Non-Vero cytotoxin-producing Enterovirulent

*Escherichia coli*

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**Introduction**

Strains of *Escherichia coli* (*E. coli*) that cause enteric disease are termed enteroinvasive (or diarrhoegenic) and, based on findings from clinical features, epidemiological evidence, phenotypic traits and defined virulence factors, they have been divided into the following six pathotypes or subclasses, Enteropathogenic (EPEC), Enteroinvasive (EIEC), Enterotoxigenic (ETEC), Vero cytotoxin-producing (VT) also referred to as (Shiga toxin-producing, STEC), Enteraggregative (EAEC) and Diffusely Adherent (DAEC). In this article, VTEC will not be discussed further.

The main factors when considering virulence in enteroinvasive *E. coli* are the ability to adhere, invade, produce toxin(s) and cause disease. Adhesion to the gastrointestinal tract is a key virulence mechanism, and three main patterns of adherence *in vitro* have been described: aggregative, diffuse and localised (see Figures 1-3). The type of adherence contributes to the definition of the assigned pathogenic sub-class.

**Enteropathogenic E. coli (EPEC)**

**Epidemiology and disease**

The EPEC pathotype was traditionally associated with *E. coli* strains causing outbreaks of infantile (children <1 year) diarrhoea; these outbreaks were often severe with both high attack and fatality rates. Bray, in 1945, reported the first outbreak in the UK associated with *E. coli* O111, followed by reports from Giles and Sanger, and Smith, of outbreaks caused by strains of *E. coli* belonging to serogroups O55 and O111, respectively. The number of outbreaks of infantile enteritis caused by EPEC in the industrialised countries has declined significantly (none reported in the UK since the early 1970s), whereas in developing countries they are one of the more frequent causes of infant diarrhoea.""
is involved in adhesion. EIEC do not decarboxylate lysine and are usually non or late lactose fermenters. However, biochemical tests are not entirely specific in identifying these organisms. Two further differences are their lower acid stomach resistance, which results in a higher infectious dose, and their inability to produce Shiga toxin (produced only by Sh. dysenteriae 1). EIEC belong to a limited number of serotypes, most of which are non-motile, and their somatic (O) antigens are identical with, or related to, certain Shigella O antigens. EIEC strains are confirmed by demonstration of invasiveness by Serény test (see below), immunologically by an ELISA, or by detection of the ipaH gene present on the chromosome and on the virulence plasmid; this gene is also present in Shigella strains. Once the invasive capacity has been established, then subsequent species confirmation by biochemical tests is essential.

**EIEC serogroups**

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**Enterotoxigenic E. coli (ETEC)**

**Epidemiology and disease**

Enterotoxigenic E. coli are an important cause of diarrhoea in both human and domestic animals. ETEC were first recognised in the late 1950s in Calcutta, India, as a cause of cholera-like diarrhoea, producing toxins that act on the mucosal cells. They adhere to the mucosa of the small intestine with no apparent histological changes and little inflammation and are non-invasive. ETEC are a common cause of diarrhoea in infants less than 2 years of age in developing countries, particularly at weaning.

The disease is usually less severe than cholera but can be fatal, especially in infants, and results in some kind of immunity which is directed against the colonisation factor antigens. ETEC may also affect adults from industrialised countries (where these pathogens are not endemic) who visit developing nations. They are considered to be the leading cause of travellers’ diarrhoea, accounting for up to 75% of these cases.

**Virulence factors**

To cause diarrhoea, ETEC require two types of pathogenic mechanisms. They produce adhesive factors termed colonisation factor antigens (CFAs) or coli surface associated (CS) antigens and two types of enterotoxin. These two toxins can be heat-labile (LT), a toxin closely related structurally, physiologically and immunologically as well as having a similar mode of action to cholera toxin, or heat-stable (ST). The two enterotoxins cause an increase in intestinal secretions by activation of adenylate cyclase (LT) or guanylate cyclase (ST). There are two types of heat-labile toxin, LT1 and LT2, and both have the same basic structure and mechanism of action. However, the two toxins do not cross-react immunologically. In addition, LT1 has a different receptor, has been found primarily in strains of animal origin and has not been associated with disease either in animals or humans. The STs are a family of small (17–19 amino acids), heat-stable and non-immunogenic toxins that are either soluble (STa or STI) or insoluble (STb or ST II) in methanol. The STa toxins produced by human and animal strains are remarkably homogeneous, yet two types have been described that are referred to as STh (produced by human strains only) and STp (produced by human and animal strains), respectively. They cause an increase in cGMP levels (in contrast to cAMP by LT) in the host cells resulting in fluid loss similar to that induced by LT. STb acts by a different mechanism from that of STa and produces diarrhoea in piglets. There is no evidence that STb-positive ETEC strains are diarrhoegenic in humans. The frequency with which LT and ST are found in ETEC isolated from persons with travellers’ diarrhoea varies between studies with usually less than 50% of the strains producing both toxins. This is important when selecting laboratory tests for the detection of ETEC because at least 25% of the cases would be missed if only tests for LT (such as the immunodiffusion test) were used.

ETEC not only produce enterotoxins but also colonise the small bowel of animals and humans. There are several types of fimbriae that mediate adhesive properties: type 1 fimbriae are usually found in E. coli strains from the resident microflora and do not seem to contribute to the pathogenic potential of ETEC strains. Pili that are important for ETEC include K88, 987P, F41, F107 and 2134P (in strains infecting pigs), K99 (strains infecting pigs, lambs and calves) and at least 22 colonisation factor antigens and coli surface antigens specific for humans have been defined. CFA/I and CFA/II are the most commonly found adhesins in human ETEC (but many strains do not produce either of these antigens and have additional ETEC adhesins).

**ETEC serogroups**

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**Enteroaggregative E. coli (EAggEC)**

Although initially isolated from adults with travellers’ diarrhoea, EAggEC are now considered to be a significant cause of bloody or protracted infantile diarrhoea in various non-industrialised countries and also a cause of sporadic cases of diarrhoea in developed countries.

Enteroaggregative E. coli show a distinct, aggregative adherence configuration when grown on HEp2 or Hela cell lines, also described as a ‘stacked brick’ pattern. They are not invasive, but bind to small intestinal cells without any obvious histological changes. Knowledge of their virulence factors is very limited, with both toxin production
The chance of detecting faecal pathogens can be increased if with previous antibiotic treatment. As specimens are collected in the acute stage of the disease. Repeat examinations are of value, especially in convalescents or patients an alternative is the use of rectal swabs.

Isolation

i) EAST for ‘enteroaggregative heat-stable toxin’ that induces fluid secretion and is antigenically related to the heat-stable toxin of ETEC; however, the genes encoding EAST have also been detected in other diarrhoegenic E. coli and non-pathogenic E. coli;

ii) a toxin similar to the α-haemolysin of E. coli associated with urinary tract infection;

iii) Pet, a high molecular weight enterotoxin belonging to the autotransporter family of secreted proteins;

iv) the Shigella enterotoxin ShET1, found predominantly in Shigella flexneri 2a strains. Four morphologically distinct types of fimbriae have been identified by Knutton et al., including so-called aggregative adherence fimbriae I (AAF/I) that are very similar to the bundle forming pil of EPEC. Other aggregative adherence fimbriae, AAF/II and AAF/III, have now also been reported.

EAggEC serogroups

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Diffusely adherent E. coli (DAEC)

Diffusely adherent E. coli were defined by the presence of a pattern of diffuse adherence when grown on Hep-2 cells and are classified as one of the six classes of enterovirulent E. coli, and yet little is known about the virulence properties or epidemiology of this group. DAEC are clearly different from EPEC strains in that they are always negative in the fluorescent – actin staining (FAS) test, which correlates with attaching and effacing lesions, and for hybridisation with the EAF probe. Their mode of adherence is also different from EPEC strains, and two adhesions have been identified: a surface fimbrial adhesin, designated F1845 confers the DA phenotype, and a second adhesin AI DA-I, a plasmid-encoded outer membrane protein. Epidemiological reports on the rates of isolation of DAEC from cases of diarrhoea and controls amongst children vary, and their significance as an enteric pathogen continues to be debated.

Isolation

The isolation of enterovirulent E. coli from patients with enteric infections is by direct culture of faecal samples onto selective media, e.g. MacConkey agar. Where stool samples are not available, an alternative is the use of rectal swabs.

The chance of detecting faecal pathogens can be increased if specimens are collected in the acute stage of the disease. Repeat examinations are of value, especially in convalescents or patients with previous antibiotic treatment. As E. coli is part of the normal intestinal flora, multiple colonies should be picked and tested for the presence of virulence markers. Cases of ETEC infection have been identified in which one colony of pathogenic E. coli was detected among 200–300 non-pathogenic isolates. Alternatively, polymerase chain reaction (PCR) targeting virulence-associated gene sequences can be performed on colony sweeps from MacConkey or other moderately selective agar plates. For the detection of toxin-producing strains, tests can be performed on colonies from agar plates or broth extracts from faecal specimens; ideally, such a procedure should be followed by subculture and identification of the responsible pathogenic strain.

Identification

Traditionally, E. coli is identified phenotypically using a variety of biochemical tests, commonly used for the identification of the Enterobacteriaceae. Problems may arise in separating certain metabolically inactive E. coli strains from Shigella, as intermediate forms may occur. E. coli is usually motile and decarboxylates lysine, Shigellae, by definition, are non-motile and do not decarboxylate lysine or hydrolyse arginine.

There are now a wide variety of biological, immunological and molecular methods available to identify the different groups of enterovirulent E. coli. Unfortunately, many of these methods are not widely used in routine diagnostic laboratories. Simple screening methods such as agglutination with specific antisera (targeting the more common serogroups associated with enterovirulence), have been employed in many laboratories. Collaboration with reference laboratories is then required to substantiate infections with enterovirulent E. coli.

Typing methods

Conventional typing methods

There are several methods available for subtyping E. coli, and they have both advantages and disadvantages. Serotyping has been the main typing method applied to E. coli, with an internationally accepted serotyping scheme. This scheme comprises somatic antigens O1–O187 and flagellar antigens H1 – 56, but typing antisera for all the E. coli antigens, both somatic and flagellar, are not readily available, with the possible exception of those used for identifying O serogroups of E. coli that are associated with certain virulence properties, e.g. traditional Enteropathogenic E. coli and Vero cytotoxin-producing E. coli. One interesting observation that can be made from the lists of serogroups associated with different pathovar groups is a commonality of serogroups belonging to the traditional‘enteropathogenic’EPEC pathovar with more than one pathovar type, e.g. O95, O86, O111, O114, O119, O125, O126 and O128 are also found in ETEC, EAggEC and VTEC. This multiple association of serogroup and pathovar has never been fully elucidated. Full serotyping (the identification of both
the somatic O-antigen and the flagella H-antigen) is usually restricted to reference laboratories and is fundamental for the identification of new pathogenic strains. Apart from serotyping, ‘conventional’ typing methods such as biotyping, phage typing, colicin typing, and resistotyping have been used and now have either been replaced by or used in combination with molecular techniques. These newer methods are often more discriminatory but are also technically more demanding, require specialised equipment and the cost of typing is an important consideration.

Molecular typing methods

Advances in molecular methods are making a significant contribution to strain typing and can be seen to complement and enhance conventional culture-based methods for the detection, identification, typing and epidemiological analysis of the wide variety of E. coli strains associated with causing disease. Plasmid profiles (resistance plasmids and small cryptic plasmids) have been widely used to further investigate the epidemiology of diseases associated with E. coli. For isolates with similar plasmid profiles, further discrimination can be achieved by the use of pulsed field gel electrophoresis (PFGE), and, when investigating outbreaks, methods that include digestion of high molecular weight chromosomal DNA with restriction enzymes followed by analysis of the resulting fragments by PFGE or by a combination of agarose gel electrophoresis, Southern blotting and hybridisation with different kinds of probes (e.g. ribotyping) may be used. With the molecular characterisation of the various virulence genes involved in the pathogenesis of enterovirulent E. coli, diagnostic tests have been developed that detect these genes by hybridisation, PCR or a combination of both; these tests have provided a greater accuracy for a more coherent identification of these groups of diarrhoeal pathogens.

Genomic molecular methods can offer more discrimination by ‘fingerprinting’ than phenotypic methods. Such methods include detection of specific genes by PCR and the use of PCR for amplification of chromosomal sequences. Examples of these techniques include restriction fragment length polymorphism typing (RFLP), restriction endonuclease analysis of chromosomal DNA by pulsed-field gel electrophoresis (PFGE), fluorescence-based amplified fragment length polymorphism (FAFLP), multilocus sequence typing (MLST) and variable number tandem repeat (VNTR) typing.

The use of multilocus sequence typing (MLST), which assigns alleles at each locus directly by nucleotide sequencing, has been proposed.[25] The main advantage of MLST is that there is no need to culture the strain as fragments for sequencing can be amplified directly from the clinical sample, but not having a culture for reference can also be a disadvantage. Also, sequence data are easy to standardise, making the creation of a ‘typing’ database relatively straightforward and readily available.

Array technology

DNA microarrays are a means by which the detection capabilities of PCR can be enhanced. They permit rapid detection of sequence variation within a defined locus and also detection of multiple products from multiplex PCR. Microarrays can also be used to fingerprint bacterial isolates. Debrindt et al.[23] compared pathogenic and commensal E. coli using DNA arrays and concluded that DNA array technology allowed them to assess the genetic diversity and genome content amongst the different types of E. coli studied.

It is unfortunate that, in spite of all the techniques mentioned above, there are still no simple, readily available methods for the identification of these enterovirulent E. coli pathogens in the routine clinical microbiology laboratory.

Acknowledgement

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References

Bacteriophages and control of animal disease

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Introduction
Animal disease remains a major problem throughout the developed and developing world. Acute, chronic and sub-clinical infections result in severe economic losses in addition to being the cause of zoonotic infections in man. The losses of production alone are enormous and are likely to be in excess of £5 billion annually worldwide (extrapolated from Bennett and Ijpe-laar, 2003). This is in addition to the losses associated with human infection.

Disease and infection in farm animals is relatively well-regulated in Europe and the UK in comparison with many parts of the world. Despite this, it remains a highly political issue which can have serious financial repercussions. Thus, recent problems with Salmonella, BSE, bovine tuberculosis and Foot and Mouth Disease (FMD) highlight some of the well-publicised problems of surveillance and control. These diseases also highlight some of the different epidemiological routes that present a range of problems for surveillance and control. These include diseases that are endemic to the natural animal populations, leading to clinically acute or chronic disease. Alternatively, new diseases have emerged as a result of changes in economics, animal husbandry, human movement and climate change. The causative agents involved include bacteria, viruses, parasites and other agents, such as the prions.

Disease may affect animals, themselves, from the point of view of welfare and economics, but food-borne and other zoonotic infections present additional problems for human disease. Food-borne bacterial pathogens remain of major significance in western countries and the rest of the world. In 2007, in the UK alone, there were more than 70,000 recorded cases of food-poisoning, much of it caused by Campylobacter and Salmonella, three times that recorded in 1985. Where human cases can be traced back, much of the infection caused by these two pathogens is associated with consumption of poultry meat and poultry products, although pig and cattle products are also implicated.

In many countries where animal production is intense and well developed, surveillance is similarly well regulated. Similarly well developed measures to control infection are also required. These should be robust, sustainable and cost effective, such that they may also be applied to those countries where surveillance is not well developed. Control measures take a variety of forms and vary enormously according to the economics and intensity of production, in addition to local environmental factors, such as ambient temperature. In the pig and poultry industries, improvements in hygiene and management may be used to control introduction of infectious agents into the population. However, the ability to house animals may not be a possibility in countries where ambient temperatures are high. Antibiotics and other chemotherapeutic agents are thus used widely to control bacterial and other infections. In many cases, the wide use of antibiotics is unregulated and is often used to stimulate growth of pigs and poultry. Chemotherapy can control bacterial infection, but the side effects of developing resistance, both in the pathogen against which the antibiotic is directed and other constituents of the normal microbial flora, are an important and unwelcome consequence. This, itself, is an important zoonotic issue which remains the source of considerable debate. Vaccines are used extensively to prevent infection and disease and are widely accepted as a biological form of infection control. This is not the place to discuss the merits and demerits of this approach.

Suffice it to say that vaccination, itself, may also induce evolutionary changes in pathogens. The use of increasingly immunogenic and virulent vaccines against Marek’s disease in poultry is required as a consequence of mutations of the virus to increased virulence as a result of earlier vaccinations.

It is clear that novel approaches to infection control should be considered. Our groups have studied novel uses of live vaccines and also the use of bacteriophages to control bacterial infection, including the control of food-borne pathogens.

Bacteriophages
Bacteriophages (phages) are viruses that attack and kill bacteria. Phages were discovered independently in the UK by Twort and in France by d’Herelle in the early 20th century. They attach to the surface of the bacterial cell via specific attachment structures, inject their nucleic acid, which replicates inside the cell, generating up to hundreds of copies of the phage particles that burst out of the cell, releasing new phages that can go on to infect neighbouring bacteria (see Figures 1-3). The exact relationship between the phage and cell genome can be highly complex. In some cases, the phage nucleic acid, rather than replicating, integrates into the bacterial chromosome and replicates with it, only to be released into the more

Figure 1. Bacteriophages attacking a bacterial cell
At this time, however, it must be remembered that microbiology was in its infancy, and the nature of viruses, the mechanisms whereby bacteria produced infection, the nature of the interaction between pathogen and phage and, indeed, the nature of phages was not understood. It was not surprising, therefore, that many of these early attempts to control disease in man and animals were unsuccessful. With the advent of antibiotics during the second world war, it was no surprise that phage therapy was largely forgotten about in the West. Interest did continue in Eastern Europe with work of sometimes indifferent quality. Phage pills against dysentery were redistributed to the Soviet army during the war, for example, despite the knowledge that there are many microbiological agents associated with diarrhoea.

New approaches for disease control in animals

The idea of phage therapy was taken up again in the West in the 1980s by the Welsh veterinary bacteriologist, H. Williams Smith FRS, who had made enormous progress in the genetics of bacterial pathogenesis. This included work on bacteriocins, antibacterial peptides produced by the bacteria, themselves, and active against related bacteria. He showed that bacteriocins could be used to treat experimental murine *E. coli* septicaemia. He had studied phages in the 1950s for the purpose of phage typing. This combination of studies led him to wonder whether phages could be used in the same way, despite the dogma suggesting that they were a waste of time. He isolated phages against septicaemic strains of *E. coli* for which the K1 capsule is a virulence determinant. These strains produce neonatal meningitis in children, in addition to being associated with septicaemia in very young calves that have received insufficient colostrum from their mothers and in poultry as a secondary consequence of viral infections. Phages attach to this capsule such that, if resistant colonies develop in the areas of lysis on a plate, most of these would be non-capsulate and, therefore, non-virulent. In rigorously controlled experiments, he showed that highly active phages were more effective than antibiotics. This was extended by our group to protecting chickens and calves in the same way. Indeed, in one experiment, infected chickens were left untreated until they showed signs of disease, and one dose of phage completely resolved the infection. Further studies on enterotoxigenic *E. coli* which produce diarrhoea in young calves, lambs and pigs also showed them to be highly effective under controlled conditions.

Following these studies, several other groups sprung up which explored phage use against a number of infections, in some cases, inappropriately. We have shown that phage treatment of *Salmonella* and *Campylobacter* infections of chickens with high titres of lytic phage can reduce the levels of colonisation of the intestine of poultry by these food-borne pathogens for short periods. To be practically useful in terms of reducing entry of these pathogens into food chains, it would require careful selection of the phage, use of the phage and management of livestock, for example the scheduling of chicken flocks for slaughter after treatment.

Our work together with that of Williams Smith has resulted in a guide to potential efficacy as follows:

Phages are most likely to be effective:

1. in *vivo* if they are also highly lytic in *vitro*
2. where there is a stage of the infection which mimics those conditions under which phages replicate most efficiently in *vitro* (e.g. blood, CSF, skin, gut mucosa)
3. When the pathogen is clonal
4. When there is no or little recirculation in the environment, which tends to accelerate the development of resistance.

In animals, a number of bacterial septicaemias of calves, sheep and poultry caused by *Pasteurella* organisms are economically important. It is likely that these would be tractable. The bacterial enterites are less likely to be practically treatable, since so many agents, including viruses, can be the cause of this disease, and clonality may be a problem here.

Phages are unlikely to be effective:
1. When the target bacteria are outnumbered by inert material, such as pus or large bowel contents
2. When the target bacteria are intra-cellular
3. When a number of agents produce the disease (e.g. enteritis).

However, phages may be used under conditions which are not optimal. Thus, although the problem of restriction means that a proportion of the target strains may be resistant as a result of nucleic acid restriction, the phages will, nevertheless, attach to the bacterial surface. Thus, application of a sufficiently high number of phages will lead to non-specific lysis through multiple cell surface perforation. This approach could be used to control pathogens on skin by spraying. This might be used to control MRSA and other such pathogens on the skin of nursing staff although there remains the risk that resistance could develop by multiplication in the gut. Resistance develops more rapidly in environments where the two agents are multiplying normally. It might also be used to control skin infections of domestic animals, which are a significant problem. Phages might be used to control contamination of skin in human burns patients, as the infected limb is generally isolated from the environment.

### Carcass decontamination in the food industry

The slaughter line in an abattoir represents another situation where contaminated skin surfaces might be treated and where there is little chance for entry of phage used for treatment back into the production houses.

Entry of bacterial pathogens into the human chain results from skin contamination with faeces before slaughter and cross-contamination after. Slaughter of food animals is becoming more industrialised with thousands of broiler age chickens slaughtered on a daily basis. The process is highly mechanised, which can lead to carcass contamination through contact between carcasses and bowel leakage.

Until recently, potable water was the only application permitted by the European Commission, but it is beginning to explore other options in an attempt to reduce entry of pathogens into the food chain.

We studied the effects of using high titre phage to decontaminate chicken skin of *Salmonella* and *C. jejuni*11,12. When levels of contamination were high, we were able to reduce *Salmonella* counts by 2 – 3 logs. When more typical levels of contamination were used (<100 cfu/sq cm) we were able to completely eliminate the pathogen (see Figure 4).

Other similar approaches might also be considered, such as the application of bacteriocins or the use of phage lytic enzymes that are produced to release the mature phage particles from the bacterial cell13. These are similar disinfection approaches, in that multiplication of the agent in the cell is not involved. The technology of phage production is simple and might be suitable where large quantities would need to be applied.

### Risks and the future

There is always a concern regarding the acceptability of introducing a new technique into biomedical science, particularly if the agent of therapy, itself, is infectious. In this case, phages present no infection risks for man or animals, and we consume large quantities of phage normally with meat as a result of normal contamination with phages which infect normal flora components in the gut. However, acceptability is an issue that needs to be addressed. Where phages might be used to control an acute infection of animals or man, there is likely to be little objection. Although treatment of food is likely to be more problematic, some phage preparations are now accepted for use in food treatment, for example to reduce *Listeria* contamination. A great deal remains to be done, but there appears to be considerable scope for using these biologically normal agents to control a number of bacterial diseases where existing methods of control are becoming less successful.

### References


