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Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC types [1]

Scientific Opinion of the Panel on Biological Hazards

(Question No EFSA-Q-2007-036)

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PANEL MEMBERS

Olivier Andreoletti, Herbert Budka, Sava Buncic, Pierre Colin, John D. Collins, Aline De Koeijer, John Griffin, Arie Havelaar, James Hope, Günter Klein, Hilde Kruse, Simone Magnino, Antonio Martinez López, James McLauchlin, Christophe Nguyen-Thé, Karsten Noeckler, Birgit Noerrung, Miguel Prieto Maradona, Terence Roberts, Ivar Vågsholm, Emmanuel Vanopdenbosch.

SUMMARY

Following a request from EFSA, the Panel on Biological Hazards (BIOHAZ) was asked to (i) identify the strains and/or serotypes of VTEC which are pathogenic to humans, (ii) to give advice regarding the analytical methods, including testing for virulence factors, to be used to detect and identify the human pathogenic VTEC strains/serotypes from food and animals, and (iii) recommend the monitoring methods in animal populations and foodstuffs that are most optimal from the public health point of view. The BIOHAZ Panel concluded that there is a wide variety of VTEC in the food-producing animal populations, of which the public health importance remains unclear. A restricted range of serotypes (i.e. O157, followed by O26, O103, O91, O145 and O111) are associated with public health risks, however isolates of these serotypes are not necessary pathogenic when recovered from food or live animals.

The main virulence factors (genes) identified for human pathogenic VTEC are: *vtx1*, *vtx2*, *vtx2c* and *eae*. There is no consensus for the optimal strategy to characterise these virulence factors (genes). The BIOHAZ Panel concluded that it is not possible at the present time to fully define human pathogenic VTEC. However, the concept of seropathotype has evolved which classifies VTEC into groups based on empirical knowledge of the typical clinical outcome of VTEC infections combined with knowledge of serotype, *vtx* subtypes and presence of additional virulence factors. This concept is likely to be further refined and will provide a valuable tool in the future for the assessment of the human pathogenic potential of different VTEC serotypes.

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Standard or validated alternative methods are available and are recommended to be used for the detection and isolation of VTEC O157 from food and animals. For the other serotypes, there are no universally accepted and validated methods, but pragmatic approaches have been produced. Improved methods for the detection and isolation of VTEC non-O157 from foods, animals and the environment should be developed and validated. There is no standard protocol for enumeration of VTEC O157 or other VTEC serotypes in food or environmental samples and such quantitative methods should be developed. Enumeration of VTEC is generally not conducted as part of routine monitoring or testing programmes, although quantitative data are essential to better understand the human health risks. Recent advances in molecular detection methods combine the traditional detection methods and target both serotype specific genes, *vtx*, and other virulence genes. However, isolation of VTEC, and subsequent strain characterisation is still needed in order to ensure that the detected genes are present on the same bacteria.

Ruminants (particularly cattle) are recognised as their main natural reservoir of VTEC, in particular VTEC O157. Comprehensive information on the occurrence of VTEC in animals other than cattle is scarce. Pigs and poultry have not been identified to be major sources of VTEC for human infection in Europe. Foodstuffs subject to faecal contamination from ruminants represent a hazard for human VTEC infection and data allowing meaningful comparisons between different Member States are currently not available. Good hygiene practices at the abattoirs and at processing plants including monitoring for microbiological indicators (*Enterobacteriaceae* and in generic *E. coli*) is likely to be the most effective method for reducing the public health risks for VTEC infection. However, compliance with the hygiene criteria does not necessarily guarantee the absence of VTEC at concentrations sufficient to cause human disease. Application of efficient validated HACCP-procedures for production of raw ready-to-eat meat, meat preparations and other foods is important to reduce the public health risks for VTEC infection.

The current monitoring activities performed as part of the Zoonoses Directive (2003/99/EC) although providing valuable data for individual Member States, in places lack harmonisation. Wherever possible, efforts should be made to apply methods which produce genuinely comparable data from different Member States. Monitoring of live ruminants, the farm environment, in water and other environmental sites may be extremely useful for targeted epidemiological investigations and for research, but will only provide single point prevalences and are unlikely to produce genuinely comparable data from different Member States. Monitoring at the abattoir represents a practical point in the meat chain, which is likely to enable comparison of results both within and between countries, while sampling of raw meat cuts or trim with harmonised methodologies would provide a representative picture of the prevalence and concentration of VTEC seropathotypes on meat as it enters the processing or distribution part of the food chain.

The BIOHAZ Panel recommended that monitoring data on the prevalence and concentration of VTEC in ruminants' faeces, coat, and carcasses after chilling at the abattoir would assist in the assessment of risk to consumers. Co-ordinated sampling of raw meat cuts or trim for the prevelance and concentration of VTEC would provide suitable comparisons between Member States. Targeted surveys, conducted on a co-ordinated basis through Member States, of foodstuffs that have been associated with illness should include ruminant meat and minced meat products (in particular those that are likely to be consumed without cooking), ready-to-eat fermented meats, fresh vegetable and salads, in addition to unpasteurised milk and dairy products derived therefrom.

The BIOHAZ Panel recommended that initially monitoring should concentrate on VTEC O157 since this serotype is most frequently associated with severe human infections (including HUS)

in the EU. Monitoring should then be extended to other serotype (e.g. those of O26, O103, O91, O145 and O111) that are identified by periodical analysis of European human disease and epidemiological data as most frequently causing for human infections. It is also recommended that all MS use harmonised methods to define VTEC seropathotypes from human and non-human sources to allow more effective monitoring by comparison of isolates from food and animals with those from humans. This should be supported through a consensus discussion involving the Community Reference Laboratory (CRL) for VTEC and other relevant reference laboratories. Further strain characterisation comparing isolates from human and non-human sources should be centrally collected using data analysis methods similar to those used by e.g. PulseNet Europe.

Key words: VTEC, virulence factors, detection methods, isolation methods, characterisation, seropathotype, monitoring, animal populations, foodstuffs, public health

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY EFSA

The Directive 2003/99/EC[2] lays down the Community system for monitoring and collection of information on zoonoses, which obligates the Member States to collect relevant, and where applicable, comparable data of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks. In addition, the Member States shall assess trends and sources of these agents and outbreaks in their territory, and transmit to the European Commission, a report covering the data collected every year. The European Food Safety Authority (EFSA) is assigned the tasks of examining the data collected and preparing the Community Summary Report.

Data collected in the framework of Directive 2003/99/EC relate to the occurrence of zoonotic agents isolated from animals, food, and feed, as well as to antimicrobial resistance in these agents. The information concerning zoonoses cases in humans and related antimicrobial resistance is derived from the structures and/or authorities referred to in Article 1 of Council Decision No 2119/98/EC[3].

EFSA published its second Community Summary Report[4] on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2005, on 14 December 2006. For the first time, the European Centre for Disease Prevention and Control (ECDC) provided the data on cases of zoonoses in humans and also the analysis of these data in this report. The data used for analysis derived from several disease networks; the Basic Surveillance Network (BSN) and two Dedicated Surveillance Networks (DSN); Enter-Net and Euro-TB.

When the data received from the Member States were analysed it became apparent that the information available on Verotoxigenic *Escherichia coli* (VTEC) was not sufficient to facilitate a proper analyses of the importance of the findings of VTEC from foodstuffs and animal populations to the human VTEC cases. This information would be crucial in order to assess the potential sources of human infections and measures to protect the public health.

There was, in particular, lack of information on the VTEC serotypes and virulence factors of the VTEC isolates from food and animals, and due to this, it was often not possible to estimate whether the VTEC isolates from foodstuffs and animals were pathogenic to humans. According to the Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on Verotoxigenic *E. coli* (VTEC) in foodstuffs[5], only a small fraction of all VTEC-types isolated from animals, food, or the environment, are associated with human illness. Also, the use of different analytical methods, some of which were only able to detect serotype VTEC O157, hampered the analyses.

VTEC infections are important zoonotic diseases which are able to cause severe and life threatening diseases in humans. In 2005, a total of 3,314 human VTEC cases were reported in 18 EU Member States (The Community Summary Report 2005).

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2 Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC (OJ L 325, 12.12.2003 p. 31)

3 Decision No 2119/89/EC of the European Parliament and of the Council setting up a network for the epidemiological surveillance and control of communicable diseases in the Community (OJ L 268, 3.10.1998, p.1)

4 The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2005, *The EFSA Journal* (2006), 94

5 Scientific Committee on Veterinary Measures Relating to Public Health on Verotoxigenic E. coli (VTEC) in foodstuffs, adopted on 21-22 January 2003

The Scientific Panels on Biological Hazards and Animal Health and Welfare concluded, in their review the Community Summary Report in 2004[6], that a clear definition of human pathogenic VTEC, in particular the serotypes and virulence factors that are of public health importance, would aid in the interpretation of the results of the Community Summary Report.

Scientific advice is needed to harmonise the analytical and diagnostic methods used to detect and characterise the VTEC isolates, as well as to specify the necessary information to evaluate the association of the VTEC findings from foodstuffs and animals to human VTEC cases. Guidance concerning the optimal monitoring schemes in animal populations and foodstuff production would also improve the quality and usability of the data received.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The Biological Hazard Panel is asked to

- identify the strains and/or serotypes of VTEC which are pathogenic to humans;
- give advice regarding the analytical methods, including testing for virulence factors, to be used to detect and identify the human pathogenic VTEC strains/serotypes from food and animals;
- recommend the monitoring methods in animal populations and foodstuffs that are most optimal from the public health point of view. These recommendations may refer to, among other things, relevant animal species and food categories to be covered, the stages of food chain to be sampled, as well as the type of sample to be collected.

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6 Opinion of the Scientific Panel on Biological Hazards and of the Scientific Panel on Animal Health and Welfare on "Review of the Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2004", *The EFSA Journal* (2006) 403, 1-62

ASSESSMENT

1. Introduction

Verocytotoxin/Shiga toxin (VT/Stx)-producing *Escherichia coli* (VTEC/STEC) are characterised by the production of potent cytotoxins that inhibit protein synthesis within eukaryotic cells. These toxins are synonymously either termed verocytotoxins (VT), because of their activity on Vero cells, or Shiga toxins (Stx) because of their similarity with the toxin produced by *Shigella dysenteriae*.

VTEC infections constitute a major public health concern, because of the severe illnesses that they can cause, such as hemorrhagic colitis (HC) and the hemolytic uremic syndrome (HUS), especially among children and the elderly. VTEC infections are of low prevelance (overall EU-total of 1.2 cases per 100,000 of the population) (EFSA, 2006a); however the high infectivity and seriousness of disease justify the inclusion of this group of bacteria as important foodborne pathogens.

The term "enterohemorrhagic *E. coli*" (EHEC) has been used to designate the subset of VTEC that is considered to be highly pathogenic to humans. The EHEC term was originally conceived to denote strains of *E. coli* that cause HC and HUS, produce VT, cause attaching-and-effacing (A/E lesions) on epithelial cells, and possess an approximately 60-MDa "EHEC plasmid" (Levine, 1987). The term "atypical" EHEC has also been used to define the VTEC strains that do not produce A/E lesions and/or do not possess the large "EHEC plasmid" (Nataro and Kaper, 1998). There is however no clear definition of the EHEC group, except that all EHEC strains by definition are considered to be human pathogens; although this is not necessarily the case with all VTEC strains. A simple definition of EHEC is therefore that it serves as a proxy for human pathogenic VTEC, albeit the clinical outcome of an EHEC infection might not necessarily be HC. For the purposes of this document the terms VTEC and VT will be used.

The majority of the cases worldwide are caused by strains of serotype O157, but infections caused by serotypes other than O157, including O26, O111, O103 and O145 have been increasingly reported; these strains are usually referred to as VTEC non–O157. Techniques for the detection of VTEC O157 and, to a lesser extent, VTEC non–O157 which are pathogenic to humans are becoming increasingly available and these organisms have been identified in association with human infection in most European countries, albeit at very different rates (see later text in section 2.1). VTEC O157 that possess the flagellar antigen 7 or are phenotypically non-motile comprise a subset of strains within the diverse O157 serotype. Strains of O157 that do not produce VT possess a range of flagellar antigens other than 7 and have been isolated from humans, animals, food and carcasses. Many are negative for other currently-known virulence factors and their pathogenic potential from humans is unknown.

1.1. Animal reservoirs

VTEC represent the only pathogenic group of *E. coli* that has a definite zoonotic origin, although not all the VTEC strains have been demonstrated to cause disease in humans. VTEC rarely cause disease in animals, and ruminants are recognised as their main natural reservoir. Cattle are considered to be the major animal source of VTEC that are virulent to humans, in particular VTEC O157, and the ecology of this microorganism in cattle farming has been extensively studied (Caprioli *et al.*, 2005). VTEC O157 and other serotypes associated with

human infections, have also frequently been isolated from the intestinal content of other ruminant species, including sheep, goat, water buffalo, and wild ruminants, while pigs and poultry have not been identified to be major sources of VTEC.

Cattle are asymptomatic excretors of VTEC O157, which are transient members of their gut microflora. The presence of VTEC O157 appears to be influenced by the age of the animals and by the season. Shedding is usually longer and more intense in calves than in adult cattle, and increases after weaning. It is also much higher during the summer period (Caprioli *et al.*, 2005). The reported prevalence of VTEC and/or VTEC O157 in cattle is also clearly influenced by the sampling and detection methods adopted in the investigations. The use of specific immuno-concentration procedures for VTEC O157 or other serotypes strongly enhances the sensitivity of the isolation methods. Faecal testing of dairy cattle worldwide showed prevalence rates for VTEC O157 (0.2 to 48.8%) and VTEC non-O157 ranging from 0.2 to 48.8% and 0.4 to 74.0%, respectively (Hussein and Sakuma, 2005). Global testing of beef cattle faeces revealed prevalence rates ranges for VTEC O157 and non-O157 VTEC of 0.2 to 27.8% and 2.1 to 70.1%, respectively (Hussein and Bollinger, 2005).

Pigs are not considered to be a major source of VTEC associated with human infections. Prevalence rates of VTEC O157 faecal carriage ranging from 0.2 to 2% have been reported in pigs slaughtered in European countries (Heuvelink *et al.*, 1999; Johnsen *et al.*, 2001; Bonardi *et al.*, 2003), Japan (Nakazawa and Akiba, 1999) and the United States (Feder *et al.*, 2003). The low carriage rate observed in those studies could be the result of accidental exposure of pigs to VTEC O157 through contamination of feedstuff or the environment with ruminant manure in farms that do not comply with good husbandry practices. However, studies conducted in South America (Borie *et al.*, 1997; Rios *et al.*, 1999) showed a surprisingly high rate of VTEC O157 faecal carriage (8-10%) in slaughtered pigs. These marked differences in prevalence may be due to differences in the pig husbandry and slaughtering practices.

There is a wide variety of VTEC in the food-producing animal populations, of which the public health importance remains unclear. A restricted range of serotypes (i.e. O157, followed by O26, O103, O91, O145 and O111) are associated with public health risks, however isolates of these serotypes are not necessary pathogenic when recovered from food or live animals. Consequently, serotyping alone when applied to VTEC isolates from food and animals is not the optimal method of identifying public health risk.

1.2. Modes of transmission

Although the ultimate source of VTEC is the faeces of ruminant animals, there are four main transmission routes whereby these organisms may be transmitted to humans.

The **food-borne route** often involves consumption of undercooked meat or meat products (usually beef) contaminated by contact with faeces at slaughter. Dairy products (milk, cheese, cream) associated with infection have included those that are unpasteurised, have had a pasteurisation failure or have been contaminated post-pasteurisation. Ready-to-eat foods have also been associated with infection, particularly cooked meats contaminated by raw materials during processing, in catering establishments, at retail sale and in the home. An increasing number of other food vehicles have been associated with human infections (Caprioli *et al.*, 2005). These include:

- low pH products including fermented salami, mayonnaise and yogurt;
- fruits and vegetables fertilized with ruminants' manure or contaminated during harvesting or processing, including bean-sprouts, lettuce, tomatoes, coleslaw, and

unpasteurized fruit juices. Such 'produce' items are now recognised in the US as a major cause of outbreaks requiring increased biosecurity and changes in processing practices.

Water is a very efficient vehicle for the dissemination of VTEC. Drinking water supplies may become contaminated by animal faeces due to poor design and inadequate treatment, particularly at times of high rainfall. This is a problem mainly in rural areas. Recreational water that has been exposed to animal faeces is also a source of infection. The use of faecally-contaminated water for irrigation may also disperse VTEC to vegetable and salad crops that act as secondary vehicles for human infection.

Direct or indirect contact with animals and their faeces results in ingestion of VTEC if hygiene (hand washing) is inadequate. VTEC may be carried superficially or may be shed in the faeces of animals. Studies have linked human infections to farm visits, particularly by children (Caprioli *et al.*, 2005). The range of implicated animals is large. Ruminants (particularly cattle but also sheep, goats, water buffalo, and wild ruminants) have most frequently been linked with human cases. Pigs and poultry, equines and other wild animals have not been identified as major sources of human pathogenic VTEC in Europe.

Person-to-person spread by the faecal-oral route is very important in the transmission of VTEC infection among family members, carers, in day-care centres, schools and in other institutional settings.

Data on transmission routes have been established by investigation of general outbreaks; however in many outbreaks more that one route may be involved, such that primary infection acquired from a food or animal source may be disseminated to secondary cases in families or the wider community. The high infectivity and intrinsic properties of VTEC (e.g. acid tolerance and the ability to survive well in the environment) have made investigation of infection increasingly complex. Case-control studies of varying design have been performed to assess risk factors for sporadic infection with VTEC (mainly O157) in several European and other countries. Results show differences between countries and the risk factors may be age-related. In several studies specific food vehicles were not identified but contact with animals and/or the rural environment were identified as the major risk factor for VTEC O157 infections (Locking *et al.*, 2001; O'Brien *et al.*, 2001; Kassenborg *et al.*, 2004; Werber *et al.*, 2007).

2. Surveillance and data sources

2.1. Human infections

Data on VTEC infections in Europe for the Zoonoses Report derive from Enter-net and from data provided by the Member States to the ECDC. Enter-net was an international surveillance network for human gastrointestinal infections which was established in 1997 to maintain and develop laboratory-based surveillance of the major enteric bacterial pathogens. The Enter-net network was funded by DG SANCO of the European Commission until September 2006 and received funding from ECDC until September 2007, when the coordination activities are transferred to ECDC. This network brings together the national surveillance leads and reference microbiologists to conduct international surveillance of salmonellosis, VTEC infections, and campylobacteriosis. Participants are from all EU Member States, WHO and non-EU countries, including EU-candidate countries, Canada, the United States, South Africa, Japan and Australia. In the Enter-net VTEC database, data for 31 countries are available, of which 21 have data for the entire period 2000-2007.

ECDC collects data about at least 40 communicable diseases in the EU. The diseases covered are specified in Commission Decision 2000/96/EC[7]. Existing surveillance data from national databases are transferred into a common EU-database. Currently, 24 Member States of the EU participate in the network, in addition to Iceland, Norway and Switzerland. The data collected reflect the content of the national databases and the surveillance strategies used and thus differs considerably between countries. While some countries routinely collect and submit data on nearly all of the diseases in the ECDC database, others have data on a subset of these. Data on VTEC are available for 17 Member States and Norway from 2004 or 2005 onwards[8]. Basic data collected include information on reporting country, the date when the case was reported, age and sex of the case as well as a case identifier, and case classification (possible, probable or confirmed). The data for the different countries are not directly comparable as the surveillance of communicable diseases is organised differently among the EU Member States. Data on the VTEC types associated with human infections are currently not collected by this method.

Standard criteria and case definitions are available to Enter-net and are available to Member States for reporting to ECDC (Appendix I); however, not all participating countries will have adopted these new criteria in their national systems. Because cases were reported to Enter-net only from national reference laboratories in each individual country, the total number of cases reported through Enter-net will usually be smaller than the total number of cases reported to ECDC. However some countries report smaller numbers of cases to the ECDC, probably because of under-reporting or local national reporting practices. It is currently not possible to directly combine Enter-net and ECDC data.

The criteria for reporting include a spectrum of clinical illnesses ranging from mild diarrhoea to HUS (Appendix I). Some of the differences between the total numbers of cases reported to both the ECDC and Enter-net therefore reflect national diagnostic and investigative practices, particularly where considerable differences in national syndrome specific reporting and investigation exist. For example, surveillance in France and Italy is largely based on reporting cases of HUS, and rarely captures data on individuals with VTEC in their stools who are asymptomatic or have uncomplicated diarrhoea (both of which are covered in the case definition). In the UK, for example, it is recommended that laboratories culture all stool samples from individuals with diarrhoea for presumptive VTEC O157. Surveillance strategies for different countries are given in the Tables II.1 and II.2 of Appendix I.

The numbers of cases, proportions of VTEC O157 and numbers of cases of HUS reported through Enter-net for 2004 are given in Table 1. These data illustrate the marked differences in the proportions of cases reported as HUS (almost 100% in Italy and <1% in Germany). These data also show considerable differences in the ratio between cases of VTEC O157 and VTEC non-O157 infection. These differences are likely to reflect that in some countries surveillance is mainly based on laboratory methods specific for VTEC O157 only Tables II. 1 and II.2 of Appendix I. For example, more than 95% of infections detected in Scotland, England and Wales were due to VTEC O157 but in Ireland this is 86% with the remainder caused by non-O157 strains. However in continental Europe, more than half of the infections was attributed to serotypes other than O157. But there are considerable national differences: O157 is the most commonly detected serotype in Belgium, France, Finland, Hungary, the Netherlands, Sweden and Spain. However in Denmark, Germany, Italy, Norway and Luxembourg, other serotypes are most commonly recognised (Enter-Net, 2004; Enter-net, 2005). Some of these differences

[7] Commission Decision of 2000/96 of 22 December 1999 on the communicable diseases to be progressively covered by the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (OJ L 28, 3.2.2000, p. 50–53).

[8] https://www2.smittskyddsinstitutet.se/BSN/tables/Contents_English.html

reflect national diagnostic and surveillance strategies; however these may also reflect natural differences in the disease. Data from Scotland (Locking *et al.*, 2001) indicate that in this Member State, even when diagnostic procedures for the detection of VTEC non-O157 are routinely applied to samples of faeces from patients with diarrhoea, VTEC non-O157 are rare.

Table 1. **Numbers of reported VTEC cases, proportions due to VTEC O157 and VTEC non-O157 and numbers of cases of HUS for 2005 (Enter-net, 2005)[9].**

Country	Number of cases	% of cases due to		Numbers of HUS cases[(a)]
		O157	Non-O157	
Austria	59	46%	54%	9
Belgium	52	60%	40%	20
Denmark	156	17%	72%[(c)]	4
England and Wales	954	100%		NK [(b)]
Finland	21	71%	29%	NK
France	108	68%	19%[(c)]	20
Germany	759	10%	64%[(c)]	8
Hungary	5	60%	40%	0
Ireland	125	86%	14%	17
Italy	22	14%	82%[(c)]	19
Luxemburg	11	18%	82%	NK
Malta	5	100%		0
Netherlands	54	100%		4
Norway	18	39%	50%[(c)]	1
Portugal	15	NK[b]	NK[b]	NK
Scotland	176	94%	6%	NK
Spain	15	100%	0%	1
Sweden	364	52%	13%[(c)]	11

(a): HUS is a clinical diagnosis, hence cases are also reported in the absence of the isolation or confirmation of VTEC infection.
(b): not known
(c): isolates from some cases not serotyped

[9] http://www.hpa.org.uk/hpa/inter/enter-net/Enter-net%20annual%20report%202005%20final.pdf

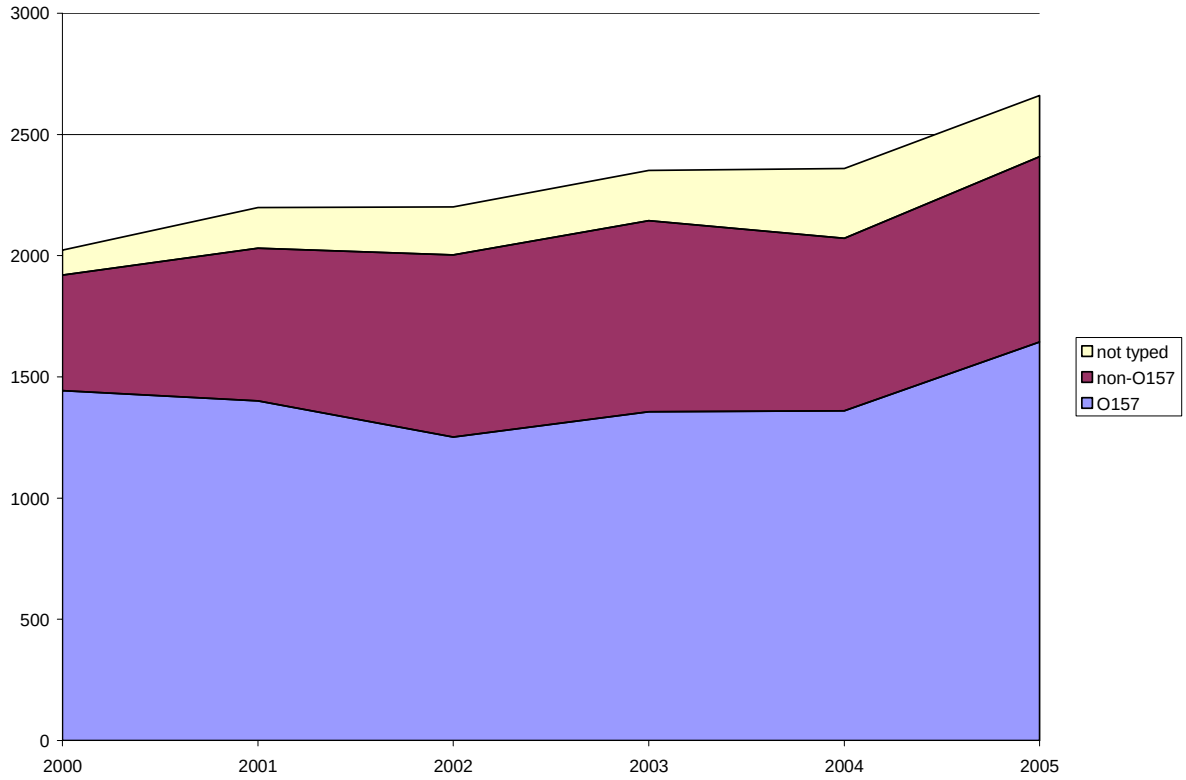

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Fig 1. **Reports to Enter-net from 21 Member States on VTEC 2000-2005 (Enter-net, 2005)**

Data from Enter-net (Figure 1) show that VTEC O157 is the most common single serotype identified in the Member States during 2000-2005, and the number of cases rose from 1,443 to 1,767. Infections caused by non-O157 serotypes rose from 476 to 775 cases over the same period. VTEC O157 infection increased by over 284 cases between 2004 and 2005, although a significant number of these (circa 200) could be attributed to the outbreak in Wales in September 2005. The range of VTEC non-O157 serotypes reported to Enter-net for 2004-2005 is shown in Appendix III. Amongst the 1441 non-O157 cases where a VTEC serotype was identified during 2004-2005, 61% were due to O26, O91, O103, O111, O145 and O146, whereas the remaining 39% were due to 110 other serotypes. Enter-net data show that there are significant differences between the proportions of the different clinical presentations of patients infected by VTEC O157 and VTEC non-O157. For example data from 2004 and 2005 show that a greater proportion of patients with bloody diarrhoea and HUS was found amongst those infected by VTEC O157 than those infected by VTEC non-O157 (Table 2).

Table 2. **Clinical presentation of VTEC cases reported to Enter-net 2004-2005 where serotypes were established (source: Enter-net data[10]).**

	Diarrhoea	Bloody diarrhoea	HUS	Asymptomatic	Total
O157	181 (31%)	231 (39%)	128 (22%)	44 (8%)	584
Non-O157	360 (69%)	69 (13%)	70 (13%)	24 (5%)	523

Enter-net data from 2002 to 2006 show a consistent pattern where in any one year at least 63% of all cases and at least 64% of all HUS cases were due to O157 (Tables 3 and 4). At least 18% of the annual totals for the remaining cases and 23% of HUS are accounted for by the serotypes O26, O103, O91, O145 and O111. Amongst this entire series of cases of HUS, there was only one due to O91 in 2003. In summary, over the period 2002-2006, of all infections where the serotype was established 66% were due to O157, 20% due to O26, O103, O91, O145 and O111, and the remaining 14% to other serotypes. During the same period, amongst the cases of HUS, 68% were due to O157, 26% to O26, O103, O91, O145 and O111, and 6% to other serotypes.

It should be noted that some non-O157 serotypes can be extremely virulent. The latter was the case in Norway, where a VTEC O103:H25 contaminated dry fermented sausage made of sheep meat and caused 18 cases of infection, of which ten were complicated by HUS (Schimmer *et al.*, 2006).A feature of the Norwegian outbreak strain is that the genes encoding VT are easily lost on subculture and VT negative variants of the outbreak strain have been only recovered from some of the HUS patients. The possibility of VT negative strains that descend from human pathogenic VTEC is a further challenge for the diagnosis of VTEC associated disease.

[10] Available at: http://www.hpa.org.uk/hpa/inter/enter-net_menu.htm

Table 3. **Most commonly reported serotypes of VTEC for all human cases amongst EU Member States where serotypes were established (source: Enter-net data[11]).**

	2002	2003	2004	2005	2006
O157	1,189 (63%)	1,262 (63%)	1,283 (66%)	1,767 (70%)	1,726 (66%)
O26	115 (6%)	143 (7%)	135 (7%)	169 (7%)	170 (7%)
O103	172 (9%)	141 (7%)	55 (3%)	119 (5%)	116 (4%)
O91	96 (5%)	86 (4%)	71 (4%)	82 (3%)	90 (3%)
O145	44 (2%)	58 (3%)	69 (4%)	55 (2%)	86 (3%)
O111	34 (2%)	34 (2%)	23 (1%)	45 (2%)	44 (2%)
O146	29	31	34	29	30
O128	17	21	15	22	18
O55	15	0	17	18	24
Other	178	238	247	236	300
Total	1889	2014	1949	2542	2604

Table 4. **Most commonly reported serotypes of VTEC causing HUS amongst EU Member States where serotypes were established (source: Enter-net data[11]).**

	2002	2003	2004	2005	2006
O157	47 (76%)	46 (70%)	52 (66%)	70 (64%)	95 (70%)
O26	9 (15%)	4 (6%)	14 (18%)	16 (15%)	9 (7%)
O145	2 (3%)	7 (11%)	4 (5%)	7 (6%)	7 (5%)
O103	1 (2%)	0	2 (3%)	3 (3%)	14 (10%)
O111	3 (5%)	5 (8%)	1 (1%)	7 (6%)	2 (1%)
O91	0	1 (2%)	0	0	0
O146	0	0	0	0	0
O128	0	1	0	0	0
O55	0	0	2	1	0
Other	3	2	3	5	9
Total	62	66	78	109	136

2.2. Animal populations and foods

There are various sources of data concerning the presence of VTEC in animal populations and food within Europe. This includes data from public health, veterinary and university laboratories. These publicly funded activities are performed for the purposes of co-ordinated food surveys, official food control, public health investigations, monitoring and research projects. The results of some, but not all of these publicly funded activities are transmitted and collected through European surveillance networks, including those collected for the Trends and Sources of Zoonoses as will be described below.

[11] Available at: http://www.hpa.org.uk/hpa/inter/enter-net_menu.htm

Food industries also test and control the foodstuffs at different stages of their production in compliance with the European and/or national regulation. Testing for VTEC is limited to O157 and such monitoring data are rarely published.

One of the primary sources of pan-European data for VTEC is the European Community system for monitoring and collection of information on zoonoses, which was established by Council Directive 92/117/ECC[12]. This Directive sets rules for the Member States of the EU to collect, evaluate and report to the Commission, each year, data on specific zoonoses and zoonotic agents in animals, foodstuffs and feedingstuffs. At the end of the 1990s, the Commission considered it necessary to revise the existing rules on monitoring and reporting of zoonoses. The aim was to improve the system, in particular regarding the comparability of data, and to extend the system to cover additional zoonoses on a mandatory basis and certain other important aspects such as antimicrobial resistance and foodborne outbreaks. The new Zoonoses Directive 2003/99/EC[13] was adopted by the Council and the European Parliament on 17 November 2003, and was enacted from 12 June 2004. Reporting according to the new rules in Directive 2003/99/EC started with data collected during 2005.

In 2004, for the first year, Member States submitted data using a new online zoonoses reporting system that was created and is maintained by the EFSA. In 2005, Member States submitted information on the occurrence of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks to the European Commission and the EFSA. Further information on zoonoses in humans was acquired from the ECDC. These data covered 16 zoonotic diseases including VTEC. Assisted by its Zoonoses Collaboration Centre, EFSA and ECDC jointly analysed the information and published the results in the 2005 Community Summary Report.

The Community Summary Report contains a compilation of data on VTEC in the food chain generated by the different Member States and it gives an important general overview of the ongoing VTEC activities in the different Member States. There are substantial differences in the amount of reported data from different countries; some Member States report little or no data and consequently there is uneven geographical coverage.

In 2006, a Community Reference Laboratory (CRL) for *E. coli*, including VTEC was established by the EC DG SANCO for the area of food safety and veterinary public health. The CRL is based at the Istituto Superiore di Sanità in Rome (www.iss.it /vtec) and is currently establishing a network with the National Reference Laboratories (NRLs) in each Member State. Such a network could be used in the future to collect data on the characteristics of VTEC isolates from non-human sources in Europe as well as for training, for the production of standardised methods and reference materials and external quality assessment activities.

[12] Council Directive 92/117/EEC of 17 December 1992 concerning measures for protection against specified zoonoses and specified zoonotic agents in animals and products of animal origin in order to prevent outbreaks of food-borne infections and intoxications. OJ L 62, 15.3.1993, p. 38–48.

[13] Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC (OJ L 325, 12.12.2003 p. 31)

3. Characterisation and typing of VTEC strains

Characterisation of VTEC strains is extremely valuable since this allows comparisons between isolates from human, animal and food origin and also provides information on changes in their prevalence over time and in different geographical locations. Characterisation of VTEC with respect to the presence of a range of virulence properties may further identify markers that confer the capacity to cause serious infections and so identify strains with increased risk of causing disease. These data are essential for an evolving definition of human pathogenic VTEC. Some typing and fingerprinting methods inform epidemiological investigations that link human cases to each other and to specific sources of infection.

3.1. Serotyping

Strains of *E. coli* including VTEC are serotyped by an internationally-recognised and evolving scheme comprising over 180 O-types (lipopolysaccharide) and 56 H-types (flagella). Several of the most recently designated types include VTEC strains. Full serotyping is generally performed in national reference laboratories although antisera for some common VTEC O-groups are available commercially. Agglutination kits, generally based on antibody-coated latex particles, are used widely in the identification of presumptive VTEC, particularly O157, isolated from human and non-human samples.

It is important to recognise that some O-groups, such as O111 and O128, are highly diverse and contain, in addition to VTEC, other strains of *E. coli* that cause diarrhoeal illness and have specific virulence traits that do not include VT. Many VTEC strains that cause human illness show specific combinations of O- and H-types (e.g. O157:H7, O26:H11, O91:H21). Data collected by Enter-net indicate that the distribution of VTEC serotypes between countries is not uniform but this may be strongly influenced by the detection methods used and criteria applied for testing (Appendix I).

Molecular 'serotyping' methods attempt to avoid the dependency on antisera and make serotype characterisation more widely available. Methods have targeted various genes involved in the biosynthesis of the O antigen by identifying sequences unique to O groups such as O157, O26, O111, O113 and O145 for specific Polymerase Chain Reactions (PCRs). PCR-RFLP (PCR-restriction fragment length polymorphism) and PCR combined with sequencing have also been used. Determination of H-type has been directed mainly at the *fli*C gene that is present even if the isolate is non-motile. The large number of O-types of *E. coli* means that in the short to medium term, DNA-based tests are unlikely to replace conventional serotyping in the reference laboratory setting for comprehensive characterisation of isolates. Such developments require sequence data to become available on a more extensive range of O-groups than the present. However detection methods for VTEC in foods are already included in DNA-based methods of O antigen determination for common serotypes together with tests for the presence of the H7 *fli*C sequence (see section 4.2). An expansion of DNA-based tests appears likely, particularly with the application of DNA and protein arrays.

3.2. Vero cytotoxin (VT) production

VTEC are defined by their ability to produce either one or both antigenically-distinct vero cytotoxins termed VT1 and VT2 (Table 5) that were first recognised by their ability to cause an irreversible cytopathic effect on Vero cells and other cell lines in culture (Konowalchuk *et al.*, 1977). The vero cell assay is performed by addition of cell-free supernatants to tissue culture monolayers and preliminary results are obtained after 24 hours with final results after 3-4 days. To demonstrate that the cytopathic effect is due to VT it is necessary to perform neutralisation assays with specific antisera that also differentiate the two toxin types (Scotland *et al.*, 1985). This assay is sensitive and regarded as the 'gold standard' to which other methods should be validated. However since maintenance of tissue culture is costly, specialised in nature and labour-intensive, for confirmation of VT production, immunological methods targeted at the VT antigens have been largely supplanted the Vero cell assay. These are suitable for both detection of VT in different sample types and confirmation of VT production from pure cultures; some assays differentiate between VT types (see section 4.1.2). Confirmation of VTEC by examining strains for the genes encoding VT using PCR is a common alternative to the Vero cell assay, although the presence of the gene sequence may not always equate with expression of functional toxin.

Table 5. **Virulence factors and putative virulence properties of VTEC for consideration in relation to prediction of serious disease**

Vero cytotoxin and VT gene		**Comment**
VT1	*vtx1*[(c)]	One amino acid difference from for shiga toxin of *Shigella dysenteriae*
VT1c	*vtx1c*[(b)]	Possible ovine association; serotype O128
VT1d	*vtx1d*[(b)]	Some bovine strains
VT2	*vtx2*[(c)]	Association with serious disease
VT2c	*vtx2c*[(c)]	Association with serious disease
VT2d[(a)]	*vtx2d*[(c)]	Activatable by intestinal mucus
VT2e	*vtx2e*[(b)]	Porcine oedema disease strains
VT2f	*vtx2f*[(b)]	Strains from feral pigeons
VT2g	*vtx2g*[(b)]	Some bovine strains
Locus of Enterocyte Effacement (LEE) pathogenicity island		
Intimin	*eae*	Present in VTEC serotypes associated with serious disease
Intimin subtypes		Sequence and antigenic differences; serotype association in VTEC and enteropathogenic *E. coli*
Other properties		
Pathogenicity island O122	*efa*1 etc	Presence linked to LEE; possible link to colonization
STEC auto agglutinating adhesion	*saa*	Limited range of LEE-negative VTEC; plasmid encoded
Enterohaemolysin (Ehx)	*ehx*A	Large plasmid of VTEC O157 and some other VTEC; role in pathogenesis not proven
Catalase/peroxidase	KatP	Other plasmid-encoded properties; potential virulence factors but roles not clear.
Extracellular serine protease	EspP	
Subtilase cytotoxin		
Sorbitol fermenting EHEC O157 fimbriae	*sfp*A	Characteristic of sorbitol fermenting VTEC O157; strains cause serious disease; plasmid-encoded
TccP	*tccp*	A recently discovered type III secretion system effector protein

(a): There are several toxins in the literature bearing the suffix 'd'. The consensus now is that this designation be reserved for the mucus activatable form. (b): vtx toxin types never or very rarely been associated with human disease, and only as a cause of uncomplicated diarrhoea. (c): associated with human disease.

3.3. Vtx gene typing and subtyping

Genes encoding VT have been designated *vtx* and this term is synonymous with *stx*, derived from the alternative nomenclature that uses the term STEC rather than VTEC (see section 1). The *vtx*1 and *vtx*2 genes encoding the main classes of VT1 and VT2 toxins have been confirmed by hybridisation with polynucleotide DNA probes in dot blot assays and do not cross-hybridise under conditions of high stringency. A large number of PCR assays have evolved for the detection of *vtx*1 and *vtx*2 genes. There is no consensus on the region(s) of the A and B subunits of VT to use as PCR targets or agreement on the primers or PCR platform to use. It is essential that PCRs are validated for use. Ring trials organised by Enter-net have been very valuable to ensure uniformity of results in centres that use different PCRs to type strains from human infections. Assays are often performed in multiplex formats and are an important part of most detection tests for VTEC (see section 4). Similar ring trials are being organised by the CRL for VTEC for the NRLs designated for the area of food safety and veterinary public health. As *vtx* genes are generally carried by bacteriophages this property may be unstable during isolation and storage of some strains or may be acquired by horizontal transmission between strains.

Association between the type of VT and particular serogoups has been demonstrated and this may differ between countries and over time. For example, O26:H11 strains have classically been positive for VT1 only, but strains producing VT1 and VT2 are now causing disease and O26:H11 producing VT2 has now been increasingly associated with HUS.

A growing number of toxin subtypes of VT2, and to a lesser extent of VT1, have been identified and designated by the addition of a letter suffix to the type name (Table 5). These differ in biological activity and may be distinguished serologically or by receptor binding. Gene subtypes, designated according to the toxin subtype, cross-hybridize under stringent conditions but there is sufficient sequence variation to permit design of subtype-specific PCRs. The nomenclature of VT subtypes and genes is complex; the list shown in Table 5 is based on Thorpe *et al.* (2002), Scheutz *et al* (2001) and Scheutz and Strockbine (2005). Strains of VTEC O157 generally possess *vtx*2 and/or *vtx*2c and may also carry *vtx*1. Non-O157 strains are highly diverse in the *vtx* sequences carried.

Several studies have indicated that the presence of some *vtx*2 subtypes is strongly associated with the development of serious disease (Friedrich *et al.*, 2002; Persson *et al.*, 2007). The combination of *vtx*2 and *vtx*2c has been found in VTEC O157 in studies of HUS and the presence of mucus-activatable toxin *vtx*2d in strains of serotypes O91:H21, O8:H8 and O113:H21 implicated in serious illness. In contrast *vtx*1c may be present in strains causing uncomplicated diarrhoea or in healthy individuals. VTEC producing *vtx*1c belong to other serotypes and are usually isolated from patients with uncomplicated diarrhoea or healthy individuals (Friedrich *et al* 2003). *vtx*2 subtypes *vtx*2e, *vtx*2f, *vtx*2g have never or very rarely been associated with human disease, and only as a cause of uncomplicated diarrhoea.

3.4. Presence of other virulence genes

The genetic locus of enterocyte effacement (LEE) is a pathogenicity island present in enteropathogenic *E. coli*, VTEC O157 and some VTEC non-O157 that contains genes required for the formation of attaching and effacing epithelial lesions. Within LEE, the most usually assayed gene is that for intimin (*eae*) that exists in a large number of sequence subtypes due to variation at the C-terminal end. PCR assays are usually directed at the conserved region of the sequence.

The presence of the *eae* gene is strongly associated with some serotypes of VTEC such as, O157:H7, O157:non-motile (NM), O26:H11, O111:NM, O103:H2, O121:H19, and O145:NM. These strains have been shown to cause serious disease such as HC and HUS. However, LEE-negative strains such as O91:H21, O113:H21 and O128 have also been associated with serious illness indicating that other factors enhance the virulence potential of these VTEC.

Several studies have investigated the role of other genomic islands identified in VTEC O157 for their potential contribution to virulence in other VTEC. One island, termed O122, is contiguous to LEE in many strains and is strongly associated with attaching and effacing *E. coli* but no other pathogroups. In VTEC O157, it contains the 5'end of the *efa*1 (EHEC factor for adherence) gene but this region is complete in many VTEC non-O157 and in sorbitol-fermenting VTEC O157 and may be linked to colonisation of the bovine intestine.

A range of plasmid-determined putative virulence factors are present in VTEC strains. The STEC autoagglutinating adhesin (Saa) has been found on the large plasmid in LEE-negative VTEC strains belonging to several serotypes including O113, O91 and O128. Production of enterohaemolysin is encoded by the large plasmid pO157 characteristic of VTEC O157 and some other VTEC. Phenotypic detection can be achieved on specialised agar (section 4.2.2.2) but expression may be difficult to detect and PCR detection of the *hly*A gene is generally employed. Other plasmid markers include *kat*P and *esp*P. Sorbitol-fermenting VTEC O157 strains that have caused outbreaks of HUS are characterised by the presence of plasmid-coded fimbriae and the plasmid-borne pilin subunit gene (*sfp*A) has been used as a potential diagnostic marker for these strains by PCR.

3.5. Phage typing

VTEC O157 strains are differentiated into about 90 types by a scheme of 16 bacteriophages (Khakhria *et al.*, 1990). The method is rapid, epidemiologically-valuable in real time and gives information about the emergence and distribution of new strains. It is performed routinely in a limited number of laboratories worldwide. There is a close association between phage type and the presence of *vtx*1 and *vtx*2 gene subtypes and some types (PT2, PT21/28) are more strongly associated with HUS than others. Generally the same phage types that cause human illness are recovered from animals, although the proportions may differ. Phage types of VTEC O157 vary between countries and information on prevalence in countries that do not perform the methods themselves may be obtained from investigation of infections in travellers. Phage typing schemes are not available for VTEC non-O157.

3.6. Subtyping and fingerprinting for epidemiology and population studies

Pulsed field gel electrophoresis (PFGE) of macro-restriction fragments of genomic DNA is widely used to compare strains for epidemiological purposes such as the investigation of outbreaks and source-tracing (Barrett *et al.*, 1994; Willshaw *et al.*, 2001). PFGE has been applied to both O157 and non-O157 VTEC: there is considerably more information on the application of PFGE to the former group. PFGE profiles are stored, analysed and exchanged electronically using commercial software (Swaminathan *et al.*, 2001). Strong epidemiological data are essential in the application of PFGE as strains that share profiles may be obtained from unlinked cases separated geographically and over long periods of time.

Sequence-based typing methods include multi-locus variable number of tandem repeat analysis (MLVA) that has been evaluated as an alternative to PFGE for VTEC O157 (Noller *et al.*, 2003a; Hyytia-Trees *et al.*, 2006). It has the advantage that the output appears as a numeric sequence that can be generated automatically but validation of protocols is required. MLVA methods have not been applied to VTEC non-O157 because of lack of sequence data. Multi-locus sequence typing (MLST) is more appropriate for study of the relationships between *E. coli* populations than for typing and epidemiological analysis (Noller *et al.*, 2003b).

3.7. Predictive markers for VTEC that may cause serious illness

Over 200 O:H serotypes producing VTEC have been identified from all sources (Scheutz and Strockbine, 2005), although many lack the full complement of known virulence factors found in strains that cause serious disease; however over 100 have been associated with disease in humans (Table 5). The role of some putative virulence factors is still uncertain and they may be detected as markers of particular strains rather than contributing to the disease process. There are substantial gaps in knowledge about the interaction between VTEC and their hosts; some VTEC, including O157, may be carried asymptomatically by both adults and children. The definition of pathogenic strains has been based on phenotypic properties and the linkage of certain serotypes to serious illness. Simple methods for identification of VTEC O157 strains and improved techniques for O26, O103, O111 and O145 may have led to a degree of skewing in the prevalence of these strains.

Although serotype may conveniently be a surrogate marker for virulence potential, the availability of molecular techniques enables simple direct detection and subtyping of virulence-associated genes. These tests can be incorporated in detection methods. The presence of LEE (Caprioli *et al.*, 2005) and of genes for VT2, particularly subtypes *vtx*2 and *vtx*2c have been identified as markers that are more closely indicative of strains associated with HUS than the serotype alone (Friedrich *et al.*, 2002; Persson *et al.*, 2007). This genetic approach can be extended to include genes such as *vtx*2d, the mucus-activatable variant associated with serious human illness in some LEE negative strains, markers on other pathogenicity islands, and the *sfp*A gene of HUS-associated sorbitol-fermenting VTEC O157 strains. An approach that incorporates virulence gene tests independent of serotype as predictors for risk may enable newly emerging VTEC threats to be identified.

All VTEC O157 possess the intimin genes on the chromosomally located LEE region. The presence of *vtx*2, either alone or with *vtx*2c, is associated with increased risk of serious disease (Friedrich *et al.*, 2002). There is a link between infection with some phage types (PT) of VTEC O157 with the development of HUS. A study in the UK and Ireland showed that PT 21/28 and PT2 that possessed *vtx*2 and *vtx*2c genes were isolated from HUS cases (Lynn *et al* 2005). Strains of

PT8 rarely cause HUS but are the second most common cause of disease in England and Wales accounting for 20-25% of confirmed isolates annually (Anonymous, 2007). Abattoir studies in England have shown that strains of VTEC O157 isolated from cattle and sheep reflect those causing human illness.

The concept of seropathotype that has emerged classifies VTEC into five groups based on the incidence of serotypes in human disease, associations with outbreaks versus sporadic infection, their capacity to cause HUS or HC, and the presence of virulence markers (Karmali, 2003; Wickham *et al.*, 2006). This approach attempts to combine these inputs to understand better the apparent differences in virulence of VTEC. **Seropathotype A** strains (VTEC O157) have a high relative incidence, commonly caused outbreaks and are associated with HUS. O26:H11, O103:H2/NM, O111:NM and O145:NM together with O121:H19 fall into **Seropathotype B**, as they have a moderate incidence and are uncommon in outbreaks but were associated with HUS. A further **Seropathotype C** included O91, O104 and O113 strains all of H-type 21 and associated with HUS, but these strains were of low incidence and rarely caused outbreaks. Groups A and B possess LEE and genes of O-island 122 but group C strains may be LEE-negative and have only some of the O122 virulence genes. **Seropathotypes D and E** are not HUS-associated and are uncommon in man or found only in non-human sources. Surveys targeting isolation of VTEC (but not specifically O157) and from non-human sources generally produce isolates from groups C and D. This classification may however be affected by differences in the relative occurrence of some serotypes in different countries (Enter-net data). The clinical criteria used for collection of data for Enter-net (Appendix I) recommend the characterisation of isolates by serotyping, phage typing, *eae* gene detection and establishment of *vtx* subtypes, which would allow the classification of isolates into these five groups.

However, even the concept of 'seropathotype' is not fully taking into account that there seem to be substantial differences in the virulence potential within the different VTEC serotypes. In recent years, a number of different investigations applying meta-analysis of the VTEC O157 genome have indicated that at least two different lineages of VTEC O157 have developed, and that one of these lineages is more commonly associated with human disease than the other (Yang *et al.*, 2004; Zhang *et al.*, 2007). Studies based on subtyping of *vtx* from VTEC O157 isolated from human patients and healthy cattle have furthermore indicated that there are differences among the relative frequency of the seropathotypes that are predominating among patients with severe disease (HC and HUS) and pathotypes that are predominating in the bovine reservoir (Roldgaard *et al.*, 2004). The same observation is made for VTEC O26. Strains of VTEC O26:H11 that cause HUS are usually identified as VT2- and *eae*-positive whereas infections caused by VT1- and *eae*-positive VTEC O26:H11 usually are characterised by causing relatively mild diarrhoeal symptoms in most patients (Ethelberg *et al.*, 2004). It is not possible at the present time to fully define human pathogenic VTEC. However, the concept of seropathotype has evolved which classifies VTEC into groups based on empirical knowledge about the typical clinical outcome of VTEC infections combined with knowledge about serotype, *vtx* subtypes and presence of additional virulence factors. This concept is likely to be further refined and will provide a valuable tool in the future for the assesment of the human pathogenic potential of different VTEC serotypes.

4. Methods for detection, isolation and identification of VTEC

The gold standard for the detection of VT employs Vero cells, but other cell lines can also be used, e.g. HeLa cells (O'Brien *et al.*, 1982). Toxin production can also be detected by the use of immunological methods and VTEC can indirectly be diagnosed by examining *E. coli* strains or samples for the genes encoding VT (*vtx*). Since a relatively small number of VTEC serotypes are responsible for the majority of human VTEC infections, serotype-specific detection methods have been developed, where strains are isolated on the basis of their O antigen and are subsequently analysed for VT production or presence of *vtx* genes. Generally, the diagnosis of VTEC is laborious, and currently there are no simple, inexpensive methods available for routine isolation of VTEC strains.

4.1. Methods to detect VTEC

4.1.1. Use of cell cultures

The Vero cell assay has been applied to the detection of free VT in faecal specimens to provide evidence of infection and to enrichment cultures inoculated with foods, animal faeces or environmental samples. However, the most usual application of the vero cell assay is for confirmation of toxin production by pure cultures which has already been described in section 3.2.

4.1.2. Immunologically based methods

Immunological methods are now widely used for the detection of VT. The methods utilize VT-specific poly- or monoclonal antibodies. There are various assay formats, several of which are commercially available. The assay formats include enzyme-linked immunosorbent assays (ELISA) and reversed passive latex agglutination (RPLA). Additionally assays have also been developed that utilize a combination of receptor-specified interactions and antibodies towards VTs (De Boer and Heuvelink, 2000; Baylis *et al.*, 2001; Scheutz *et al.*, 2001; Bettelheim and Beutin, 2003). Assays can be applied to pure and mixed cultures (enrichment broth inoculated with food or faeces, usually after overnight incubation). When VT is detected, the broth can be subcultured onto isolation media and pure or pooled colonies can be further examined. Immunoassays are generally reliable and most assays are easy to implement in laboratories and do not require expensive equipment.

4.1.3. DNA-based methods

An alternative approach to immunoassays for detection of VTEC is the demonstration of *vtx* and other virulence associated gene fragments as well as serotype specific gene fragments. For serotype specific targets, the majority of methods have targeted the rfb_{O157} gene of O157 (Paton and Paton, 1998). Detection of these targets is achieved by the use of DNA-DNA hybridization probes or by amplification of VT-specific DNA. Numerous DNA-DNA hybridization assays, using oligo- or polynucleotide probes have been described and different formats have been used including dot-blot and replica assays, liquid-based assays, and more recently micro-array chips. Amplification of specific DNA is most frequently achieved by PCR, but other DNA amplification techniques like Nucleic Acid Sequence-based Amplification (NASBA) are also applicable for VTEC detection. A

range of different PCR formats is being used, and additional techniques to ensure the identity of the amplicon beyond measurement of the size of the amplified DNA may be necessary. These techniques include the use of internal sequence-based probes (particularly in real-time PCR), full DNA sequencing, and the use of fragment analysis following restriction endonuclease digestion. The DNA-based detection methods can be applied to nucleic acid from pure or mixed cultures (enrichment broth inoculated with food or faeces), as well as colonies growing on solid isolation media. DNA probes are widely used to detect VTEC by the use of replica plating techniques, whereas PCR-based methods can be used to investigate single colonies or pools of colonies.

Generally, the amplification-based techniques are rapid and will give a result within hours. One of the advantages of DNA-based methods is that it is possible to simultaneously investigate cultures for several genes at the same time. However, when testing mixed cultures the detected genes might not originate from the same VTEC strain. By using DNA-based methods it is also possible to differentiate between the different *vtx* subtypes. Furthermore, several quantitative PCR methods can be used to assist in subculturing of selected enrichment broths with priority given to the highest target concentration since there is a correlation between the number of *vtx* copies and the success of isolation of VTEC from an enrichment broth. DNA based methods have the disadvantage og being unable to distinguish between DNA from viable and non-viable cells, although this may only be important in specific situations.

4.2. Methods to detect specific serotypes and immuno-magnetic separation

Studies conducted on both human infections and animal reservoirs have consistently shown that VTEC found in the gastrointestinal tract of ruminants belong to a large variety of serotypes, but only a limited number of those have been associated with the majority of human infections. These include VTEC O157, and to a lesser extent other serotypes like O26, O111, O103, O91 and O145 (see section 2.1).

This observation has prompted the development of detection methods based on the specific detection of VTEC strains belonging to serotype O157, and also to some of the most common serotypes considered to be pathogenic to humans. These methods can be based on:

- the use of selective/differential media (see section 4.2.1.1): these include Sorbitol MacConkey (SMAC) agar and/or media supplemented with 4-methylumbelliferyl-β-D-glucuronide (MUG), detecting non-sorbitol fermenting and β-glucuronidase-negative *E. coli* O157 strains, or the rhamnose MacConkey agar to detect VTEC O26.
- serotype-specific enrichment procedures, based on immunocapture techniques: these include the use of serotype-specific LPS-antibodies coated to paramagnetic beads (immuno-magnetic separation, IMS) or other supports. Originally developed for VTEC O157, these reagents are now available for other main VTEC serotypes, including O26, O103, O111, and O145. The presence of specific serotypes can be also detected by PCR amplification of O-antigen-specific DNA sequences (section 3.1).

IMS has been shown to be a sensitive method for isolation of VTEC O157 from artificially mixed bacterial cultures, inoculated meat samples, and inoculated as well as naturally contaminated bovine faeces (Fratamico *et al.*, 1992; Chapman *et al.*, 1994; Wright *et al.*, 1994; Bennett *et al.*, 1996;

Heuvelink *et al.*, 1997). When applied to artificially contaminated samples the methods usually enable the detection of less than 1 CFU per gram of sample.

4.2.1. Isolation and enrichment of VTEC O157

4.2.1.1. Isolation

VTEC usually possess phenotypic characteristics which are indistinguishable from those of the other *E. coli*. However, an important exception to this is that VTEC O157 are usually both unable to ferment sorbitol within 24 hours of incubation and lack β-glucuronidase activity (March and Ratnam, 1986; Ratnam *et al.*, 1988; Thompson *et al.*, 1990). These characteristics are utilised in the routine selective isolation of VTEC O157. The most widely used solid medium for the detection of non-sorbitol fermenting VTEC O157 is sorbitol MacConkey (SMAC) agar. Media that simultaneously indicate sorbitol fermentation and β-glucuronidase activity have also been developed, including different chromogenic media. A range of selective indicative media is commercially available. The selectivity of the different solid media can be improved by the use of selective supplements, the most frequently used being cefixime, a third generation cephalosporine, and potassium tellurite (e.g. CT-SMAC) (Zadik *et al.*, 1993). However, some VTEC O157 strains are sensitive to cefixime and potassium tellurite and therefore may not be detected on CT-SMAC agar (MacRae *et al.*, 1997).

Following incubation of the isolation media, individual colonies suspected to be VTEC O157 should be tested for the O157 antigen by using VTEC O157 antiserum or latex agglutination reagents. Isolates agglutinating with O157 antiserum should be confirmed as *E. coli* by biochemical reactions, since other species and VTEC non-O157 can cross-react with O157 antiserum. Since not all VTEC O157 strains produce VT, it is necessary to confirm VT production or the presence of *vtx* genes, as described in the previous section.

In recent years, human infections with sorbitol fermenting (SF) VTEC O157 have been increasingly recognised in many EU Member States. Although the methods used for VTEC O157 are not validated for the detection of SF VTEC O157, these organisms can be detected by testing sorbitol fermenting colonies grown on solid media that do not present typical VTEC O157 colonies for the O157 antigen.

4.2.1.2. Enrichment techniques

While human clinical stool specimens are examined by direct plating onto selective and differential agars, animal faeces, food and environmental samples usually contain low numbers of VTEC O157 together with an abundant microbial flora, and therefore require a selective enrichment step. Enrichment methods are also applied to human faeces which can contain low levels of VTEC. The most widely used media for the enrichment of VTEC O157 are tryptone soya broth (TSB) (mainly for food) and buffered peptone water (for human and animal faeces). These broths may be supplemented with different selective agents such as novobiocin, vancomycin, cefsulodin, cefixime, and bile salts (Doyle and Schoeni, 1987; Chapman *et al.*, 1994). There is currently no consensus on optimal incubation temperature (37°C versus 42°C) and time (6-8 hours incubation versus overnight incubation) for all types of samples.

The incubation period required will depend on the competing microflora. Standard methods for food include the analysis of both the 6- and 18-h incubation enrichment cultures. A 6-8 hour incubation of the enrichment broth increases the sensitivity when analysing matrices with a high number of background flora. However, when stressed or sublethally-injured VTEC O157 are present there are difficulties in reaching a detectable level after 6-8 hours of enrichment. Therefore, this short period of incubation can only be recommended when testing matrices where *E. coli* has a short-lag time before onset of growth, as for example with minced meat products.

Following incubation, enriched culture are subcultured directly onto selective indicative solid media. However, the sensitivity of culture-based detection is improved by the use of IMS, in which *E. coli* O157 cells are affinity-purified by the use of paramagnetic beads coated with O157-specific antibodies.

4.2.2. Isolation and enrichment of VTEC non-O157

4.2.2.1. Isolation

It is possible to examine material specifically for the presence of other VT-associated serotypes of *E. coli*. In its most simple form this comprises subculturing onto solid media where single colonies are then tested for the presence of different O antigens by slide-agglutination with O-specific sera or pools of sera. This procedure is suitable for the detection of many serotypes that are not too heavily encapsulated.

4.2.2.2. Enrichment and immuno-separation techniques

The growing concern over the association between VTEC non-O157 and human infections, together with the limited specificity of culture methods for these other serotypes, has led to the provision of beads coated with antibodies to the O antigen of other VTEC, including O26, O103, O111, and O145. IMS-based detection of serotypes other than O157 are similar to those for the detection of *E. coli* O157; enrichment, IMS followed by seeding onto selective indicative agars although these have not yet been sufficiently validated. Selective agents to improve the isolation of VTEC O157 (e.g. novobiocin) may inhibit the growth of some VTEC non-O157 (Vimont *et al.*, 2007). However, many VTEC non-O157 serotypes (O5:H-, O26:H-, O26:H11, O91:H21, O111:H-, O111:H8, O104:H11, O113:H21 and O157:H8) are capable of growing on media supplemented with vancomycin, cefixime, and cefsulodin.

MacConkey agar with lactose replaced by rhamnose and supplemented with cefixime and potassium tellurite has been described to be the optimum agar for recovery of VTEC O26, giving the most effective suppression of background microflora (Catarame *et al.*, 2003; Murinda *et al.*, 2004). Serotype-specific detection of VTEC O26 was achieved by selecting cefixime-tellurite-resistant, MUG-fluorescent, rhamnose-non-fermenting colonies.

A nonselective, but differential plating medium is enterohaemolysin agar (Oxoid) (washed sheep blood agar supplemented with calcium) which may be suitable for isolation of all VTEC strains including O157 (Beutin *et al.*, 1989). Nearly all (ca. 90%) VTEC O157 strains and a significant proportion of VTEC non-O157 strains (ca. 70%) produce enterohaemolysin. Enterohaemolytic *E. coli* are characterised on this medium by small turbid zones of haemolysis around the colonies occurring after 18 to 24 h incubation at 37°C. Alpha-haemolytic *E. coli* form large, clear zones of

hemolysis after only 3 to 6 h of incubation. To improve the selectivity of the medium, antibiotics such as novobiocin and cefsulodin may be used.

In summary there is currently insufficient data on the use of selective agents as well as differential phenotypic characteristics to design selective media suitable for culturing all of the VTEC non-O157.

4.3. Alternative methods to detect specific serotypes

There are a number of non-culture-based approaches that can be applied to detect specific VTEC serotypes. Apart from the use of IMS techniques, there is a range of other immunological assays targeting O-specific antigens. These assays are available commercially in a variety of formats e.g. ELISA, immunoblots and dipsticks. There is a very limited range of immunoassays which specifically target VTEC non-O157 serotypes. An inherent problem of such immunoassays is that false-positive results may be generated because of cross-reaction with surface antigens of other bacteria. This means that it is not possible to rely solely on these rapid immunological tests to identify VTEC serotypes without cultural isolation and further characterisation.

There are also PCR methods which target a limited number of other VTEC serotypes identified as clinically important to humans. These include O26, O111, O145 and O103 (Paton and Paton, 1999; DebRoy *et al*, 2004; O'Hanlon *et al*, 2004 and Perelle *et al*., 2007). PCR-based detection kits for *E. coli* O157 are commercially available.

4.4. Standard methods available for food

4.4.1. Standard methods for VTEC O157

The Nordic Committee on Food Analysis (NMKL) and the International Organization for Standardization (ISO) have issued horizontal methods applicable for culture-based detection of VTEC O157 in all types of foods and feeding stuffs (NMKL No. 164, 1999, and ISO 16654: 2001). Both methods prescribe the use of TSB supplemented with bile salts and novobiocin (mTSBn) for the pre-enrichment step, with an incubation period of 6-8 hours as well as 18-24 hours at 41.5°C. Further, both methods prescribe to perform an immunomagnetic affinity purification step and to subculture the immunomagnetic particles with adhering bacteria onto CT-SMAC and the user's choice of a second selective isolation agar. These two standard methods are widely being used. For the detection of *E. coli* O157:H7 in France, the ISO method (IMS) or the AFNOR[14] validated protocol is also used.

4.4.2. Standard methods for non-VTEC serotypes

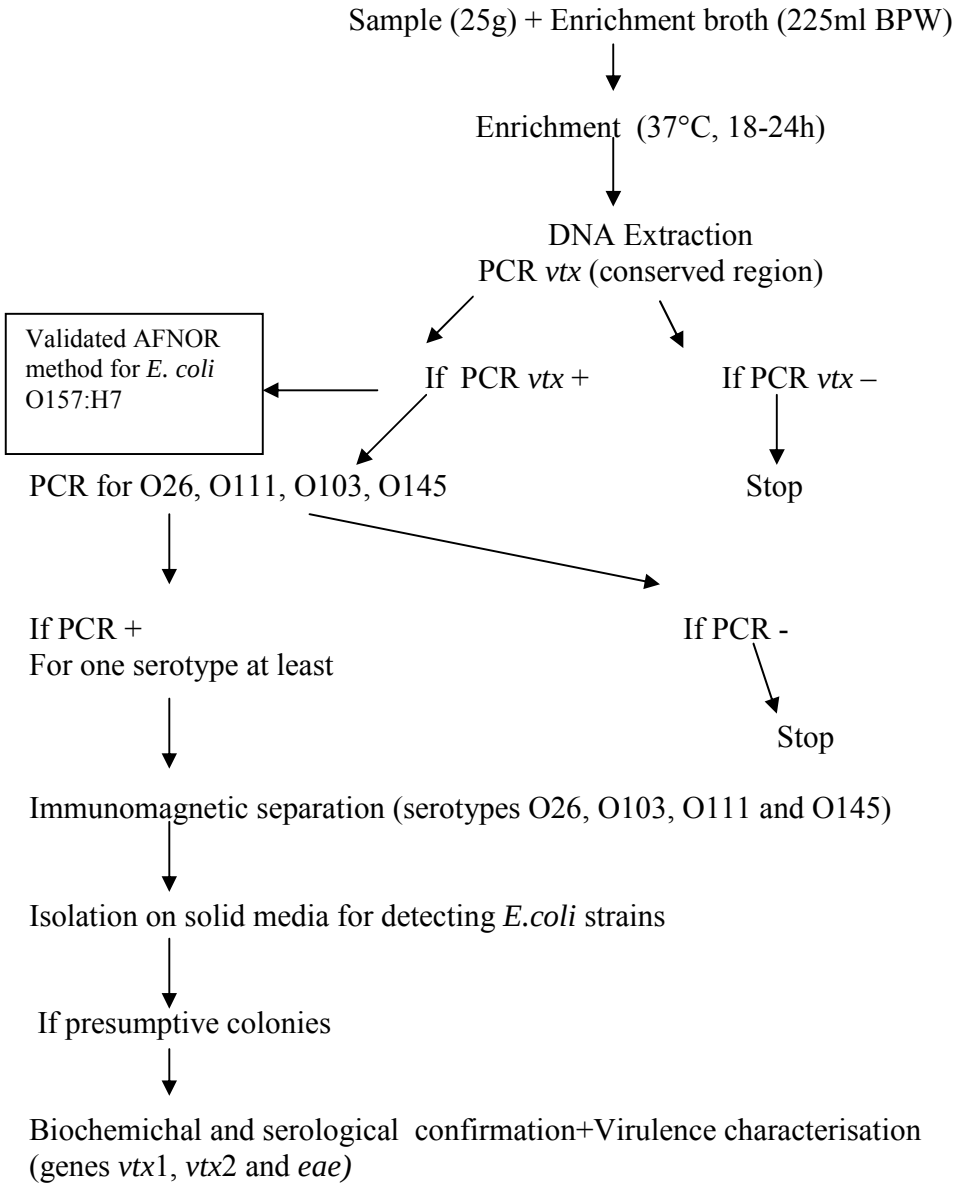
Currently, there is no international standard method for the detection and isolation of VTEC non-O157. However, under the authority of Working Group 6 of the Technical Committee 275 of the European Normalisation Committee (CEN TC275/WG6) is currently preparing a European Standard proposal based on a PCR-based horizontal method.

[14] Available at: http://www.afnor-validation.org/afnor-validation-methodes-validees/e-coli-o157.html

The German organisation for standardisation (DIN) has published two documents on the detection of VTEC in food derived from animals. DIN 10118:2004 specifies a method based on immunochemical detection of VTs in enrichment cultures and subsequent isolation of presumptive VTEC from VT-positive cultures by means of an immunoblot procedure, followed by confirmation of the positive colonies. The technical rule BVL L 07.18-1 (2002) describes a method for detection, isolation and characterisation of VTEC from minced meat by PCR and colony DNA hybridisation.

The French Food Safety Agency (AFSSA) recommends a PCR-based method (Figure 2) that, beside VTEC O157, is suitable for detecting VTEC belonging to the main pathogenic serotypes. The method used is based on the definition by AFSSA: *E. coli* strains has to (i) harbour *vtx* genes (*vtx*1 and or *vtx*2) and the *eae* gene and (ii) belong to one of the following serotypes: O157, O26, O111, O103, or O145. Screening is based on two PCR steps: the first one allows the detection of the *vtx* genes, and the second one the genes encoding the LPS of the five major VTEC serotypes associated with human diseases: O157, O26, O103, O111 and O145. The samples positive for *vtx* genes and one or more of these O antigen genes are further submitted to an IMS step specific for the respected O antigen(s) detected by PCR. Although it is not an official standard method, the method is described in order to provide an example from a Member State to detect a range of VTEC in foods.

Figure 2. **AFSSA method for detecting pathogenic VTEC in foods**



4.5. Methods for detection in animal faeces and environmental samples

There is no internationally recognised standard method for the isolation of VTEC from animals. However, for the detection of VTEC O157, the ISO 16654 and the NMKL No. 164 methods based on the IMS enrichment are recommended for food analysis and can be adapted to faeces and have been used in many studies published in the literature.

An IMS-based method for screening animal faeces for VTEC O157 is described in detail in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004, Chapter 2.10.13. It includes details on strain identification and confirmation, as well as the detection of the presence of *vtx* and *eae genes*.

The detection of VTEC in drinking, source, waste and recreational waters is preformed for specific purposes such as source tracing and epidemiological studies and is carried out by filtration followed by enrichment and immuno-magnetic separation in an analogous method to that for food described previously. However, transient contamination that caused disease may not be detected at the time of sampling. The presence of VTEC O157 in treated water should result in investigation and assessment of control measures to protect public health.

4.6. Quantification of VTEC

There is no standard protocol for enumeration of VTEC O157 or other VTEC serotypes from food or environmental samples. Enumeration of this group of pathogens is generally not conducted as part of routine monitoring or testing programmes, although quantitative data are essential to better understand the human health risks. Observations suggest that some animals excrete very large numbers of VTEC O157 at certain times and these are referred to as super-shedders. It is been suggested that these r-shedders are of prime importance of transmission and maintenance within herds and the wider environment (Low *et al.*, 2005).

For bovine/beef samples, there are limited published studies in the literature reporting successful enumeration of VTEC O157 (Arthur *et al.*, 2004; Cagney *et al.*, 2004; Robinson *et al.*, 2004; O'Brien *et al.*, 2005; Carney *et al.*, 2006; LeJeune *et al.*, 2006). The overall approaches used include subculturing dilutions of the sample directly onto SMAC or CT-SMAC. The detection limit of this technique is generally low and stressed or sublethally injured bacteria may not be recovered. An alternative approach is to use an MPN (most probable number) method (Fegan *et al.*, 2004).

Some studies have attempted to estimate numbers of VTEC non-O157 (O111, O26, O145, O103) from bovine and ovine samples. However, the absence of an agar that clearly differentiates colonies of different serotypes morphologically, means that this presents considerable technical difficulties since many colonies must be identified using serological or molecular methods. This indicates that enumeration of VTEC as part of a routine monitoring programme would be very difficult.

5 Monitoring schemes

5.1. Monitoring

Monitoring is defined in the Zoonosis Monitoring Manual as the "system of collecting, analyzing and disseminating data on the occurrence of zoonoses, zoonotic agents and antimicrobial resistance related thereto. As opposed to surveillance, no active control measures are taken when positive cases are detected (Dir 2003/99)". Microbiological testing in monitoring can therefore be used for (i) identifying trends in human illness caused by foodborne pathogens e.g., sentinel studies, (ii) establishing baseline prevalences in primary production and in later stages of the food chain, i.e., testing foods in distribution or at retail, (iii) estimating the load of bacterial pathogens in foods reaching the consumer (e.g., when assessing exposures of a pathogen), (iv) measuring compliance with good hygienic practices, and (v) measuring the effect of intervention measures such as control programs.

The current monitoring activities performed as part of the Zoonoses Directive (2003/99/EC) although providing valuable data for individual Member States, in places lack harmonisation. Wherever possible, efforts should be made to apply methods which produce genuinely comparable data from different Member States. Results from the application of such methods should identify differences and show trends in the epidemiology of the various diseases and distribution of their respective agents which are independent of the characteristics of the monitoring used in different Member States.

Criteria to set priorities in monitoring programs should be defined by applying the principles indicated by the Zoonoses Directive. According to the article 4 of this Directive, the following criteria should be taken into account: (i) the occurrence of the agent in animal and human populations, feed and food, (ii) the gravity of the effects for humans, (iii) the economic consequences for animal and human health care and for feed and food businesses and (iv) epidemiological trends in animal and human populations, feed and food.

There are a number of targets for improvement for monitoring of VTEC which include not only the infections in humans but also the occurrence of human pathogenic VTEC in:

- animal populations,
- animal carcasses and meats,
- environmental sites subject to faecal contamination from animals including water,
- ready to eat foods subject to faecal contamination from animals

The purpose for monitoring in animals and in foodstuffs is to establish comparable data on the occurrence of human pathogenic VTEC in animal populations and food such that epidemiological relationships can be established to link reservoirs of human infection (particularly for VTEC non-O157) to their sources as well as measuring the effects of any interventions designed to prevent this group of bacteria from entering the food chain. If genuinely comparable data from different Member States are to be obtained, microbiological methods and sampling plans must be standardised.

5.2. Human infections

There is currently insufficient information collected on human VTEC infections to meaningfully compare disease rates between different Member States or to identify trends in the incidences of the disease. It is also not currently possible to use the data collected to estimate health burden statistics, including the extent of serious sequelae.

5.3. VTEC serotypes

The application of VTEC monitoring to animals and foods as part of the Zoonoses Directive should be responsive to surveillance data on the prevalences of the VTEC serotypes causing human disease. Initially monitoring should concentrate on VTEC O157 since this serotype is most frequently associated with severe human infections (including HC and HUS) in the EU. Moreover, standard, and sensitive methods are widely available for this serotype, and many laboratories throughout Europe are accustomed to the use of these methods.

Monitoring should then be extended to other serotypes that are identified as pathogenic for humans, based on the periodical analysis of human disease and epidemiological data. Based on current surveillance activities and given methodological advances, monitoring should be extended to VTEC O26, O103, O91, O145 and O111 which would cover the majority of serotypes associated with human infection. Information on the reservoirs and sources of these serotypes are currently scarce. It should be taken into account that there are geographical differences in the prevalence of the different VTEC serotypes in human infections between Member States. For some countries it would be advantageous to establish specific monitoring programs for certain VTEC serotypes as soon as possible, for example O103 in Norway, O121 in Sweden and O91 in Germany.

Isolates of VTEC from animals, food and the environment, as well as those from human infections, should be characterised by serotyping (including molecular serotyping), phage typing for VTEC O157, *vtx* gene subtyping and detection of *eae* genes using harmonised methods. Strain characterisation could be completed by molecular typing (PFGE, MLVA) and comparison of isolates from human and non-human sources may be useful in providing data on the spread of specific strains in the future. Data derived from humans, foodstuffs and animals should be centrally collected.

5.4. Animal population

5.4.1. Animal species

Serotypes of VTEC, many of which have been associated with human disease, have been isolated at some time from a broad range of domesticated, wild and pet animals. However this probably reflects the widespread occurrence of VTEC in the environment and that many species act as passive vectors which contribute to the spread of these organisms through the environment, these species are probably not the primary reservoir for this group of bacteria.

As previously outlined in section 1.1, ruminants (particularly cattle) are recognised as the main natural reservoir of VTEC, particularly VTEC O157 and therefore monitoring of this species is of primary importance (Hussein and Bollinger, 2005). After cattle, goats and sheep probably

represent the second most important reservoirs for monitoring (Rey *et al.*, 2003; Ogden *et al.*, 2004; Lenehan *et al.*, 2007). Since data on the occurrence VTEC non-O157 is scarce, cattle, sheep and goats should be initially investigated as possible reservoirs for these serotypes.

Additional monitoring of other species, such as water buffalo, game animals and wild ruminants, should take place according to the national differences in animal populations, farming practices and food consumption patterns.

Pigs and poultry have not been identified to be major sources of VTEC in Europe and where these have yielded this group of bacteria, these have not been associated with the seropathotypes associated with human disease (Heuvelink *et al.*, 1999; Johnsen *et al.*, 2001; Bonardi *et al.*, 2003; von Müffling *et al.*, 2007; von Müffling T. *et al.*, 2007).

5.4.2. Stages of the food chain

Data on the presence of VTEC O157 in the different types of cattle operations are abundant in the literature (Sofos *et al.*, 1999; Hancock *et al.*, 2001; Caprioli *et al.*, 2005) and indicate that faecal shedding of VTEC O157 appears to be transient, has quantitative differences between animals and is likely to be influenced by several factors, including the age of the animals and their diet. The prevalence also depends on the season, as increased rates of faecal carriage have been repeatedly reported in warmer months. There are insufficient data to determine whether the same phenomena occur in VTEC non-O157. Based on the above considerations, monitoring to obtain comparable data on the prevalence of all VTEC in different countries is likely to be difficult. There are less data available on factors affecting the prevalence of VTEC in small ruminants but it could be assumed that similar difficulties will apply.

5.4.2.1. At farm level

Monitoring at farm level is likely to provide information on the spread of VTEC in the environment; however these studies are labour intensive and expensive and should be focused on specific high risk situations such as open farms that are visited by the general public including school visits. Monitoring at farm level in other situations is not recommended but may provide useful data for targeted research.

5.4.2.2. At the abattoir

Good hygiene practices at the abattoir including monitoring for microbiological indicators (*Enterobacteriaceae* and in generic *E. coli*) is likely to be the most effective method for reducing the public health risks for VTEC infection. However, compliance with the hygiene criteria does not guarantee the absence of VTEC at concentrations sufficient to cause human disease.

Monitoring at the abattoir represents a practical point in the meat chain, which is likely to enable comparison of results both within and between countries. At this stage of the food chain, standardised sampling is readily achievable. Objective sampling plans which select statistically representative selection population (EFSA, 2006b) should be applied.

Because of the considerations given in section 5.4.1 sampling plans should take into account season and different animal production systems. The types of samples that could be considered for monitoring are faeces of the animals after the slaughter, hide and fleece and pre-chill carcasses. In particular, data on the presence of VTEC on hides or fleece provide a starting point for quantitative microbial risk assessment models (Duffy, 2006).

Data generated should preferably be both qualitative and quantitative in nature. However, it should be noted that the background flora of these sample types presents a challenge for the enumeration, particularly for routine testing.

5.4.2.2.1. Faeces from cattle and small ruminants

Sampling of the faeces of the animals at the abattoir will reflect what is present in the animal population being presented for slaughter. Optimal sampling will be achieved by collecting rectal samples. Monitoring to provide quantitative data at this point will generate valuable information on the occurrence of super-shedder animals.

5.4.2.2.2. Cattle hide and small ruminants fleece

Ruminant coats (cattle hide and small ruminants fleece) represent a key source of VTEC contamination into the slaughter plants (Elder *et al.*, 2000; O'Brien *et al.*, 2005; Arthur *et al.*, 2007; Lenehan *et al.*, 2007; Mather *et al.*, 2007).

Sampling of a defined area of the hide or fleece (e.g. brisket or ears) will reflect the level of VTEC contamination of animals being presented for slaughter and will reflect cross-contamination during transport and lairage. The brisket area of the hide is cut during dehiding and is thus a likely source of contamination onto the carcass. Ongoing studies in Sweeden indicate that ears may represent a convenient sampling material which is likely to reflect the general level of contamination of the animal (EFSA, 2006a).

5.4.2.2.3. Carcasses

Sampling of cattle and small ruminant carcasses at the abattoir will provide an indication of VTEC contamination on muscle derived from the animal. Sampling at this stage of the chain has major advantages since this is the first sample point in the chain where the animal is converted to meat and reflects the level of contamination which has come from the live animal. There is already a defined EU microbiological sampling procedure for fresh meat carcasses (Commission Decision 2001/471[15] and Regulation (EC) No 2073/2005[16]) which can be applied equally well to VTEC. This EU protocol outlines the carcass sites to be sampled and the method permitted to take the samples i.e. either by excision or swab based procedure.

[15] Commission Decision of 8 June 2001 laying down rules for the regular checks on the general hygiene carried out by the operators in establishments according to Directive 64/433/EEC on health conditions for the production and marketing of fresh meat and Directive 71/118/EEC on health problems affecting the production and placing on the market of fresh poultry meat. OJ L 165, 21.6.2001, p. 48–53.

[16] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ L 278, 10.10.2006, p. 32–32.

5.4.2.3. At the processing of meat including deboning and trimming

Sampling raw meat cuts or trim with harmonised methodologies would provide a representative picture of the prevalence and concentration of VTEC seropathotypes on meat as it enters the processing or distribution part of the food chain. The advantage of taking samples here is that this is the end of the fresh meat production chain and will be representative of the effect of washing, chilling etc. It will also reflects cross-contamination which occurs during deboning and trimming meat from the primal cuts. There is no standardised approach to take samples at this part of the chain. It is suggested that samples of a standard size are taken after deboning from either the meat trays or bins and include the superficial tissue surfaces of muscle directly under the hide or fleece.

5.5. Foodstuffs subjected to faecal contamination from animals

Foodstuffs subject to faecal contamination from ruminants represent a potential hazard for VTEC infection and comparative data are currently not available for meaningful comparisons between different Member States.

Good hygiene practices at processing plants including monitoring for microbiological indicators (*Enterobacteriaceae* and in generic *E. coli*) is likely to be the most effective method for reducing the public health risks for VTEC infection. However, compliance with the hygiene criteria does not guarantee the absence of VTEC at concentrations sufficient to cause human disease. Therefore, monitoring should take into account compliance with the criteria of the Regulation (EC) No 2073/2005, the presence of VTEC in high risk foodstuffs and other risk-based supporting data. Application of efficient validated HACCP-procedures for production of raw ready-to-eat meat, meat preparations and other foods is important to reduce the public health risks for VTEC infection.

5.5.1. Raw minced meat or meat products

Sampling raw minced meat or meat preparations, gives a representative picture of the prevalence and concentration of VTEC at the point it is leaving the production process and entering the distribution part of the chain to the consumer. Monitoring data should be collected on minced meat products that are likely to be consumed without cooking such as raw beef used for tartare and those that are likey to be subjected to under or minimal cooking.

5.5.2. Ready-to-eat fermented meats

VTEC O157 and some other VTEC serotypes have a tolerance to acid which can allow VTEC to survive in acidic foods. Such foods have been associated with food poisoning outbreaks and include ready-to-eat fermented meats such as salami and pepperoni type products. Thus, unless intervention steps are included during processing to specifically reduce any potential VTEC on the raw meat, these products pose a higher risk to consumers.

5.5.3. Fresh produce

Fresh vegetables and salads and fruits can be contaminated with VTEC from direct contact with faecally contaminated soil, agricultural run off or irrigation water. All these commodities have been implicated as transmission routes for VTEC to humans, most notably in a recent large spinach related outbreak in the USA (Anonymous, 2006). However, prevalence studies have only rarely detected the presence of the pathogen on fresh produce (Johannessen *et al.*, 2002; Robertson *et al.*, 2002; Lukasova *et al.*, 2004; Fahey *et al.*, 2006).

5.5.4. Unpasteurised milk and derived dairy products

Outbreaks of VTEC infection in humans have been frequently linked to consumption of unpasteurised (raw) milk. The potential for VTEC to survive in dairy products made from raw milk, particularly in soft and semi-soft cheeses, also make these of greater potential risk. Therefore any monitoring of milk and dairy products should focus on raw milk and dairy products made using raw milk.

5.6. Water and environmental sources

Data on the presence of VTEC in water and other environmental samples may be extremely useful for epidemiological investigations. However, as outlined above for farm environments, the detection of the presence of VTEC will provide in most cases only a single point prevalence. Therefore, water and other environmental samples do not appear to be a suitable stage for VTEC monitoring.

CONCLUSIONS

Strains and/or serotypes of VTEC which are pathogenic to humans

1. VTEC infections continue to constitute a major public health concern, because of the severe illnesses that they can cause.
2. There are four main transmission routes whereby these bacteria may be transmitted to humans, which are food-borne, water-borne, direct or indirect contact with animals, and person-to-person spread. More than one route may be involved in a single outbreak.
3. Over 200 O:H serotypes producing VTEC have been identified from all sources, although many lack the full complement of known virulence factors found in strains that cause serious disease; however over 100 have been associated with disease in humans.
4. Over the period 2002-2006, of all reported human infections where the serotype was established, 66% were due to O157, 20% due to O26, O103, O91, O145 and O111, and the remaining 14% to other serotypes. During the same period, amongst the cases of HUS, 68% were due to O157, 26% to O26, O103, O91, O145 and O111, and 6% to other serotypes.
5. There is a wide variety of VTEC in the food-producing animal populations, of which the public health importance remains unclear. A restricted range of serotypes (i.e. O157, followed by O26, O103, O91, O145 and O111) are associated with public health risks, however isolates of these serotypes are not necessary pathogenic when recovered from food or live animals. Consequently, serotyping alone when applied to VTEC isolates from food and animals is not the optimal method of identifying public health risk.
6. The main virulence factors (genes) identified for human pathogenic VTEC are: *vtx1*, *vtx2*, *vtx2c* and *eae*. There is no consensus for the optimal strategy to characterise these virulence factors (genes).
7. It is not possible at the present time to fully define human pathogenic VTEC. However, the concept of seropathotype has evolved which classifies VTEC into groups based on empirical knowledge about the typical clinical outcome of VTEC infections combined with knowledge of serotype, *vtx* subtypes and presence of additional virulence factors. This concept is likely to be further refined and will provide a valuable tool in the future for the assesment of the human pathogenic potential of different VTEC serotypes.

Methods for detection, isolation and identification of VTEC

8. The detection of VTEC in the different matrices (foodstuffs, animal faeces, environmental samples) have traditionally been based on two different approaches:
    a. the detection of VT- producing strains present in the sample using assays aimed at detecting VT, and/or *vtx* genes. In subsequent steps, the VTEC strains are isolated in pure culture and characterised by serotyping and detection of accessory virulence genes. This approach has the advantage of detecting all VTEC but is of low

specificity for the detection of the predominating human pathogenic VTEC when dealing with ruminant faeces or foodstuffs. This approach is appropriate for laboratory diagnosis of human infections, since any strain detected in clinical cases may have an etiological significance and the identification of new emerging pathogenic serotypes is of paramount importance. The approach is also valuable to establish basic knowledge about the prevalence of different VTEC serotypes in different reservoirs.

b. The detection of a defined range of VTEC serotypes, which have been selected using human surveillance data. The isolated strains belonging to the target serotypes will have to be confirmed as VTEC by using the above mentioned approach (8a). This approach has the advantage of increased specificity but will only detect VTEC serotypes covered by the reagents used. This approach is more appropriate for examining food and animal reservoirs, where high sensitivity and serotype-specificity are required.

9. Recent advances in molecular detection methods combine the above detection approaches and target both serotype specific genes, *vtx*, and other virulence genes. However, isolation of VTEC, and subsequent strain characterisation is still needed to ensure that the detected genes are present on the same bacteria.

10. There are standardised (ISO and NMKL) and sensitive methods to detect and isolate VTEC O157 from food, and animals. For the other serotypes, there are no universally accepted and validated methods, but pragmatic approaches have been produced.

11. There is no standard protocol for enumeration of VTEC O157 or other VTEC serotypes in food or environmental samples. Enumeration of VTEC is generally not conducted as part of routine monitoring or testing programmes, although quantitative data are essential to better understand the human health risks.

Monitoring in humans, animal populations and foodstuffs

12. European surveillance of human VTEC infection is based on nationally collected data which are further compiled by ECDC using the Enter-net. There has been considerable progress in the use of coordinated surveillance networks (with standardised case definitions), but there are considerable national differences in the methods used for the diagnosis and surveillance.

13. It is not currently possible to use the data collected to estimate health burden statistics, including the extent of serious sequelae.

14. Ruminants (particularly cattle) are recognised as the main natural reservoir of VTEC, in particular VTEC O157. Comprehensive information on the occurrence of VTEC in animals other than cattle is scarce. Pigs and poultry have not been identified to be major sources of VTEC for human infection in Europe.

15. Foodstuffs subject to faecal contamination from ruminants represent a hazard for human VTEC infection and data allowing meaningful comparisons between different Member States are currently not available.
16. The current monitoring activities performed as part of the Zoonoses Directive (2003/99/EC) although providing valuable data for individual Member States, in places lack harmonisation. Wherever possible, efforts should be made to apply methods which produce genuinely comparable data from different Member States.
17. There are a number of targets for monitoring of VTEC which include not only infections in humans, but also the occurrence of VTEC seropathotypes in environmental sites subject to faecal contamination from ruminants including water, ruminant populations, ruminants' carcasses and meats, and ready-to-eat foods subject to faecal contamination from ruminants.
18. Monitoring of live ruminants, the farm environment, in water and other environmental sites may be extremely useful for targeted epidemiological investigations and for research, but will only provide single point prevalences and are unlikely to produce genuinely comparable data from different Member States.
19. Monitoring at the abattoir represents a practical point in the meat chain, which is likely to enable comparison of results both within and between countries.
20. Sampling of raw meat cuts or trim with harmonised methodologies would provide a representative picture of the prevalence and concentration of VTEC seropathotypes on meat as it enters the processing or distribution part of the food chain.
21. Good hygiene practices at the abattoirs and at processing plants including monitoring for microbiological indicators (*Enterobacteriaceae* and in generic *E. coli*) is likely to be the most effective method for reducing the public health risks for VTEC infection. However, compliance with the hygiene criteria does not necessarily guarantee the absence of VTEC at concentrations sufficient to cause human disease.
22. Application of efficient validated HACCP-procedures for production of raw ready-to-eat meat, meat preparations and other foods is important to reduce the public health risks for VTEC infection.

RECOMMENDATIONS

Strains and/or serotypes of VTEC pathogenic to humans

1. It is recommended that all MS use harmonised methods to define VTEC seropathotypes from human and non-human sources to allow more effective monitoring by comparison of isolates from food and animals with those from humans. This should be supported through a consensus discussion involving the Community Reference Laboratory (CRL) for VTEC and other relevant reference laboratories.

2. Further strain characterisation comparing isolates from human and non-human sources should be centrally collected using data analysis methods similar to those used by e.g. PulseNet Europe.

Methods for detection and isolation

3. Standard or validated alternative methods are available and are recommended to be used for the detection and isolation of VTEC O157 from food and animals.
4. Improved methods for the detection and isolation of VTEC non-O157 from foods, animals and the environment should be developed and validated.
5. Quantitative methods should be developed for the enumeration of both VTEC O157 and VTEC non-O157 in samples from animals, food and the environment.
6. For detection and isolation of VTEC, it is recommended that the CRL continues to coordinate standardisation and harmonisation of procedures between laboratories in all Member States through the network of National Reference Laboratories and other laboratories.

Monitoring of animal populations and foodstuffs

7. Initially monitoring should concentrate on VTEC O157 since this serotype is most frequently associated with severe human infections (including HUS) in the EU. Monitoring should then be extended to other seropathotypes (e.g. those of O26, O103, O91, O145 and O111) that are identified as pathogenic for humans, based on the periodical analysis of human disease and epidemiological data.
8. Monitoring data on the prevalence and concentration of VTEC in ruminants' faeces, coat, and carcasses after chilling at the abattoir would assist in the assessment of risk to consumers. Co-ordinated sampling of raw meat cuts or trim for the prevelance and concentration of VTEC would provide suitable comparisons between Member States.
9. Targeted surveys conducted on a co-ordinated basis through Member States, of foodstuffs that have been associated with illness should include ruminant meat and minced meat products (in particular those that are likely to be consumed without cooking), ready-to-eat fermented meats, fresh vegetable and salads, in addition to unpasteurised milk and dairy products derived therefrom.

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APPENDICES

Appendix I: Criteria for reporting of case of VTEC infections in humans.

Clinical Criteria

STEC/VTEC diarrhoea

Any person with at least one of the following:

- Diarrhoea
- Abdominal pain

HUS

Any person with sudden onset of symptoms followed by at least two of the following three*:*

- Haemolytic anaemia
- Thrombocytopenia
- Renal impairment

Laboratory Criteria

At least one of the following two*:*

- Isolation of Verotoxin producing *E. coli* (VTEC) from stool
- Detection of *vtx*1 or *vtx*2 gene(s) from stool

Only for HUS:

- *E. coli* serotype-specific antibody response

Isolation and additional characterisation by serotype, phage type, *eae* genes, and subtypes of *vtx*1/*vtx*2 should be performed, if possible

Epidemiological Criteria

At least one of the following five epidemiological links:

- Human to human transmission
- Exposure to a common source
- Animal to human transmission
- Exposure to contaminated food/drinking water
- Environmental exposure

Additional information

Incubation period 2-10 days, most often 3-4 days

Case Classification

A. Possible case of HUS

Any person meeting the clinical criteria for HUS

B. Probable case

Any person meeting the clinical criteria and with an epidemiological link

C. **Confirmed case:** Any person meeting the clinical and the laboratory criteria

Appendix II: Table II.1: National strategies for surveillance of human VTEC infections in different European countries

Country	Human data predominantly obtained from	Comments
Austria	Mandatory national notification of cases of bacterial foodborne disease by clinicians and reports of laboratory-confirmed cases by the microbiological laboratories.	
Belgium	Sentinel network of laboratories; 60% of the laboratories participate, but coverage is higher because these include all major laboratories.	A small minority of cases of diarrhoea are tested for VTEC. This is due to the financial system for microbiology of stools which does not cover the VTEC screening costs.
Denmark	Reports of laboratory-confirmed cases by the clinical microbiological laboratories through the laboratory surveillance system and outbreak reporting. Mandatory national notification of all cases of VTEC infection and HUS. All to the Statens Serum Institut.	
England and Wales	Referral of presumptive VTEC O157 isolates from primary labs to National Reference Laboratory for confirmation and typing and subtyping where required. Few (<6) other VTEC confirmed annually.	Data collated annually on confirmed isolates. Serotype, phage type and vtx gene type for all isolates. Possible to identify general outbreak and foreign travel cases. Clinical data supplied and also available locally.
Estonia	National notification of laboratory-confirmed *E. coli* infections	
Finland	National notification of VTEC	
France	HUS surveillance system based on 31 paediatric nephrology departments for children <15 years of age	
Germany	National notification system for VTEC, and (since 2003 separately) for HUS; in Enter-net only VTEC cases typed at the National Reference lab are included. Data from this source are not primarily used for the Zoonosis Report (only to study details not available in the national notification, such as antibiotic resistance).	Serotype of notified cases known for about 40% of VTEC cases and about 80% of HUS cases.
Hungary	National notification of infectious enteritis, laboratory-confirmed as VTEC. Some lab-confirmed cases reported without the clinical status. HUS cases due to VTEC registered in "Illness caused by pathogenic *E. coli*" (so not all VTEC cases).	
Ireland	Statutory notification of VTEC infections by both clinicians and laboratory directors. Outbreaks (family and general) are also notifiable. National enhanced surveillance for all VTEC cases notified. HUS of possible infective aetiology (suspected VTEC infections) are reported on a voluntary basis.	

Country	Human data predominantly obtained from	Comments
Italy	HUS surveillance system on paediatric patients.	
Luxembourg	In theory, mandatory reporting of VTEC cases by physicians.	In practice, almost all reporting of laboratory-confirmed VTEC cases is done by the Microbiology unit of the National Health Laboratory.
Malta	Mandatory notification by medical practitioners of all suspected cases, and of all laboratory-confirmed cases by the medical diagnostic laboratories.	VTEC infection is notifiable.
The Netherlands	Enhanced Laboratory-surveillance for VTEC O157, national coverage (rarely detected cases of non-O157 will be included as well)	
Norway	Mandatory national notification of VTEC infections by microbiological laboratories and clinical doctors.	
Portugal	Number of isolates sent voluntarily by microbiology laboratories (mainly hospital laboratories) to the National Centre of Bacteriology National Institute of Health in Lisboa.	
Scotland	(1) Enhanced national laboratory-based surveillance of statutory reports of *E. coli* O157 and voluntary reports of non-O157 serotypes; (2) voluntary clinical reporting of HUS; (3) voluntary public health reports of general outbreaks of infectious intestinal disease. (All systems report to Health Protection Scotland).	VTECs are distinguished in Reference Laboratory reports, but serotype O157 is reportable by statute irrespective of VT status. Few local laboratories can identify non-O157 serotypes. Reference Lab also reports seropositives.
Slovakia	Mandatory national notification of VTEC infections by microbiological laboratories and clinical doctors.	
Spain	Number of isolates sent voluntarily by microbiology laboratories (mainly hospital laboratories) to the National Centre of Microbiology.	
Sweden	Mandatory national notification of EHEC infections by microbiological laboratories and clinical doctors.	
Switzerland	Mandatory national notifications of VTEC by laboratories and physicians.	

Source: Anonymous, 2007, Enter-net annual report: 2005 – surveillance of enteric pathogens in Europe and beyond. Enter-net surveillance hub, HPA, Centre for Infections, Colindale, London.

Table II.2: Laboratory practices with regard to VTEC in different European countries providing data to Enter-net in 2005.

Country	Main testing strategy	Main diagnostic tool(s) used in routine diagnosing laboratories	Comments
Austria	Screening for VTEC (few); screening for non-sorbitol fermenters and O157 (some).	Few laboratories routinely examine stools for VTEC (e.g. diarrhoeal and bloody stools from children <7, like the reference centre), some test only for non-sorbitol fermenters (not considering SF O157) followed by testing for O157, missing out all non-O157.	
Belgium	Few laboratories routinely examine stools for VTEC.	Peripheral laboratories use mainly SMAC-based methods for O157; many analyses are done in the reference laboratory, with a PCR-based screening of colonies grown on SMAC, followed by testing individual colonies if positive.	
Denmark	Half of the Danish laboratories test selected diarrhoeal stools (primarily bloody stools and from children less than 4-7 years (is meant "from children 4-7 years old" or is meant "from children less than 4 years old" or less than 7 yrs and HUS cases for VTEC.	Laboratories testing samples from about 50% of the Danish population use molecular detection methods (PCR or dot blot hybridisation) targeting the *vtx* genes, followed by slide agglutination and typing. Most of the remaining laboratories use slide agglutination of suspect colonies, with OK-antisera against the most common VTEC serotypes. At a few laboratories VT-specific ELISA detection is used.	
England and Wales	Culture of all diarrhoeal stools for presumptive VTEC O157 then referral of isolates to National Reference Laboratory. The NRL offers primary diagnosis of appropriate culture-negative stools by enrichment/IMS and PCR.	HPA National Standard Method for VTEC O157 by culture on CT-SMAC; agglutination reagents. Probably followed in non-HPA labs but not obligatory. No methods for other serotypes in routine use.	National Reference Laboratory performs serological testing (saliva and serum) for evidence of infection with VTEC O157 and a limited range of other VTEC groups
Estonia	No information received.	No information received.	
Finland	Testing strategy unknown.	Almost all of about 25 clinical microbiology routine laboratories use SMAC plates to screen non-SF VTEC O157 strains. A few laboratories test also the VT toxin by commercially available methods and one routine laboratory uses also an in-house test for the *vtx*1 and *vtx*2 genes.	

Country	Main testing strategy	Main diagnostic tool(s) used in routine diagnosing laboratories	Comments
France	Most medical laboratories do not routinely examine stools for VTEC.	Those clinical laboratories that look for STEC O157 most often use SMAC, SMID, or Chromagar O157 media (report available at http://www.invs.sante.fr/publications/2006/enquete_e_coli_2003/rapport_e_coli.pdf)	Serological testing also performed in National Reference Laboratory.
Germany	Most peripheral labs will not routinely test diarrhoeal stools for VTEC, only on request.	The routine labs mostly use EIA (ELISA) commercial kits of different companies. But it is also possible (and allowed) to use PCR and similar systems like PCR-ELISA and hybridization with DNA or oligonucleotide probes. It is also demanded to perform further investigation in case of VT/*vtx*-positive results. At first the serotyping of the 5 most common types (O157, O26, O103, O145, O91) is performed. These results are reported to the surveillance system.	
Hungary	No information received	Mainly culture is performed on EMB, SMAC, CT SMAC media in the countyLaboratories.	
Ireland	Testing diarrhoeal samples for VTEC O157	Most labs use CT-SMAC and latex agglutination to screen for VTEC O157, some labs screen for VTEC non-O157 using slide agglutination on suspect colonies. All positive isolates are sent to the PHL-HSE-DML for toxin and virulence gene testing by PCR. Some labs send bloody or HUS stools directly to PHL-HSE-DML for testing by IMS, culture and PCR.	
Italy	Most laboratories not routinely examine stools for signs of VTEC infection	Some clinical laboratories look for VTEC O157 using SMAC and slide agglutination reagents.	
Luxembourg	Unknown	Unknown	The lab is planning a survey to collect this information.
Malta	All stool samples are tested for *E. coli* O157.	SMAC agar used to culture *E. coli*. Subculture performed on nutrient agar medium. Serological tests carried out for *E. coli* O157. VT is tested with commercial available kits.	
The Netherlands	Mainly testing bloody stools, HUS and HC cases (80% of labs), children (10% of labs), routine screening of all stools minority of labs (8% in 2000)	Culture on (CT-)SMAC: in 2000 used by 88% of the laboratories. From 2007/2008 onwards, real time PCR and commercial assays targeting vtx/VT are/will be introduced in some labs.	Serological testing for O157 LPS performed in one university hospital laboratory.

Country	Main testing strategy	Main diagnostic tool(s) used in routine diagnosing laboratories	Comments
Norway	All laboratories: All bloody stools (actual or anamnestic) and all cases of HUS A few laboratories: All children < 2 years with diarrhoea.	All laboratories: culture on SMAC, serological tests for O157. In addition: 4 out of 5 regional laboratorties are using PCR for *vtx* genes, 1 out of 5 regional labs is using a commercial ELISA for VT.	All suspected VTEC strains from all labs are forwarded to NIPH/ NRL for verification and further characterization.
Portugal	Most medical laboratories do not routinely examine stools for STEC	Some clinical laboratories look for VTEC O157 using SMAC and slide agglutination reagents.	
Scotland	All routine laboratories screen all diarrhoeal stools for *E. coli* O157. Only one lab screens routinely for *E. coli* non-O157 and a couple of labs screen occasionally.	The majority culture directly onto CT-SMAC, with the remaining labs culturing directly onto SMAC. A small number of laboratories use slide agglutination using polyvalent antisera to identify the presence of *E. coli* non-O157. All laboratories should forward culture negative faeces from patients in high risk groups (HUS/TTP, bloody diarrhoea, contact of case with diarrhoea or asymptomatic contact <10y or >60y) to the Scottish *E. coli* Reference Lab for more sensitive, molecular testing.	Serological testing also performed in Scottish *E. coli* Reference Laboratory
Slovakia	Few laboratories routinely examine stools for VTEC	Those clinical laboratories that do test, test for VTEC on the selective agars and use PCR, immunofluorescence and immunochromatography.	
Spain ** Data only available for a small number of diagnosing laboratories.	Routine screening for VTEC of all stools only performed in minority of labs. The majority tests only bloody stools, and stools from HUS and HC cases.	SMAC agar used to culture *E. coli.* Serological tests carried out for *E. coli* O157.	Serotyping (O157,O26 O111,O103 O145), phagetyping (O157), antimicrobi-al resistance profile and PCR targeting the virulence genes (*vtx*, *eae*, *hly*), performed in National Reference Laboratory.
Sweden	Varies between different labs. Most of them test bloody stools, HUS and if there is an epidemiological-link. Some labs test all children with diarrhoea (sometimes depending on season).	All clinical laboratories (that perform VTEC testing) use PCR to detect *vtx* genes in primary cultures (broth or plate) of patient samples. Attempts to isolate and type strains made on PCR-positive samples.	

Country	Main testing strategy	Main diagnostic tool(s) used in routine diagnosing laboratories	Comments
Switzerland	Few laboratories routinely test stools for VTEC. Eight children's hospitals take part in a long term project aimed at testing stools of cases with bloody diarrhoea and/or HUS: analysis and pathogen isolation are performed at NENT.	Primary labs use ELISA toxin detection systems and/or PCR. At NENT, additional methods are performed: strain isolation through culture techniques as well as genetic pathotyping by hybridization techniques. Further typing is done by serotyping (O157) and PFGE.	

Source: Anonymous, 2007, Enter-net annual report: 2005 – surveillance of enteric pathogens in Europe and beyond. Enter-net surveillance hub, HPA, Centre for Infections, Colindale, London.

Appendix III: VTEC non-O157 cases reported to Enter-net 2004-2005 (Enter-net data)

	Country																				
serotype	AT	BE	CH	DE	DK	EE	ES	FI	FR	GB	GZ	HU	IE	IT	JP	LU	NO	NZ	SE	SI	Total
1				1	3																4
2				9	1		1														11
3		1		4																	5
4				1																	1
5	2	1		17															1		21
6				3	1				1												5
7				2																	2
8				18	1														2		21
9				2	2																4
11					1														1		2
13				1																	1
14				1																	1
15		1		2	2																5
16				2																	2
17	1																				1
19					1																1
20					2																2
21				2									1								3
22	1			4																	5
23				8																	8
25	1																				1
26	10	6		158	29	2			40		2	4	16	16		1	1		17	2	304
28				1																	1

38		1	3	3								7
40			1	1								2
43		1										1
45				1								1
49				1								1
55	1		27	2		2			3			35
63			5									5
69			2									2
74			5									5
75	1		7				2					10
76			20	7								27
77			4									4
78			8									8
80			1									1
82			1									1
84				1								1
86			4							1		5
87			3								1	4
88			2									2
90			1									1
91	1	1	146	5								153
98			4	1								5
99			2									2
100	1		2	1								4
101			1									1
103	7	4	113	37			1		3	4	5	174

104	1			1		1										3
105			1													1
106			10													10
110			1											1		2
111	3	2	42	9		2			2	6		1		1		68
112			3													3
113	4	1	25	3												33
114	1		1													2
115			8													8
117		1	4	16		2								2		25
118	1		6				1									8
119	1		2	2								1				6
121			5	2						1				10		18
123			1	1					1							3
126				1												1
127	2		2	1		1					1					7
128			26		2	2					7					37
132			2													2
136			5	1												6
138			2													2
139				1												1
142			2													2
143	1															1
145	6	4	89	9			1	3	1	6		2		1	2	124
146	4	3	37	17					1					1		63
148			2				1									3

150			1					1
152			1			1		2
153		1	6					7
154			5	1				6
156			5	3				8
158	1		1					2
159			1					1
161			1					1
163			1					1
165			1					1
166		1	4	2				7
168				1				1
169			2					2
170			1					1
174	1		10	5				16
175			3	3				6
176				1				1
177	3		4	2			4	13
178				1				1
179				1				1
180				1				1
181		1		1				2
182				1				1
Ungroupable						2		2
112ab	1							1
125ac	1							1

128ab		2			14															16
19a											1									1
IF1					3															3
Non-O157	4	1	11					12		4	9		1		498		2	11		553
X176				2																2
X177				11																11
X178				3																3
X179				2																2
X181				1																1
X182		3			1															4
X184					1															1
Total	61	36	11	940	209	4	1	12	51	6	17	6	26	32	498	12	12	11	47	4	1,996

GLOSSARY/ ABBREVIATIONS

AFNOR: Association Française De Normalisation.

CRL: Community Reference Laboratory.

CT-SMAC: SMAC containing cefixime and tellurite.

eae: Gene encoding intimim, The gene is situated on the locus of enterocyte effacement – LEE pathogenicity island. Presence of eae is indicative of the presence of the LEE gene cluster.

EHEC Enterohemorrhagic *E. coli*, a subset of serotypes of VTEC that has been firmly associated with bloody diarrhoea and HUS in industrialized countries.

ELISA: Enzyme-Linked Immunosorbent Assay.

Enter-net: an international surveillance network for human gastrointestinal infections which was established in 1997 to maintain and develop laboratory-based surveillance of the major enteric bacterial pathogens. The Enter-net network was funded by DG SANCO of the European Commission until September 2006 and receives funding from ECDC until September 2007, when the coordination activities was transferred into ECDC. This network brings together the national surveillance leads and reference microbiologists to conduct international surveillance of salmonellosis, VTEC infections, and campylobacteriosis. Participants were from all EU Member States, WHO and non-EU countries, including EU-candidate countries, Canada, the United States, South Africa, Japan and Australia. In the VTEC database, data for 31 countries are available, of which 21 have data for the entire period 2000-2005.

HC: hemorrhagic colitis

HUS: hemolytic uremic syndrome

IMS: Immuno magnetic separation.

ISO: International Organization for Standardization.

LEE: The genetic locus of enterocyte effacement (LEE) wich is a pathogenicity island present in enteropathogenic *E. coli*, VTEC O157 and some VTEC non-O157 that contains genes required for the formation of attaching and effacing epithelial lesions.

MLVA: Multiple-Locus Variable-Number Tandem Repeat Analysis

Monitoring: According to the Directive 2003/99/EC on the monitoring of zoonotic agents, monitoring means a system of collecting, analysing and disseminating data on the occurrence of zoonoses, zoonotic agents and antimicrobial resistance related thereto.

MPN: Most Probable Number.

NMKL: Nordic Committee on Food Analysis

NRL: National Reference Laboratory.

PCR: Polymerase Chain Reaction.

PFGE: Pulsed field gel electrophoresis.

SF VTEC: Sorbitol fermenting VTEC.

SMAC: Sorbitol-MacConkey agar.

Survey: According to the Report on Guidance on Good Practices for Design of Field Surveys (EFSA, 2006b), survey is a study involving a sample of units selected from a study population. This type of study is often known as a *descriptive survey*. Its main objective is that of *estimating* the mean level of some characteristics in a defined population including a measure of the precision for those estimates. A secondary objective of surveys often is the measurement of the relationship between two or more variables measured at the same point in time. These are *analytical surveys*.

VTEC: Verotoxigenic *Escherichia coli* which produce potent cytotoxins, termed verocytotoxin(s) (VT), that inhibit the protein synthesis within eukaryotic cells. These VTs are synonymously named Shiga toxins (Stx). The terms VTEC and STEC are synonymous.