosomal DNA from each bacterial strain was performed as described in Maniatis *et al.* (1982). Samples of chromosomal DNA (~5 μg) *from various bacterial species* were digested with *Eco*R1 and *Xa*b1 and DNA fragments separated by 1% (w/v) gel electrophoresis (Fig. 6).

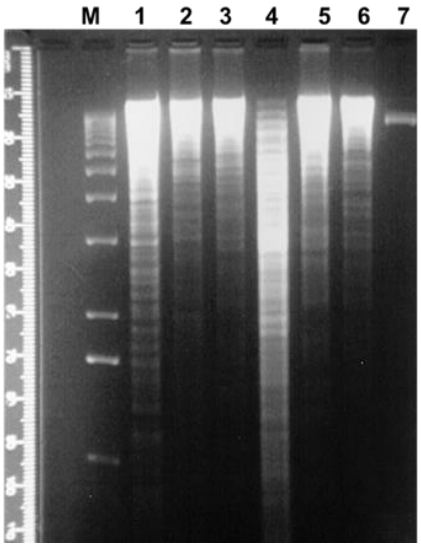

Fig. 6 Restriction digests of chromosomal DNA from selected bacterial species analysed by 1% (w/v) agarose gel electrophoresis. Chromosomal DNA samples from various bacterial species were digested with *Eco*RI. The bacteria species analysed are indicated below. Key to tracks: Lane: M. 1Kb ladde 1. Salmonella enteritidis. 2. Aeromonas hydrophila 3. *E. coli* O157:H7 (*slt*2). 4. *E. coli* K12.5. *E. coli* O157:H7 (VT1+VT2). 6. *Klebsiella luteus*. 7. pNTP 707 (*slt* 2 gene)

Probed with DIG labeled *Sma*I and *Pst*I fragments of the plasmid NTP707 contain the VT2 gene (Random Primed DNA Labelling). The visible bands are due to hybridisation of probe to homologous stretches of nucleotide sequence from the chromosomal DNA. Duplicate gels were blotted onto a nylon filter (Boehringer Mannheim's, Nylon Me. The 850 bp DNA fragment was recovered from the gel, DIG labelled and used to probe the chromosomal digest of a number of bacterial species and plasmid pNTP707. The hybridization was carried out under low stringency conditions at 64°C. After hybridization to the filter and subsequent washing steps the filter was examined using a DIG-Nucleic Acid Detection Kit as described previously. Signals were detected with the specific 850 bp DNA probe, two faint bands from *E. coli* O157:H7 (Fig 7. track 1), and a weak signal was obtained with the DNA from *K. luteus* (track 4). A strong hybridization signal was obtained from the positive control plasmid NTP707 (track 7). No signal was visible on the DNA from any other strain even at low stringency (2x SSC, 64°C). This was the first step to proving that part of the *slt* gene sequence is present in *K. luteus*.

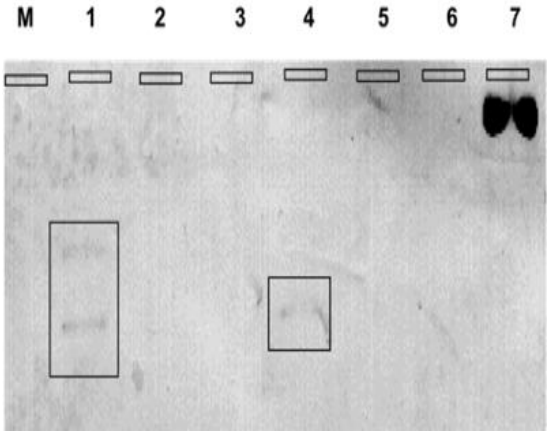

Fig 7. Restriction digests of chromosomal DNA from selected bacterial species analyzed by 1% (w/v) agarose gel electrophoresi Chromosomal DNA from various bacterial species was digested**: Lane: 1**. *E. .coli* O157:H7 (VT2). **2.** *Yersinia enterocolitica*. **3.** *Pseudomonas aeruginosa*. **4.** *Klebsiella luteus*. 5. *Serratia marcescens*.. **6.** *Listeria monocytogenes*. **7.** Plasmid pNTP707 (VT2 gene).

The Polymerase Chain Reaction (PCR)

The effect of annealing temperature on the PCR Annealing temperature

It was important to choose a relatively high annealing temperature 58°C, to guarantee binding of the primers despite minor sequence variations in the target sequence. Different temperatures were used in the PCR experiment, starting with the first pair of primers (VTa /VTb) to select the optimal annealing temperature. Different annealing temperatures were used in the PCR and the resultant products obtained from amplification of *Klebsiella luteus* and *E. coli* O157:H7 chromosomal DNA after a pair of oligonucleotide primers (VTc-VTd) were used, were analysed by 1% (w/v) agarose gel electrophoresis (fig. 8)

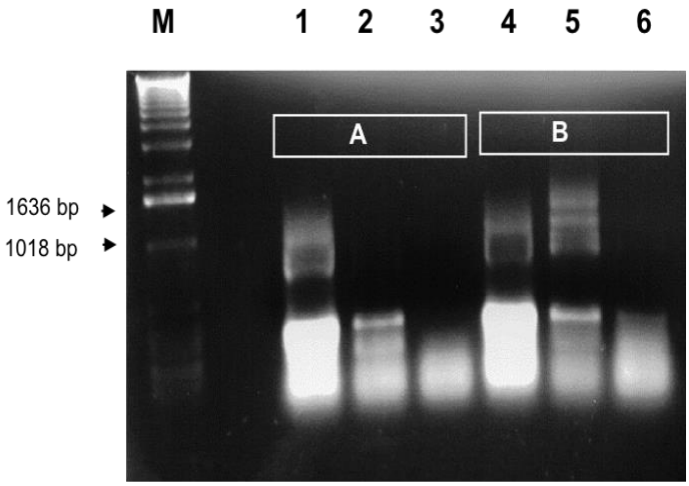

Fig. 8. Effect of annealing temperature on PCR products using oligonucleotide primers (VTc-VTd).
M. molecular weight marker; (A) Lane 1 *Klebsiella luteus*; Lane 2 *E. coli* O157:H7; Lane 3 negative control (no DNA). (B) Lane 4 *Klebsiella luteus*; Lane 5 *E. coli* O157:H7; Lane 6 negative control (no DNA). Annealing temperatures 52°C and 55°C were used respectively.

However, temperatures 52 °C and 55 °C gave extra bands when used with *K. luteus* chromosomal DNA (Fig. 9: lane 1 and 3), but other bands disappeared when the temperature was increased giving a single band at 58 °C (Fig. 10, lane 1). Chromosomal DNA from *K. luteus, E.coli* K12 and *E.coli* O157:H7 were digested with *Eco*RI and analysed by agarose gel electrophoresis. Key to tracks : Lane: M. ladder 1kb DNA as a molecular size marker. 1. *Klebsiella luteus*. 2 *E. coli* K12 negative control. 3 *E. coli* O157:H7 (VT2).

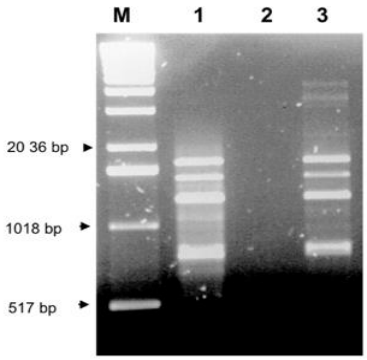

Fig 9 lanes 1and 3 are the products of the PCR reaction of *Klebsiella luteus* DNA at an annealing temperature of 52°C (Lane 1), 55°C (Lane 3) respectively. Lane 2: negative control (no DNA).

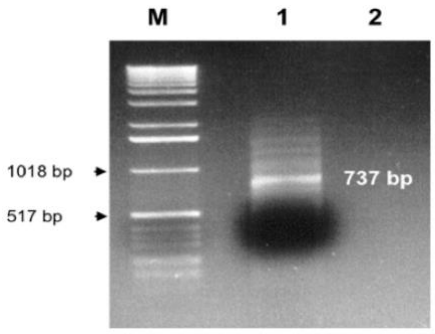

Fig 10 Lane 1. the products of the PCR reaction of *Klebsiella luteus* DNA at an annealing temperature of 58°C. Lane 2: negative control (no DNA). Lane**:** M. is a λ ladder 1kb DNA as a molecular size marker.

The same annealing temperatures of 52°C and 55°C were used to select the optimal temperature for the second pair of primers (VTc / VTd). The result shows that at an annealing temperature of 52°C, *K. luteus* produced very faint bands whilst O157:H7 gave none. There was no PCR product in the negative control (Lane 3) but at 55°C there were several bands obtained from O157:H7 data not shown.

The Polymerase Chain Reaction (PCR)

The PCR technique was used to amplify the *slt* -II gene of interest from chromosomal DNA samples, using a DNA Thermal Cycler (Perkin-Elmer, Norwalk, USA). and to make non-radioactively labelled DNA probes for Southern blot hybridisation studies. A typical program, comprised of an initial denaturing step, followed by primary and then secondary extension, and then final extension. Chromosomal DNA was extracted from the strains described above and gene amplification using PCR was carried out as described in the material and methods. Gene amplification using PCR was carried out as described in the material and methods. To generate a *slt* II gene specific probe by PCR, two pairs of oligonucleotide primers were designed to the termini of the *slt*-II A-subunit coding region. These primers were designed from published sequences for

the *slt* II gene. The primers were designed to produce sequences in the 5′ to 3′ (+sense). The open reading frame for the *slt* II A gene is between 239 and 1193 bp, (with the *N*-terminal arganine of the processed polypeptide depicted as +1). The sense primer, VT-a (5′-GCATAGCTCATCGGAACAAG) was taken from position 4 to 23 and the antisense primer VT-b (5′-CTGAACTCCATTAACGCCAG) was taken from position 722 to 741 bp. The *slt* II B open reading frame is between base pairs 1207 and 1474, (with the N-terminal alanine depicted as +1), and the sense primer, VT-c (5′-GGTTCGAATCCAGTACAACG) was taken from position 124 to 143 and the antisense primer VT-d (5′-CAGCGACTGGTCCAGTATTC) designed from open reading frame for B-subunit was taken from position 1345 to 1364. The reverse oligonucleotide primers, designated VTb and VTd, are listed in the 5′ to 3′ orientation with respect to the noncoding strand. Therefore, these primers appear as the reverse complement of the sequence given at position 722 to 741 and 1345 to 1364 in the *slt*-II A gene Table1. In Fig 11 lane 2. the expected size of the amplified PCR product fragments (1240 bp) were generated from total chromosomal DNA of *Klebsiella luteus* after a pair of oligonucleotide primers (VTc-VTd) were designed for the amplification of the VT2 gene. The products were obtained with an annealing temperature of 58°C. The negative control was a standard PCR mix with only one primer and no chromosomal DNA (Fig. 11 lane 1).

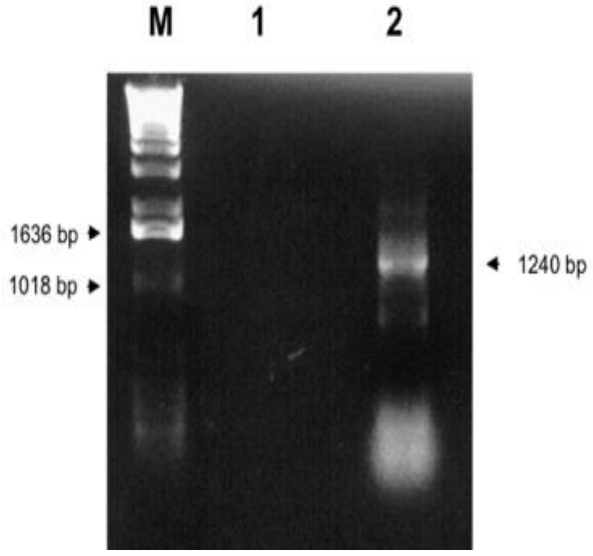

Fig. 11 *Slt* II gene fragments generated from *E. coli* O157:H7 chromosomal DNA and amplified by PCR. Key to tracks : Lane: M. l ladder 1kb DNA as a molecular size marker. 1. Negative control (no DNA). 2. The expected size of PCR fragment (1240 bp) from *Klebsiella* genomic DNA.

Verocytotoxin II gene fragments generated from *E. coli* O157:H7 chromosomal DNA and amplified by PCR.. Oligonucleotide primers (VTc-VTd) were designed to amplify a fragment of (approx. 1240 bp), fig. 12 lane 2 the *slt* 2 gene from the chromosomal DNA of *E.coli* O157:H7. The PCR amplified fragment was labelled by incorporating digoxigenin-11-dUTP by *Taq* DNA polymerase during PCR. The probe was used in Southern blot hybridisation experiments with DNA from various bacterial species. The PCR product (50µl) was electrophoresed on a 1% (w/v) TBE agarose gel. The expected size of fragment (1240 bp) was excised from the gel and subjected to the Gene clean procedure, prior to use as a probe in hybridisation. The products were obtained with an annealing temperature of 58°C.

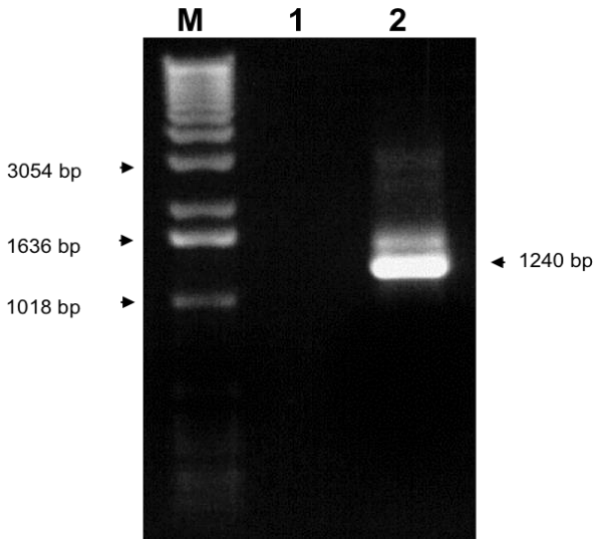

Fig. 12 *slt* II gene fragments generated from E. coli O157: The products were obtained with an annealing temperature of 58°C. **Key to tracks:** Lane**:** M. l ladder 1kb DNA as a molecular size marker 1kb ladder. 1. Negative control (no DNA). 2. The expected size of PCR fragment (1240bp) from *E.coli* O157:H7 genomic DNA.

Incorporation of Digoxigenin-11-dUTP during PCR

Digoxigenin-11-dUTP (DIG-dUTP) was incorporated by Taq DNA polymerase during the polymerase chain reaction by using a PCR DIG Probe according to the manufacturer's instruction. When total chromosomal DNA was used as the PCR template, the generation of even minor amounts of non-specific by-products influenced significantly the specificity of hybridisation to total genomic target DNA. This is due to the high sensitivity of the labelled probes generated during the PCR. The labelled specific PCR product was purified before using it as a hybridisation probe in Southern blot analyses. The PCR mixture was separated by agarose gel 0.7% (w/v) electrophoresis, and the correct band was cut from the gel and extracted as described previously. When using plasmid as a template no purification of the PCR DIG probe was necessary because the minor amount of by-product do not influence the specificity of hybridisation to the chromosomal target. Incorporation of DIG in to the PCR mixture significantly increased the sensitivity of the PCR assay and permitted visualisation of the amplified DNA fragment.

Sensitivity and specificity of PCR and PCR-DIG probes

Sensitivity and specificity of the PCR-DIG probes in hybridisation analysis were evaluated for their capacity to detect the *slt*-II A gene sequences. The sensitivity of the PCR probe was determined using genomic DNA from *E. coli* 0157:H7 (ATCC 43889) that produces *slt*-I and *slt*-II and genomic DNA from *E. coli* 0157:H7 (ATCC 43894) that produces *slt*-II only. These two strains were considered as positive controls in Southern blot analyses. Two sets of oligonucleotide primers were used, with the first pair homologous to the *slt*-II -A subunit gene and the second pair homologous to the *slt*-II-A and part of the B-subunit genes. This level of homology might provide high specificity and equivalent sensitivity among *slt*-II genes in PCR detection. Strains of *E. coli* O157:H7 designated VT positive in Vero cells assay were found to harbour one or both of the VT gene sequences as detected by hybridisation using the PCR reaction (Table .3). The VT2 gene from *E. coli* O157:H7 ATCC (43894) was found to be positive with normal amplification of the *slt*-II gene sequence when total DNA was used as template in the PCR assay (data not shown).

Furthermore, the amplified fragment specific for the *slt*-II gene was clearly detected when the nucleic acid template used in the PCR was less than 1ng. However, no PCR products were obtained with DNA from other strains that did not produce SLT such as *E. coli* K12 and Salmonella. Negative amplification was also obtained from the two strains and these were considered as negative controls (data not shown).

Southern hybridisation with a *slt*-II PCR probe

A *slt*-II gene specific probe was generated by PCR as mentioned previously. Oligonucleotide primers were designated to the termini of the *slt*-II coding region and designated VTc and VTd and the amplification was carried out using the O157:H7 chromosomal DNA as template. The PCR *slt* II DIG labelled product was used to probe the chromosomal digest of a number of bacterial species. Samples of chromosomal DNA (~5 μg) prepared from a selection of bacterial strains were digested with *Eco*R1 (no *Eco*R1 sites within *slt*-II). Restriction digests of chromosomal DNA from selected bacterial species analysed by 1% (w/v) agarose gel electrophoresisa Chromosomal DNA samples from various bacterial species were digested with *Eco*RI. The bacteria species analysed are indicated below Fig 13

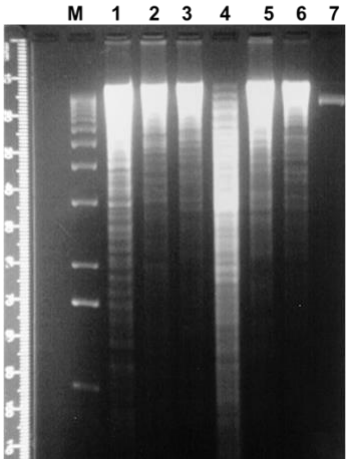

Fig.13 Restriction digests of chromosomal DNA from selected bacterial species analysed by 1% (w/v) agarose gel electrophoresis a Chromosomal DNA samples from various bacterial species were digested with *Eco*R I. The bacteria species analysed are indicated below. Lane**:** M. 1Kb ladder . **1**. *Salmonella enteritidis*..2. *Aeromonas hydrophila* **3**. *E. coli* O157:H7 (VT2) **4.** *E. coli* K12 **5**. O157:H7 (*slt* 1 and *slt* 2 gene). **6**. *Klebsiella luteus*. **7**.pNTP 707 (*slt* 2 gene)

DNA fragments were separated by 1% (w/v) gel electrophoresis (Fig. 13) and blotted onto a nylon filter (Boehringer Mannheim). The 1240-bp DIG -labelled fragment was used in Southern hybridisations of the digested chromosomal samples, and subsequent washing steps were carried out as described previously using low stringency washes. A larger collection of Gram-negative bacteria was analysed by hybridisation to determine which strains carried homologous regions to the verocytotoxin gene. Under the conditions described previously, an interesting result was obtained by probing *Klebsiella luteus* chromosomal DNA with the PCR probe, homologues were detected, as can be seen from Figure.14. (lane 6). *Samples of chromosomal DNA from various bacterial species were digested with EcoRI (Fig.14) and probed with the DIG labelled PCR amplified VT2-gene fragment (1240 bp) from E .coli O157:H7. The visible bands are due to hybridization of probe to homologous nucleotide sequences from the chromosomal DNA present. The bacterial species challenged are indicated below.*

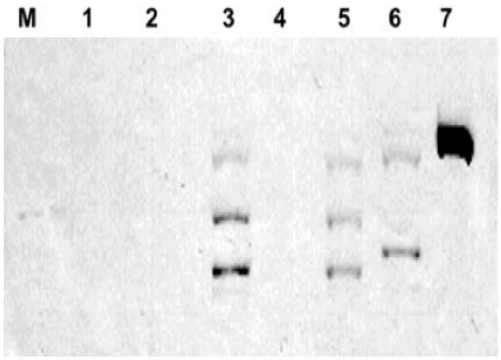

Fig. 14 Southern blot hybridization to detect the presence of homologues of the VT2 gene in various bacterial species. Key to tracks: Lane M. 1Kb ladder 1. *Salmonella enteritidis* (slt 1+slt 2) 2. *Aeromonas hydrophila*. 3. *E. coli* O157:H7 (*slt* 2). 4. *E. coli* K12. 5. *E. coli* O157:H7 6. *Klebsiella luteus* 7. pNTP 707 (*slt*2 gene)

Only, *Klebsiella luteus* was observed to hybridise with the toxin gene probe, all other strains were negative. Chromosomal DNA from *Klebsiella luteus* generated two strong signals of different sizes, 4.7 and 5.5 kb. This implies that this strain has two copies of a nucleotide sequence similar to *slt*-II or related genes. The results in Figure 14 (tracks 3 and 5) also showed that hybridisation to genomic DNA of two *E. coli* O157:H7 (ATCC 43894 and ATCC 43889) strains generated three chromosomal *Eco*R1 fragments (~4.5 kb, 4.9 kb and 5.5 kb) which hybridised to the *slt*-II PCR probe. These bands appeared to be a positive marker in the hybridisation challenge to the other strains of bacteria. Three copies of the toxin gene related to VT2, which hybridised with the VT2 probe, were identified in both strains of *E. coli* O157:H7. This may be due to integration of *slt*-II carrying phage at different locations on the chromosomal DNA. This was to be expected, and might reflect the presence of the VT2 gene in different size fragments. Schmitt *et al.* (1991) also reported that *E. coli* O157: H7 (E32511) contained two copies of a VT2-related gene. The importance of multiple copies of the *slt*-II operon in *E. coli* associated with disease remains to be determined. So far there has been no evidence of multiple copies of VT1 related genes in a single strain. *E. coli* K12 and *Salmonella* showed no hybridisation with either of the DNA probes nor with the PCR probe. pNTP707, containing the *slt*-II A gene, and *E. coli* O157: H7 were used in every hybridisation analysis as control. Finely, a summary of the hybridisation results after stringent washes with 0.1x SSC, 0.1% SDS, obtained by probing chromosomal DNA of the strains indicated with DIG-labelled probe. .

Table .3 Summary of organisms , toxin profiles, DNA and PCR probe result.

Species probed	Toxin type	DNA probe	PCR probe	Size of *Eco*RI fragment (kb)	Source
E.coli O157:H7	VT1and VT2	+	+	3 fragment4.5,4.9 & 5.5 kb	ATCC (43889)
E.coli O157:H7	VT2	+	+	3 fragment4.5,4.9 & 5.5 kb	ATCC (43894)
E.coli K12		-	-		Warwick Univ.
*E.coli*K12(60R363)ContainpNTP707	VT2	+	+		B. Rowe (Willshaw *et al.* 1985)
Yersinia enterocolitica		-	-		Warwick Univ.
Listeria monocytogenes					Warwick Univ.
Psedomans aeruginosa		-			Warwick Univ.
Serratia marcescens			-		Warwick Univ.
Aeromonas hydrophila		-	-		Warwick Univ.
Klebsiella luteus		+	+	2 fragment4.7 & 5.5 kb	Warwick Univ.
Salmonella enteritidis		-	-		Warwick Univ.

+ = Hybridisation - = No hybridisation

Blot analysis of DIG-labelled PCR products of *K.luteus*

To confirm the presence of a nucleotide sequence in the chromosomal DNA of this strain homologous to sequences of the *SLT*-IIA gene, Southern blot analysis was carried out using DIG-labelled PCR products amplified from purified chromosomal DNA of *K. luteus*. Figure.15 shows the expected size of the PCR product band (1240-bp) after a pair of oligonucleotide primers have been used (VTc/VTd).

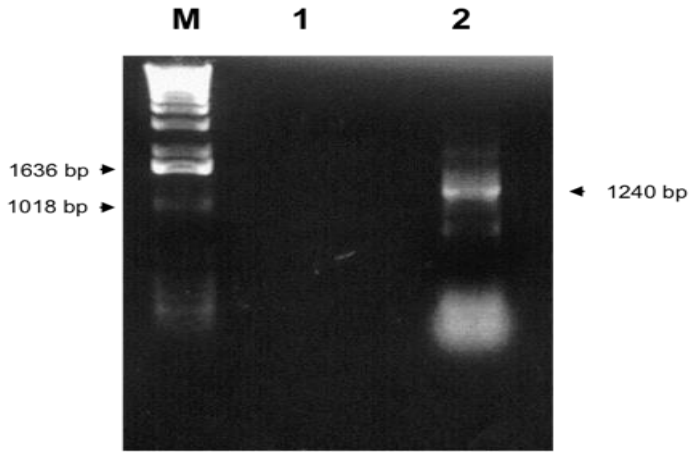

Fig 15 Figure.7a shows the expected size of the PCR product band (1240-bp) after a pair of oligonucleotide primers have been used (VTc/VTd).

The annealing temperature was 58°C. Samples of chromosomal DNA (~5 μg) prepared from *K.luteus*, K12 and O157: H7 were digested with *Eco*R1. DNA fragments were separated by 1% (w/v) gel electrophoresis (Fig.16) and blotted onto a nylon filter (Boehringer Mannheim). The specific probe 1240-bp DIG -labelled fragment was used in Southern hybridisation experiments and washing steps were carried out as described previously using low stringency. Hybridisation challenges with the PCR DIG-labelled probe, generated from genomic DNA of *K.luteus* and challenged against the later strain, produced a very weak single band (Fig. 17: lane 1). *E.coli* O157:H7 producing VT2 was used as a positive control and this generated two bands, one was very strong and the other one was faint. K12 was used as a negative control.

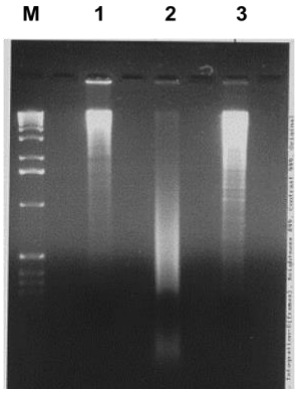

Fig. 16. Restriction digests of chromosomal DNA from selected bacterial species analysed by 1% (w/v) agarose gel electrophoresis. Key to tracks Lane: M. l ladder 1kb DNA as a molecular size marker. 1. *Klebsiella luteus. 2 E. coli* K12 negative control. 3 *E. coli* O157:H7 (*slt*2).

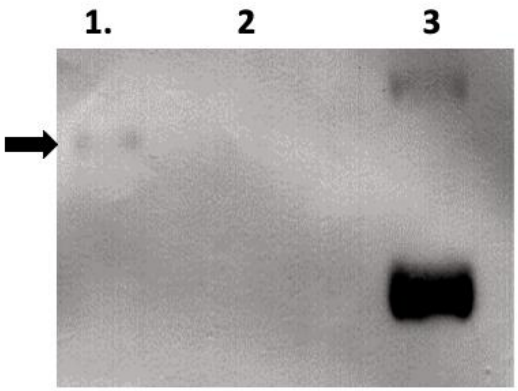

Fig 17 Restriction digests of chromosomal DNA from selected bacterial species analysed by 1% (w/v) agarose gel electrophoresis) Chromosomal DNA from *K. luteus*, *E.coli* K12 and *E.coli* O157:H7 were digested with *Eco*RI and analysed by agarose gel electrophoresis**..** 1.*Klebsiella luteus.* **2** *E. coli* K12 negative control. **3** *E. coli* O157:H7 (slt 2)

Amplification of PCR products from E. coli O157:H7 and Klebsiella luteus chromosomal DN

The expected size of amplified PCR fragments (737bp) were generated from total chromosomal DNA of *Klebsiella luteus* and *E. coli* O157:H7 after a pair of oligonucleotide primers (VTa-VTb) were designed for the amplification of the VT2 gene. No amplification products were obtained from the other bacterial strains analysed. Fig 18

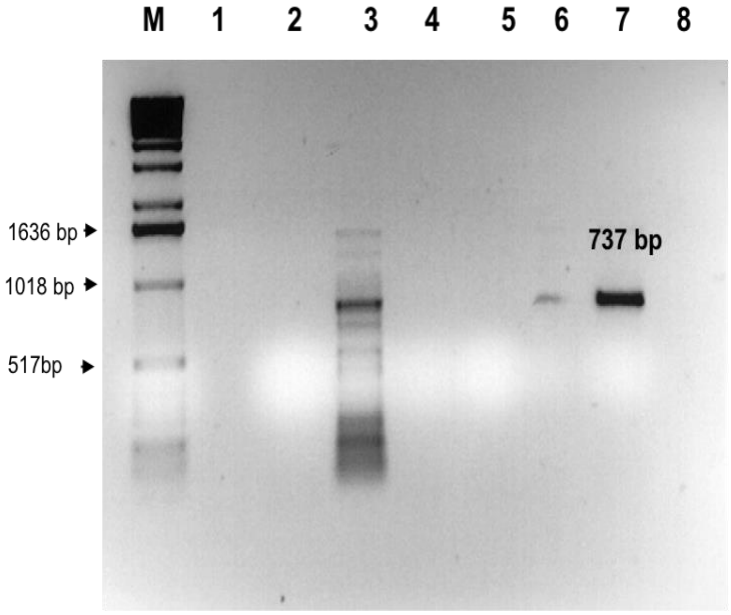

Fig. 18. **Amplification of PCR products from E. coli O157:H7 and *Klebsiella luteus* chromosomal DNA** Key to tracks : Lane: M. l ladder 1kb DNA as a molecular size marker 1kb ladder.1. *Serratia marcescens*. 2. *Salmonella enteritidis.* 3. *Klebsiella luteus.* 4. Listeria monocytogenes. 5. Yersinia enterocolitica. 6. *E. coli* K12. 7. E. coli O157:H7 (VT2). 8. Negative control no DNA.

PCR product DNA direct sequencing

Both strands of the PCR product from *K.luteus* were sequenced directly using the Applied Biosystems 373A automated sequencer. Two internal sequencing primers VTc and VTd were used, the nucleotide sequences of the 5′end and the 3′ end of the PCR fragments genomic. Fig 19

```
   1    ATCGCATAGC TCATCGGAAC AAGCTCAAGC GGTCTCCGGT CGAGTCCTCA TGCGTCCATT ATCTGCATTA TGCGTTGTTA 80
  81    GCTCAGCCGG ACAGAGCAAT TGCCTTCTGA GCAATCGGTC ACTGGTTCGA ATCCAGTACA ACGCGCCATA TTTATTTACC 160
 161    AGGCTCGCTT TTGCGGGCCT TTTTTATATC TGCGCCGGGT CTGGTGCTGA TTACTTCAGC CAAAAGGAAC ACCTGTATAT 240
 241    GAAGTGTATA TTATTTAAAT GGGTACTGTG CCTGTTACTG GGTTTTTCTT CGGTATCCTA TTCCCGGGAG TTTACGATAG 320
 321    ACTTTTCGAC CCAACAAAGT TATGTCTCTT CGTTAAATAG TATACGGACA GAGATATCGA CCCCTCTTGA ACATATATCT 400
 401    CAGGGGACCA CATCGGTGTC TGTTATTAAC CACACCCCAC CGGGCAGTTA TTTTGCTGTG GATATACGAG GGCTTGATGT 480
 481    CTATCAGGCG CGTTTTGACC ATCTTCGTCT GATTATTGAG CAAAATAATT TATATGTGGC CGGGTTCGTT AATACGGCAA 560
 561    CAAATACTTT CTACCGTTTT TCAGATTTTA CACATATATC AGTGCCCGGT GTGACAACGG TTTCCATGAC AACGGACAGC 640
 641    AGTTATACCA CTCTGCAACG TGTCGCAGCG CTGGAACGTT CCGGAATGCA AATCAGTCGT CACTCACTGG TTTCATCATA 720
 721    TCTGGCGTTA ATGGAGTTCA GTGGTAATAC AATGACCAGA GATGCATCCA GAGCAGTTCT GCGTTTTGTC ACTGTCACAG 800
 801    CAGAAGCCTT ACGCTTCAGG CAGATACAGA GAGAATTTCG TCAGGCACTG TCTGAAACTG CTCCTGTGTA TACGATGACG 880
 881    CCGGGAGACG TGGACCTCAC TCTGAACTGG GGGCGAATCA GCAATGTGCT TCCGGAGTAT CGGGGAGAGG ATGGTGTCAG 960
 961    AGTGGGGAGA ATATCCTTTA ATAATATATC AGCGATACTG GGGACTGTGG CCGTTATACT GAATTGCCAT CATCAGGGGG 1040
1041    CGCGTTCTGT TCGCGCCGTG AATGAAGAGA GTCAACCAGA ATGTCAGATA ACTGGCGACA GGCCTGTTAT AAAAATAAAC 1120
1121    AATACATTAT GGGAAAGTAA TACAGCTGCA GCGTTTCTGA ACAGAAAGTC ACAGTTTTTA TATACAACGG GTAAATAAAG 1200
1201    GAGTTAAGCA TGAAGAAGAT GTTTATGGCG GTTTTATTTG CATTAGCTTC TGTTAATGCA ATGGCGGCGG ATTGTGCTAA 1280
1281    AGGTAAAATT GAGTTTTCCA AGTATAATGA GGATGACACA TTTACAGTGA AGGTTGACGG GAAAGAATAC TGGACCAGTC 1340
1341    GCTGGAATCT GCAACCGTTA CTGCAAAGTG CTCAGTTGAC AGGAATGACT GTCACAATCA AATCCAGTAC CTGTGAATCA 1420
1421    GGCTCCGGAT TTGCTGAAGT GCAGTTTAAT AATGACTGAG GCATAACCTG ATTCGTGGTA TGTGGGTAAC AAGTGTAATC 1500
1501    TGTGTCACAA TTCAGTCAGT TTGACAGTTG CCTGTCAGAC TGAGCATTTG TTAAAAAAAT TTCGCATGGT GAATCCCCCT 1580
1581    GTGTGGGGCG ACTGGTGAAA AATCCTTGCT TGTGATTCAT TATCGACACG GGTTCGGTGG TACC 1644
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Fig.19 Nucleotide sequence of the slt-II operon cloned from *E.coli* strain 933

DNA from *K.luteus* was compared with the nucleotide sequence of the *slt*-II operon from bacteriophage 933W using the pile-up programme of the GCG data base, and the result displayed via the Gene-Doc programme. This showed the homology percentage of the fragment (Fig. 20). These results showed significant homology from the 316-bp ending at the 805-bp giving similarity in 489-bp. Only 35 nucleotide were different giving about 92.9% similarty. From the result (Fig..21)**,** the direct sequence of this PCR fragment exhibits a very high level of identity. However, to investigate this further, it would be necessary to clone the PCR fragment and sequence this clone. Due to time limitation sequencing of the cloned PCR products was not undertaken.

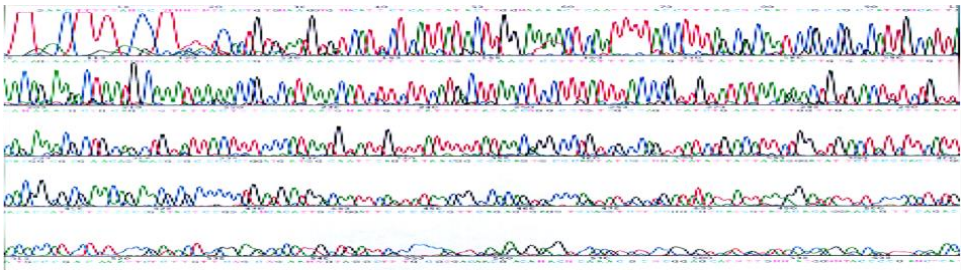

***Fig 20** The nucleotide sequence of the PCR product generated from the K.luteus chromosomal DNA*

Alignment of the nucleotide sequence of the downstream region of *slt*-II

Nucleotide sequences were compared between the bacteriophage 933W carrying *slt*II gene and part of the chromosomal DNA sequence of *Klebsiella luteus* as displayed by the Gene-Doc programme. The black colour indicates the residues conserved Fig 21 . The numbers in the right side indicate the start homology of each sequence line for both organisms, which is listed on the left.

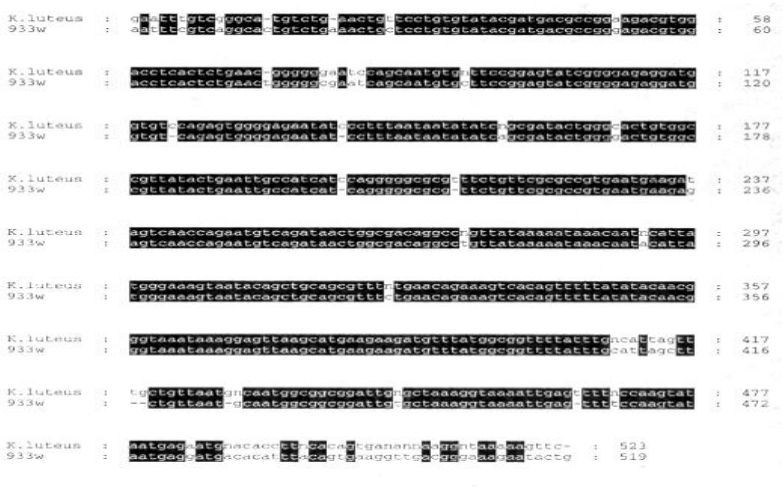

Fig 21. Alignment of the nucleotide sequence of the downstream region of slt-II.

Discussion

A molecular genetic approach was taken using Southern blot hybridisation to gain a clearer picture of the distribution of nucleotide sequences *homology* of the *slt*-II gene in different strains of Gram-negative bacteria. To identify and distinguish those strains of gram-negative bacteria carrying sequences encoding *slt*-II gene -A and B subunits cloned DNA fragments and Polymerase chain reaction (PCR) amplification fragments were used as specific probes. Large scale isolation of chromosomal DNA from different bacterial species was performed, and chromosomal DNA samples were digested with. The cloned DNA specific gene probe hybridised to the chromosomal DNA of the bacterial strains was a 850-base-pair *Sma*1-*Pst*1 (*Ava*1-*Ps*t1) fragment present in the A-subunit of the Shiga-like toxin II (*slt*-II) gene (Newland et al., 1987). This fragment, obtained from the recombinant plasmid NTP707 (Willshaw et al., 1987), carries a 4.7 Kb *Eco*RI fragment encoding the *slt*-II operon. A *slt*-II specific probe was generated by PCR when pure chromosomal DNA of *E. coli* O157:H7 (VT2 producer) was used as template DNA in PCR protocols, positive amplification was obtained, resulting in a band of the expected size on an agarose gel and this was used as a probe. Two sets of oligonucleotide primers were used, the first pair was homologous to the *slt* II-A subunit gene and the second pair were homologous to the *slt* II -A and part of the B-subunit genes. The Boehringer Mannheim DIG system was used for the detection of DNA and PCR probes used in Southern blot hybridisation. About 90% nucleotide sequence homology was identified in a *Klebsiella luteus* strain by Southern hybridisation and direct sequence analysis of the PCR product. The focus of this study, was the identification of *slt* II gene homologue in various bacterial species. Studies have shown that *E. coli* O157:H7 strains containing only the *slt* II gene

are more frequently associated with HUS and TTP than strains containing VT1 or both VT1 and VT2 (Ostroff et al., 1989; Tarr et al., 1989). Scotland et al. (1987) also noted that strains producing *slt* II alone were more frequently associated with HUS. Different bacterial strains were initially examined by Southern hybridisation analysis of chromosomal DNA using a *slt*-II cloned DNA probe digestion of plasmid DNA from NTP707 Sma1 and Pst1 digestion giving a 850-bp restriction fragment used. Willshaw et al. (1987) reported identifying restriction fragments from *slt* 1 and *slt* II clones that may be used as *slt* gene probes. The above strains were selected for further analysis because a previous study had shown that PCR is useful in confirming the presence of *slt* genes in VTEC strains recovered from clinical specimens. Two pairs of primers were used in PCR for amplification of a *SLT*-II gene fragment subsequently used as a specific probe in hybridisation. Other investigators have used a single pair of primers for detection of VT genes in clinical isolates of *E. coli* (Karch and Meyer, 1989a). In this study the sensitivity and specificity of probes created by using PCR and labelling with DIG to detect the *slt* gene sequences in different bacterial species were compared with a DNA probe from the recombinant plasmid PNT707. From the results it was observed that the sensitivity of the assay was increased by incorporation DIG into the PCR mixture. In this study the purified DNA used for identification of homology gave equivocal results. In contrast, the whole-cell lysates, which were used in a previous study may have caused background staining due to non-specific binding of the anti-DIG antibody or endogenous alkaline phosphate activity. Thirty PCR cycles were used to amplify 737-bp and 1240-bp fragments of DNA specified by two pairs of synthesised oligonucleotide primers homologous to the slt-IIA gene sequence and the annealing temperature was 58°C. The sense primer, (VT-a) was homologous to region 4-23 upstream of the gene coding for the A subunit and the antisense primer (VT-b) was complementary to a region 722-741-bp downstream and the sense primer of the second pairs, (VT-c) was homologous to region 124-143 upstream of the gene coding the A subunit and the antisense primer VT-d was complementary to a region 1345-1364-bp downstream. Thus, these primers direct the amplification of 737-bp and 1240-bp DNA fragments. The 1240-bp PCR product contains the *slt*-II A subunit and part of the B subunit, including part of the promoter region. It can be seen from the results (Fig.14 lane) that three *Eco*RI fragments approximately 4.5, 4.9 and 5.5 kb from the chromosomal DNA of both E. coli O157:H7 strains hybridised with the VT2 gene probe under stringent conditions. This was not unexpected since the two strains contain the *slt*-II gene and produce verocytotoxin. These three bands were almost identical in size, and may be due to integration of the *sl*t-II carrying phage at identical sites in the chromosomal DNA. This implied that these strains carried of multiple copies of the VT2 gene within a single cell since none of the *slt* operons sequenced to date have contained an *EcoRI* site within or between the structural genes for the A and B polypeptides and might reflect the presence of the VT2 gene in different size fragments.

Schmitt et al 1991 also reported that E. coli O157: H7 (E32511) contained two copies of VT2-related genes when a VT2 probe was used. This was the first published demonstration of multiple copies of toxin genes in *E. coli* O91:H21 related to the VT2 gene within a single strain (Oku et al., 1989). These data strongly support our results. So far there has been no evidence of multiple copies of VT1 or related genes in a single strain. The importance of multiple copies of the *slt*-II operon in E.coli strains associated with disease remains to be determined and may become a useful tool for epidemiological investigations of infections with E. coli O157:H7 producing VT2. The above supports the results obtained from O157:H7 (E32511) DNA digested with *Eco*RI which gave two bands of approximately 4.8 and 5.5 kb which showed hybridisation with the VT2 probe

(Schmitt et al., 1991). The values obtained from our results are slightly higher than the values reported for cloning of VT2 (E32511) from a 4.7 kb EcoRI fragment derived from phage DNA of E32511 (Willshaw et al., 1985). Rietra et al (1989) reported that a 5.0 kb *EcoR*I fragment hybridised with specific sequences. A significant result was obtained by hybridising an EcoR1 fragment digest from *K. luteus* chromosomal DNA, which gave two bands in different size ranges, approximately 4.7 and 5.5 kb. This also suggested that this strain might have two copies of nucleotide sequence homologous to the *slt*-II gene. The results in this study suggest that the oligonucleotide probes can provide important information about the diversity of the *slt*-I and *slt*-II genes. Also the nucleotide sequences of both strands of the PCR product from *K. luteus* are about 805-bp, similar to the *slt*-II nucleotide sequence from bacteriophage 933W i.e. 489-bp, with more then 90% similarity. It would have been necessary to clone and sequence this fragment to determine its identity and relationship with *slt* II but this work was not carried out because of time constraints. Successful amplification of DNA segments from *K.luteus* suggests that the binding sites of the primers represent conserved regions of the VT2 gene and that these are probably present in this species. Using the PCR fragment as a probe in Southern blot analysis (Fig .12), and comparing the data with the result of hybridisation of sequences nucleotides of this strain with the DIG-PCR probe generated from chromosomal DNA of wild-type *E. coli* 0157:H7, suggests that there is a homologous sequences to the VT2 gene of E. coli 0157:H7 in chromosomal DNA of *K. luteus* .

The PCR product amplified from chromosomal DNA of *K. luteus* was used as a probe to determined whether the amplified segments were indeed derived from genomic DNA of this strain (Fig. 12 lane 2). The purpose of this was to confirm the positive PCR results obtained from *K. luteus* which gave a hybridisation signal when challenged in Southern blot analysis using the same PCR product as probe. This study demonstrates that the techniques used in these experiments are sensitive and specific methods for the detection of the homologoue sequences of the genes that encode SLT in non-VTEC strains and the methods can also be used to differentiate *slt* II- from *slt* II -producing *E. coli* isolates. Use of conventional PCR for the detection of *slt* II genes from VTEC isolates has been reported by Thomas et al. (1994). These authors all found that the technique was sensitive and specific and may be useful for rapidly screening of clinical specimens for VTEC. It was important to chose a relatively high annealing temperature of 58°C to guarantee binding of the primer despite minor sequence variations in the target sequence. Increasing the annealing temperature resulted in increased specificity. High sensitivity of detection by hybridisation was achieved by using the PCR product as a probe comparing with the DNA probe. Although DNA probes provide satisfactory results in Southern blot analysis in this study the DNA probe was less sensitive than the PCR product. The PCR fragments generated from *K.luteus* chromosomal DNA and used in Southern blot hybridisations gave very weak signals, with approximately 90% similarity. The explanation as to why *K.luteus* is a non-toxin producing bacterium, may be the inability of this strain to express the toxin, which in part may be explained by the absence of the promoter, or the inability to produce VT mRNA which may be because the transcripts, once produced, are being degraded very rapidly by the cell. It is an important characteristic of prokaryotic mRNA that its half-life is short compared to other types of RNA molecules. For bacteria, the half-life of a typical mRNA molecule is a few minutes. This feature has an important regulatory function. If a protein is no longer required, a cell need only turn off synthesis of the mRNA that encodes the protein, soon afterwards, none of that particular mRNA will remain and synthesis of the protein will no longer occur. It was not known whether *slt* mRNA synthesis occurs

throughout growth of this organism or only at specific stages of the growth cycle. It is, however, assumed that changes in transcription of the genes reflect changes in the rate of transcription and hence changes in toxin production. Finally, the toxin genes may not express sufficient toxin, (even if *slt* II mRNA synthesis was occurred) under the chosen environment conditions.

Concluding remarks

This brief review shows how the knowledge of VTEC and their importance in human and animal disease has advanced very rapidly in the last 5 years. There are, however, still many unanswered questions and also a need for improvements in methods of detection of VTEC. Comparisons of VTEC of human and animal origin have provided several interesting observations. VTEC of serogroup 0157 are clearly the most important in human disease whereas these strains have usually been isolated from healthy, rather than diseased, animals and have been detected in a range of foods, particularly raw meats. However, experiments have shown that 0157 VTEC can cause disease in a range of animal models. VTEC strains belonging to serogroups other than 0157 appear to be isolated quite frequently from cattle and pigs and there is a significant association with disease. In many cases the serogroups of these animal VTEC differ from those of VTEC associated with human disease. Some VTEC from animals may also produce heat-stable or heat-labile enterotoxins whereas, so far, VTEC of human origin have not produced enterotoxins. The production of both cytotoxin and enterotoxin may well affect the clinical symptoms observed in the animal hosts. The differences in biological activity and genetic control of VT in porcine strains compared with human and bovine strains may be important in explaining why VT production by porcine strains is much lower, in general, from that observed with other VTEC. Further understanding of the Vero cytotoxins from different origins should result from analysis at the amino-acid or nucleotide level. This will provide information on the structure and functions of different regions of the toxin molecules. Sequence data have also produced interesting suggestions about the evolution of bacteria and Verocytotoxins. Jackson et al. (1987a) proposed that the structural genes for VT1 and VT2 diverged from a common ancestral gene before the evolution of *E.coli* and *S. dysenteriae* serotype 1 as separate genera. Comparison of sequence data showed that the A subunit of VT1 had homology with the A subunit of ricin, a toxin from the castor-bean plant (Calderwood et al., 1987). The two toxins share similar mechanisms of activity on eukaryotic cells and the homology may represent conservation of an active site in the two molecules. The presence of cytotoxins related to VT1 or VT2 in bacteria other than *Shigella* and E. coli has also been investigated (see O'Brien and Holmes, 1987). There is still much to learn about the precise role of VT in disease. The wide range of clinical symptoms associated with VTEC infections in man and animals suggests there are differences in the response of the host. For example, neurological disorders but not bloody diarrhoea are characteristic of oedema disease in piglets. In addition to the role of VT, the mechanisms of adhesion of VTEC in the early stages of pathogenesis are unclear at present. The association of fimbriae or other surface structures with adhesion is being investigated and will require improved in-vitro techniques as well as the use of animal models. A variety of methods for detecting VTEC has been described but rapid, simple tests suitable for clinical laboratories are needed. Immunological techniques with ELISAs or latex-particle agglutination should provide convenient tests for these laboratories. For large scale epidemiological surveys, the use of DNA probes may prove to be appropriate. These types of study should lead to a better understanding of the sources and vehicles of VTEC infection. In July 1987 the first International Symposium

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