Challenges in the detection and isolation of Verotoxigenic *Escherichia coli* on fresh meats

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Introduction

The Gram negative bacterium Escherichia coli, is a common commensal inhabitant of the gastrointestinal tract of mammals and birds. It is an adaptable organism that can persist for extended periods outside of a host in water, soil or on plants. Specific E. coli strains may be pathogens and there are six recognised enteric pathotypes of E. coli, Enterotoxigenic, Enteropathogenic (EPEC), Diffusely Adherent, Enteroaggregative (EAEC), Enteroinvasive and Verotoxigenic (VTEC are also known as Shigatoxigenic, STEC or Enterohemaorrahgic E. coli) (Kaper *et al.,* 2004). Many virulence factors are located on genetically mobile elements and may be distributed between pathotypes (Figure 1) (Table 1).

VTEC are distinguished from other E. coli pathotypes by the possession of the genes for one or more verotoxins (Kaper *et al.,* 2004). In addition some VTEC strains carry a virulence factor common to the EPEC pathotype, the locus of enterocyte effacement (LEE) (Kaper *et al.,* 2004). Recently verotoxin expressing EAEC have been identified as a significant public health issue (Beutin and Martin 2012).

Foodborne VTEC is most commonly associated with two types of food: salad greens and fresh meats, particularly beef (Rangel *et al.,* 2005; EFSA 2007). Though E. coli does not grow at temperatures below 7 °C, it can survive indefinitely on refrigerated aerobically or anaerobically packed fresh meats, and can reproduce if temperature abuse occurs. The estimated infectious dose for VTEC is low, with estimates of less than 100 cells in two outbreaks (Tilden *et al.,* 1996; Tuttle *et al.,* 1999). Health hazards may arise from the consumption of under cooked meat or cross contamination of other foods.

VTEC of the serogroup O157:H7 and O157:NM have been the focus of public health and regulatory activity in North America since the first recognised out breaks in the early 1980s. It is relatively easy to differentiate and isolate VTEC O157 from other E. coli, as this serogroup possess two traits that are relatively unusual in E. coli, they do not ferment the sugar sorbitol and lack the enzyme β-Dglucuronidase. VTEC belonging to serotypes other than O157 (non- O157 VTEC) have come to be recognised as responsible for a significant proportion of illness (Brooks *et al.,* 2005; EFSA 2007). In North America it has been generally assumed that the majority of VTEC illness is caused by E. coli O157, but this view is changing as testing for non-O157 VTEC is being expanded in clinical settings. In 2010, non-O157 VTEC accounted for 50% of VTEC illness reported to the CDC FoodNet program (CDC 2011). Meat products have been identified as the vehicle of non-O157 VTEC infection in a number of outbreaks (Table 2).

In response to concerns about non-O157 VTEC in the USA, the USDA-FSIS began a testing program on 4 June 2012 for the presence of an additional six VTEC serogroups in beef trim. The USDA defines the target VTEC as E. coli with the verotoxin and LEE genes (stx and eae), that belong to one of the following O-types, O26, O45, O103, O111, O121 or O145 (USDA-FSIS. 2012a). However, testing foods for non-O157 VTEC is relatively expensive and time consuming compared to testing for E. coli O157. These problems arise due to challenges in differentiating non-O157 VTEC from non-pathogenic E. coli.

Qualitative methods for the detection of bacterial pathogens in foods, including VTEC, consist of the following four stages. 1. enrichment. 2. screening. 3. isolation. 4. confirmation. The challenges associated with each of these stages are discussed below.

1. Enrichment

Methods for the detection of VTEC must achieve a limit of detection approaching 1 cell per analytical unit (325 g or 65 g)(USDA-FSIS. 2012a, 2012b). To achieve this limit of detection the sample must undergo enrichment to raise the number of target cells to a density such that isolation is not dependent upon chance and that screening methods will be able to reliably detect its presence. Ideally, the enrichment should be selective, so that the target organism forms the major proportion of the total flora, which increases the probability of isolation.

Selective enrichment for E. coli O157 in the presence of other E. coli is possible due to its relatively high resistance to certain antibiotics, such as novobiocin, potassium tellurite and cefixime. However, this resistance is not common to other VTEC and there is no evidence that VTEC as a group are more resistant to any selective conditions than other E. coli (Vimont *et al.,* 2006; Vimont *et al.,*2007; Baylis 2008; Hussein and Bollinger 2008; Gill *et al.,* 2012). Consequently, the use of enrichment conditions developed for E. coli O157 may inhibit the growth of non-O157 VTEC or, if more permissive enrichment conditions are used, non-pathogenic E. coli and other enteric bacteria can be expected to form a major part of the enrichment flora.

2. Screening

Enrichment broths are screened to detect the potential presence of pathogens. Screening reduces the cost of testing by allowing the rapid release of product which tests negative and focusing isolation efforts on potential positive samples.

Enrichment broths can be screened for VTEC by polymerase chain reaction for the verotoxin genes (stx) and other molecular markers associated with pathogenicity (LEE, O or H antigen) or by testing for verotoxin production can be detected by ELISA or immunoprecipitation. Commercially available kits make such tests easy to perform.

Though screening tests are not technically challenging, predicting whether or not a pathogenic VTEC is present remains a challenge. This issue is particularly relevant to the beef industry as the proportion of enrichment broths testing positive is expected to be substantially greater than for E. coli O157. The reported proportion of ground beef or trim enrichments that test positive for stx alone ranges from 5.5% to 36% (Perelle *et al.,* 2007; Cobbold *et al.,* 2008; Bosilevac and Koohmaraie 2011; Hill *et al.,* 2011). The rate for samples confirmed positive for E. coli O157 in the USDAFSIS ground beef testing program from 2008 to 2010 was 0.24 to 0.46% per year (USDA-FSIS 2012c).

The number of potential positive samples can be reduced by testing for multiple traits, as the USDAFSIS method MLG 5B.02 requires (USDA-FSIS 2012a). However, a high false positive rate can be expected, as it is not possible to determine whether genes are present in a single cell and eae and Oantigen synthesis genes can be found in verotoxin negative E. coli. The predictive potential of these traits is also limited. Serious illness has been reported in infections by VTEC which do not possess the LEE or one of the six US top serotypes, such as the E. coli O104:H4 strain responsible for the VTEC outbreak with the largest number of cases, reported to date (Beutin and Martin 2012).

The predictive value of the O- and H-antigens for virulence is limited, as they are not virulence factors nor are they genetically linked to known virulence factors. The presence of a serotype associated with previously reported pathogenic strains only indicates a potentially greater risk and does not confirm pathogenicity. Similarly the presence or absence of specific serotypes does not indicate that a strain is harmless.

3. Isolation

Once potential positive enrichment broths have been identified by screening tests, the organism must be isolated into a pure culture so that its identity can be confirmed.

Immunomagnetic separation (IMS) may be used to enhance the probability of the recovery of specific E. coli serotypes. But IMS has no value in recovering novel or unexpected serotypes and the efficiency of recovery is dependent upon the length of the enrichment incubation (Verstraete *et al.,* 2010.).

The most difficult challenge in the isolation of VTEC is that no selective and/or differential agar medium for VTEC has yet been created. This necessitates that a very large number of individual colonies must be tested for stx or verotoxin production, without reference to colony appearance. This is a laborious process and greatly reduces sample throughput. Cells may be isolated onto a membrane and VTEC identified by colony hybridisation and colony immunoblot methods (Todd *et al*., 1999; Atalla *et al*., 2000), but these approaches are currently too technically complex and time consuming for adoption outside of research laboratories.

A wide range of differential and selective agar for E. coli O157 exist based on the absence of sorbitol or rhamnose fermentation, the absence of β-Dglucuronidase activity, hemolysin production and antimicrobial resistance. No set of similar traits have be identified for all VTEC. Specific serogroups have carbohydrate fermentation patterns that can used to aid identification, but this requires multiple agar media for even a small set of serogroups (Possé *et al.,* 2007). An agar medium CHROMagar™ STEC is being marketed as a differential/selective agar for VTEC. However, an independent performance study of CHROMagar™ STEC reported that it is in fact a selective agar for E. coli with tellurite resistance (Tzschoppe *et al*., 2011). Tellurite resistance genes were more common (87.2% of 235) in VTEC strains than other E. coli (12.5% of 567) tested. But the presence of resistance genes does not necessarily result in a resistant phenotype and the media was found to be inhibitory to some VTEC belonging to serogroups of known public health significance (Tzschoppe *et al*., 2011).

4. Confirmation

Once presumptive VTEC isolates have been recovered a series of confirmatory test are required to verify the species of the isolate and the presence of indicator traits. Identification of species is performed by biochemical testing and the presence of other indicator traits may be by PCR or serological methods. There are no significant challenges with this process once a pure culture of the isolate is established.

Conclusions

The challenges associated with VTEC detection and isolation are the use of suitable enrichment media, the need for better molecular markers for pathogenicity and the need for higher throughput methods for the isolation of VTEC colonies. Though improving the selectivity of enrichment for VTEC is desirable this issue can largely be resolved by the adoption of enrichment media which are permissive for VTEC growth, rather than using enrichment media originally developed for E. coli O157.

The other two challenges cannot be easily resolved without major research efforts. The identification of additional molecular markers for the identification of VTEC pathogens will require extensive genomics study of large populations of clinical isolates, ideally with data on patient symptoms and outcomes. A relationship between certain verotoxin subtypes and the risk of severe patient outcomes has been proposed (Persson *et al*., 2007), but further study is required.

To increase the throughput of isolation there are two potential approaches, selectively capturing VTEC or improved colony screening. Due to the limitations of IMS capture based on the O-antigen, the development of a capture system based on a cell surface antigen directly linked to virulence would be an improvement. Capture based on the LEE protein intimin is a possibility (Horner *et al*., 2006). The rapidity of colony screening for verotoxin could potentially be improved by the adaption of ELISA, colony hybridisation or colony blot methods into an easy to use kit format. But the ideal remains a differential agar on which verotoxin expressing colonies can be easily identified.

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Table 1. Major characteristics of *E. coli* pathotypes.

Pathotype	Characteristics
Enterophathogenic (EPEC)	Locus of enterocyte effacement
Verotoxigenic (VTEC)	Verotoxins. Some strains have the locus of enterocyte effacement.
Enterotoxigenic (ETEC)	Heat liable and/or heat stable enterotoxins
Enteroaggregative (EAEC)	Biofilm formation, secretory enterotoxins and cytotoxins.
Enteroinvasive (EIEC)	Invasion of colonic epithelial cells
Diffusely adherent (DAEC)	Induces cellular projections from small bowel enterocytes which wrap around the bacteria

Table 2. Selected outbreaks of non-O157 VTEC illness associated with meat.

Year	Country	Scope	Implicated Food	Number of cases (deaths)
2011	Japan	O111	Raw Beef	180(5)
2010	USA	O45	Smoked meat	7
2010	USA	O26	Ground beef	3
2009	France	O123:H-	Ground beef	2
2007	Denmark	O26:H11	Beef sausage	20 (0)
2007	USA	O111	Ground beef	23 (0)
2006	USA	O111:K58	Beef steak	9
2006	Norway	O102:H25	Mutton sausage	17 (1)
2004	Canada	O111:NM	Ground beef	2
2004	USA	O111:K58	Ground beef	11
2002	USA	O111:K58	Ground beef	9
2000	Germany	O26:H11	Beef	11
1999	USA	O111:NM	Ground beef	24
1995	Australia	0111:NM	Fermeted sausage	158 (1)

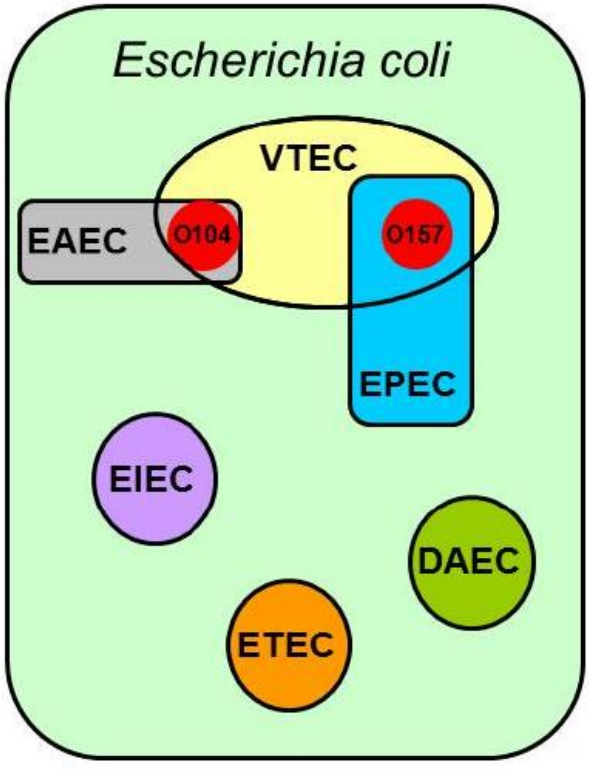

Figure 1. Relationship between *Escherichia coli*enteric pathotypes