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CapE-Capture, Extract, Amplify:
A Rapid Method for Monitoring Large Water Volumes for Pathogenic Contaminants

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Executive Summary

The incidence of human infection with verocytotoxigenic *Escherichia coli* (VTEC) in Ireland increases every year. Ireland consistently has the highest incidence of human infection with VTEC across Europe, with 558 cases notified to the Health Protection Surveillance Centre (HPSC) in 2012, at a crude incidence rate of 8.9/100,000 population, significantly higher than the European Union (EU) average of 1.15/100,000. The spectrum of human illness associated with VTEC is wide from self-limiting gastroenteritis to haemolytic uraemic syndrome (HUS), which is associated with fragmentation of red blood cells, renal failure and risk of death. Approximately 10% of VTEC cases develop HUS, with those most at risk being children under 5 years of age. Those who survive may require long-term renal dialysis or kidney transplant. Water is recognised as an important transmission route.

Currently, there is no legislation requiring specific monitoring of drinking and recreational waters for VTEC. Screening for the presence of VTEC occurs only in outbreak situations, in which a 1-l sample is collected and examined. The microbiological quality of drinking and recreational water is assessed mainly by cultivation of faecal indicator bacteria – *E. coli* and *Enterococci* from small-volume (100 ml) samples taken at specific time points. Absence of *E. coli* and *Enterococci* from a 100-ml sample does not assure absence of pathogens and is limited in the ability to evaluate for low-level contamination with virulent organisms such as VTEC for which the infectious dose is very low. The approach is also limited in that it does not differentiate between different categories of *E. coli* (based on virulence characteristics or antimicrobial resistance) although these may be important for public health.

This 2-year research and development project focused on validating a rapid and convenient method for screening large volumes of water for the presence of VTEC.

A large proportion of publicly managed drinking water supplies in Ireland is now routinely monitored for the presence of *Cryptosporidium*, using commercially available filtration systems designed specifically for this purpose. These can be located on-site and can filter up to 1,000 l of water. This study examined the ability of the IDEXX Filta-Max® System (IDEXX Laboratories, Inc., Westbrook, ME, USA) to capture bacteria using spiked sterile water samples (10 l) and concluded that the system in its current format could not effectively capture bacteria. The authors considered a number of modifications to the system and determined that the addition of an in-line, 142-mm, 0.45-µm filter was optimal for the capture of VTEC from large volumes of water. Validation experiments to assess the impact of a mixed microbial population, the impact of other particulate matter in source waters, and the limit of detection of the method were assessed initially in the laboratory using 10-l volumes and subsequently on-site at various water sources. A limit of detection of 1 CFU/100 ml was achieved in line with EU regulations for monitoring drinking water supplies for the presence of *E. coli*. Up to 30 l of raw water and larger volumes of treated water could be screened at one time, depending on the levels of particulate matter.

Molecular detection and quantification of pathogens offer a number of advantages over conventional culture-based methodologies:

- More than one species or group of organisms can be identified concurrently;

- Results are available within a number of hours; and

- DNA and RNA once extracted can be assessed for numerous genetic determinants.

This study examined a number of options for the extraction of microbial DNA and it determined that direct extraction from enrichment broths following 24 h incubation at 42°C was optimal. The European Food Safety Authority (EFSA) recently proposed a molecular classification scheme to determine pathogenicity and,

using this scheme, VTEC of particular serogroups that harbour a verotoxin (*vtx*) gene in combination with the *eae* gene (encoding intimin) OR the *aaiC* (encoding a type VI secretion system) and *aagR* (encoding a transcription regulator) genes represent a high risk for diarrhoea and HUS. The authors therefore applied previously developed real-time polymerase chain reaction assays to detect VTEC virulence (*eae*, *vtx1* and *vtx2*) and O-antigen determinants (O157 and O26) and validated this approach using spiked water samples.

The optimised Capture, Extract and Amplify for the detection of *E. coli* (CapE) method was evaluated in parallel with standard methods at six group water schemes (GWSs) over a 3-month period. Source (groundwaters) and treated waters were sampled on-site using the CapE method and 1-l samples were collected in parallel and examined using standard methods (ISO 16654:2001). Sixteen samples were collected and the CapE method detected VTEC in 13 samples within 24 h in contrast to the detection of VTEC in two samples using standard methods, with the time to result ranging from 48 to 96 h. The

incorporation of molecular methods into standard culture-based methodologies increased the detection rate to eight of 16 samples. The participating GWSs were located in East Galway. VTEC was consistently detected in the groundwater sources of five of the six participating GWSs, indicating widespread contamination of groundwater with VTEC in this region.

The CapE method will be of value to environmental health officers charged with the collection of samples in outbreak investigations and laboratories engaged in monitoring the microbial quality of water, and, overall, the results inform regulatory authorities with regard to human exposure to VTEC and vulnerability of groundwaters to contamination.

The CapE method is validated as effective for the detection of VTEC and could be readily extended to the detection of other *E. coli* variants of public health significance and a range of other bacterial and protozoan pathogens of concern, e.g. *Salmonella* spp., *Campylobacter* spp., etc.

1   Introduction

1.1   What Is VTEC?

There are a number of different pathogenic types of *Escherichia coli* associated with gastrointestinal infection (enteric pathogenic *E. coli*). Currently in Ireland and Europe, the most significant of these pathogenic *E. coli* is verocytotoxigenic *E. coli* (VTEC). The risk posed to public health by VTEC and some of the complexity of detection and management was dramatically illustrated in 2011 by the major outbreak of food-borne VTEC, which originated in Northern Germany and which caused 3,821 cases in 14 countries across Europe, Canada and the United States and 47 deaths (ECDC, 2011). The spectrum of human illness associated with VTEC is wide. Some people exposed to VTEC may remain asymptomatic, some develop self-limiting gastroenteritis, and some develop bloody diarrhoea and painful abdominal cramps (haemorrhagic colitis). The most serious complication of VTEC infection is haemolytic uraemic syndrome (HUS), which is associated with fragmentation or red blood cells within the circulation system (haemolysis) and kidney failure (uraemia). HUS is associated with a risk of death and among those who survive some will require long-term renal dialysis or kidney transplant. Approximately 10% of VTEC cases develop HUS, with those most at risk being children under 5 years of age (HPSC, 2012a). Of the 3,821 cases in the outbreak of VTEC originating in northern Germany in 2011, 793 cases developed HUS, and 47 died (ECDC, 2011). The incidence of human infection due to VTEC in Ireland has been the highest in Europe since 2008 (with the exception of 2011), increasing from 1.4/100,000 in 2004 to 12.07/100,000 in 2012 (National VTEC Reference Laboratory Service, 2011; HPSC, 2012b). Data suggest the incidence rate is continuing to rise, with 711 cases notified in 2013 (HPSC, 2013). The public health challenge is heightened by the low infectious dose, the ability to survive in the environment and the potential for large-scale outbreaks as a result of contaminated food or water (McDowell and Sheridan, 2004). The importance of VTEC from a public health perspective is reflected in that the local director of public health must be informed of each case of VTEC infection (notifiable disease) and that the Department of Public Health is required to follow up and investigate every single case of VTEC infection.

1.2   Where Does VTEC Infection Come from?

VTEC can be detected in the gastrointestinal tract contents of a variety of animal species, including cattle, sheep and goats in which they frequently are associated with no illness whatever. Among the targets and recommendations of *Food Harvest 2020* (Department of Agriculture, Fisheries and Food, 2010) is the aim to increase cattle and dairy herds, which will increase the vulnerability of groundwaters to contamination in areas with a high density of livestock farming. These are very significant concerns both in terms of public health and in economic terms given the potential impacts of VTEC transmission and infection on the tourism, agriculture and food industries. Companion animals have also been reported as a source of transmission of VTEC to humans. Cafferty et al. (2006) reported the detection of VTEC in dog faeces on a football pitch, and in squirrel faeces at a picnic area. Transmission of VTEC to humans can occur via direct contact with animals, exposure to VTEC-contaminated faecal material such as soil, vegetation or water, during recreational activities, through consumption of VTEC-contaminated food or water or as a result of close contact with an infected person. There is reason to believe that contamination of water is a significant contributor to the burden of VTEC infection in Ireland and a number of outbreaks have been associated with contaminated water. In 2012, 12 outbreaks of VTEC were reported as being associated with water, affecting 57 people, seven of whom were hospitalised (Garvey et al., 2012a,b; HPSC, 2013).

1.3   Monitoring Water for the Presence of VTEC

The drinking waters distributed by local authorities in Ireland are termed public water supplies (PWSs).

Recent data suggest that the proportion of the population receiving their water from PWSs is 82%, with the remaining 18% supplied by public and private group water schemes (GWSs) or small private supplies (7.4%) and private wells (10.6%) (EPA, 2012). Provision of potable water 'fit for human consumption' requires compliance with strict standards set for 48 microbiological, chemical and indicator parameters by the European Union (Drinking Water) Regulations, 2014 (SI 122 of 2014). The microbiological parameters measured include *E. coli* and *Enterococci* and detection of either species in a 100-ml sample is deemed non-compliant and warrants immediate investigation of the water supply and remedial action. Small group water supplies serving fewer than 50 persons and private wells are exempt from these regulations. Most recent data indicate that, in 2012, 5.6% of monitored water supplies failed to meet the standard for *E. coli* and *Enterococci* on one or more occasions during the year, with small private supplies accounting for 81% of all exceedances (EPA, 2011). It is estimated that 30% of private wells in Ireland are contaminated with *E. coli* as a result of contamination with human or animal waste. Analysis of cases of human infection with VTEC in Ireland finds that patients are up to four times more likely to have consumed untreated water from private wells. The risk of contamination is, to a considerable degree, related to inadequacies in relation to location, protection and construction of wells. Such is the level of concern regarding contamination of private wells with VTEC that the Environmental Protection Agency (EPA) and the Health Service Executive (HSE) of Ireland recently (June 2014) launched a public awareness campaign on private wells (http://www.epa.ie/water/dw/hhinfo/protprivwell/).

The majority of water-borne outbreaks of VTEC infection are associated with small GWSs or private wells (HPSC, 2012b). Currently there is no legislative requirement in either Ireland or Europe to monitor water supplies for the presence of VTEC, and waters are typically only sampled in an outbreak situation. In these instances, generally a single 1-l sample is collected. Therefore, the extent of contamination of waters with VTEC in Ireland is largely unknown, as is the contribution this makes to human infection, despite this being recognised as the second most important route of transmission of VTEC infection in Ireland. There is evidence that ruminants are an important source of *E. coli* contamination of rural group water supplies and, therefore, it is reasonable to suppose that VTEC is likely to be present at least intermittently (Cormican et al., 2012). Furthermore, VTEC has been detected in water supplies during the investigation of outbreaks by the Health Protection Surveillance Centre (HPSC) and the HSE Public Health Laboratory, Cherry Orchard Hospital (National VTEC Reference Laboratory Service, 2011; Garvey et al., 2012a,b; HPSC, 2012b, 2013).

1.4   The Biology and Detection of VTEC

Initially human infection with VTEC was identified as being associated with specific phenotypic markers that were relatively easy to detect by conventional means. These markers included the presence of the O157 antigen and the loss of the ability to ferment sorbitol. Conventional microbiological systems for the detection of VTEC with these phenotypic characteristics are widely applied in clinical laboratories. However, as understanding of the phenomenon has evolved, it has become apparent that not all O157+ *E. coli* are verotoxigenic and that many VTEC strains do not have the O157 antigen and may ferment sorbitol. The importance of non-O157 VTEC is illustrated in the outbreak associated with Germany in 2011. In this case, the VTEC was of type O104. Therefore comprehensive evaluation of water samples for VTEC based on conventional microbiology has proved difficult. In 2012, the majority of VTEC infections were of serogoup O157, but an increasing proportion are non-O157, in particular VTEC O26 (HPSC, 2012b). This increase has been attributed, in part, to the more widespread use of methods that can detect non-O157 VTEC. Common methods for the detection of VTEC are based on phenotypic characteristics (surface antigens and or biochemical variation) that are not comprehensive in their coverage of known VTEC strains and which are not specific for VTEC. Although immunomagnetic separation improves sensitivity of detection of VTEC variants, it is limited to detection of the specific antigenic variants targeted. In addition, traditional cultivation and quantification-based methods for the detection of VTEC in water can take between 48 and 96 h from time of sample receipt to

result, and involve analysis of very small sample volumes (typically 100–1,000 ml). Consequently, current recommended methodologies are limited for the detection of pathogens in drinking and recreational waters. The defining characteristic of VTEC is the presence of a series of genetic elements (*vtx* and *eae* genes) that are associated with the potential to cause disease and, therefore, the most comprehensive approach to detection of VTEC is based on the application of molecular methods to the detection of these genetic targets (Persson et al., 2007). In 2014, the Laboratory Sub-Group of the VTEC Sub-Committee of the HPSC Scientific Advisory Committee published a guidance document for laboratory diagnosis of human VTEC infection and advocated the incorporation of molecular methods into testing protocols (HPSC, 2014). Molecular detection and quantification of viral, bacterial and protozoal pathogens offer a number of advantages:

- More than one species or group of organisms can be identified concurrently;

- Results are potentially available within a number of hours; and

- DNA and RNA once extracted can be assessed for numerous genetic determinants: virulence, pathogenesis, invasiveness, antimicrobial resistance, adherence, microbial biodiversity, etc.

A number of studies have examined molecular methods for detection of pathogens, including VTEC, in water. Gene targets for polymerase chain reaction (PCR) assays include the lipopolysaccharide (*rfbE*), the H7 flagellin (*fliC*$_{H7}$) and the virulence factors, verotoxin 1 (*vtx1*), verotoxin 2 (*vtx2*), haemolysin (*hly*$_{933}$), and the intimin gene (*eae*). These approaches typically involve the following steps:

- Sampling and concentration of bacteria from water;

- Extraction of DNA and/or RNA;

- Detection and quantification of the pathogen of interest; and

- Assessment of virulence.

Sen et al. (2011) successfully combined an enrichment step in presence/absence medium with a specific probe-based multiplex quantitative PCR (qPCR) assay for the detection of viable *E. coli* O157:H7 in source and drinking water. Good sensitivity and specificity were reported for 1-l water samples spiked with *E. coli* O157:H7, and one of six surface waters screened was positive for the presence of *E. coli* O157:H7. Bonetta et al. (2011) also combined an enrichment step with a multiplex PCR assay for the specific detection of *E. coli* O157:H7, virulence genes (*stx1, stx2, eae*) and *Salmonella* spp. in surface waters, and again reported good sensitivity for spiked samples.

1.5 VTEC Monitoring – an Emerging Regulation?

VTEC is emerging as a focus of growing concern for regulatory authorities in several countries. The United States Environmental Protection Agency (USEPA) recently placed VTEC on its *Drinking Water Contaminant Candidate List* (USEPA, 2009). In 2010, the Food Safety Authority of Ireland (FSAI) published a major report on the prevention of food-borne VTEC infection, with recommendations on how best to control and manage the pathogen throughout the food chain (FSAI, 2010). These included a recommendation that focused "*surveillance and research on VTEC in food animals, derived foods and water should be carried out so as to inform stakeholders of the risk posed by such exposure and to develop appropriate prevention and control strategies*". In 2005, the HPSC published a guidance document on the prevention and control of VTEC, including recommendations with regard to environmental control, which advocated monitoring of drinking and recreational waters by appropriate authorities (HPSC, 2005).

It is possible that, in the coming years, other European countries, including Ireland, will be required to monitor all drinking and recreational waters for *Cryptosporidium*, VTEC and other pathogens that represent a risk to human health. The EPA has recognised this through funding the development of a national *Cryptosporidium* monitoring programme led by Dr Theo DeWaal, University College Dublin, in collaboration with the Department of Agriculture, Food and the Marine's laboratories on the Backweston

Campus. Similar programmes are also warranted for other pathogens that pose a significant risk to public health, including VTEC. Key to the success of these monitoring programmes is the availability of sensitive and specific detection methods for individual pathogens.

1.6 Project Aim and Objectives

The overall aim of this project was to develop, validate and implement a convenient and effective method for harvesting microbial nucleic acid from large volumes of water and for the molecular detection of human pathogens in the nucleic acids. The model organism chosen for molecular detection was VTEC because of the importance of this pathogen for human health in Ireland and the EU. The developed Capture, Extract and Amplify for the detection of *E. coli* (CapE) method will be of value to laboratories engaged in monitoring the microbial quality of water and the overall results will inform regulatory authorities with regard to human exposure to pathogenic *E. coli.*

Specific objectives included:

- Evaluation of the effectiveness of filters used for the concentration of *Cryptosporidium* from large volumes (1,000 l) of water for capture of *E. coli*;

- Examination of modifications that may enhance the performance of these systems to optimise the capture of *E. coli*;

- Evaluation and optimisation of methods for extraction of nucleic acid from (a) filter membranes and (b) concentrate generated post-filtration;

- Evaluation of nucleic acid amplification tests (NAATs) for the detection of VTEC in extracted material; and

- Monitoring of selected drinking water and recreational water sources and supplies for the presence of VTEC over a period of time.

2   Methodology

2.1   Development of the CapE Protocol

The challenge of detection of low-level intermittent contamination of water by pathogens has previously been addressed successfully for *Cryptosporidium* spp. by filtration of large volumes of water. These systems are designed for sampling water on-site at the drinking water supply. One example is the IDEXX Filta-Max® System (IDEXX Laboratories, Inc., Westbrook, ME, USA) which consists of a filter (nominal pore size 1 μm) enclosed in a filter housing connected to a sampling rig backpack with a submersible pump (Hydraulic Modelling Services Ltd, Swindon, UK) (Fig. 2.1) After filtration, the Filta-Max® filters are subjected to manual washing for retrieval of captured *Cryptosporidium*. This system is convenient, easy to transport, negates the need to transport water samples to the laboratory and, as large volumes are concentrated, is very specific for the detection of *Cryptosporidium*. Although cryptosporidia are much larger than bacteria, the authors considered that this approach provided a platform that could be modified for detection of VTEC. The USEPA method 1623 and the UK drinking water inspectorate have approved the use of two commercial filters for this purpose – the IDEXX Filta-Max® and Pall
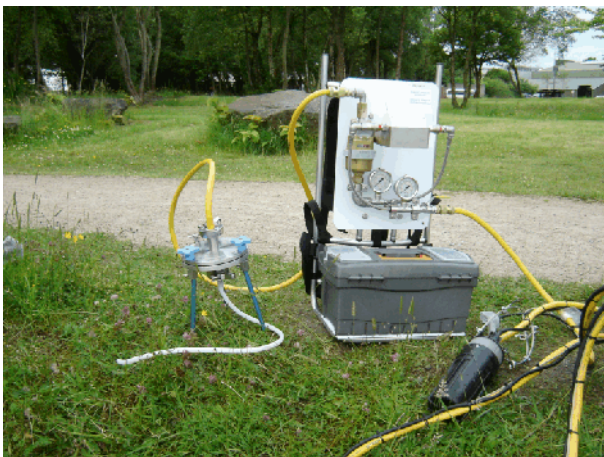
Figure 2.1. Modified filtration system for the capture of verocytotoxigenic *Escherichia coli* (VTEC).

Envirochek® filters. The authors chose to evaluate the IDEXX Filta-Max® filter alone for the purposes of this project, as this is the most commonly used system in Ireland for monitoring for the presence of *Cryptosporidium*; however, as the IDEXX Filta-Max® was essentially acting as a pre-filter for the developed CapE method, the material of this pre-filter is not critical to the successful capture of bacteria as demonstrated below.

The IDEXX Filta-Max® module comprises multiple layers of open-cell foam discs that are compressed to create a filter with a nominal pore size of approximately 1 μm to effectively trap *Cryptosporidium* and *Giardia*. After sampling, the filter is processed by releasing the compression on the foam discs and washing in a solution of phosphate-buffered saline (PBS) plus Tween 20 optimised for recovery of *Cryptosporidium*. The washing process uses expansion/compression cycles, enabling all captured material to be recovered. The volume of buffer is then reduced by concentrating through a 3-μm membrane.

Initially, the Filta-Max® system was assessed for capture of bacteria by filtration of 10 l of sterile water spiked with 10^3 CFU/ml of a non-toxigenic strain of *E. coli* O157:H7 NCTC 12900. The Filta-Max® filters, the wash buffers, the 3-μm membrane and the filtrate were assessed for the presence of bacteria by the IDEXX Colilert® system.

The Filta-Max® system was modified by the addition of an in-line high-pressure stainless steel 142-mm filter holder (Fig. 2.1) (Millipore Corp., Bedford, MA, USA) attached to the outlet hose of the sampling rig. Individual 0.45-μm MF™ membrane filters (Merck Millipore Ltd, Cork, Ireland) were used with the filter holder for the capture of bacteria.

Following filtration, the 0.45-μm filter was enriched in buffered peptone water (BPW) overnight at 37°C, DNA was extracted from the enrichments and screened for the presence of virulence (*eae, vtx1* and *vtx2*) and serotype-specific genes (O26 and O157) by real-time

PCR. The time from sample collection to result using the CapE method was less than 24 h ([Fig. 2.2](#)).

2.2 Validation of the CapE Protocol

All validation experiments were performed in triplicate. Initial validation experiments were carried out in the laboratory by spiking 10 l sterile water with known concentrations of a non-toxigenic strain of *E. coli* O157:H7 NCTC 12900 with and without a mixed microbial background ([Table 2.1](#)). Spiking concentrations were verified by standard methods. All

validation experiments outlined below were repeated in triplicate.

2.2.1 Assessment of the Filta-Max® system for capture of bacteria

Ten litres of sterile water spiked with 10^3 CFU/l of *E. coli* O157:H7 NCTC 12900 were filtered using the IDEXX Filta-Max® system unmodified. The spiked water pre-filtration, the filtrate post-filtration and the Filta-Max® filter wash waters were examined for presence of *E. coli* using the Colilert®-18 Quanti Tray 2000 (IDEXX, Technopath, Limerick, Ireland) method in accordance with the manufacturer's instructions.
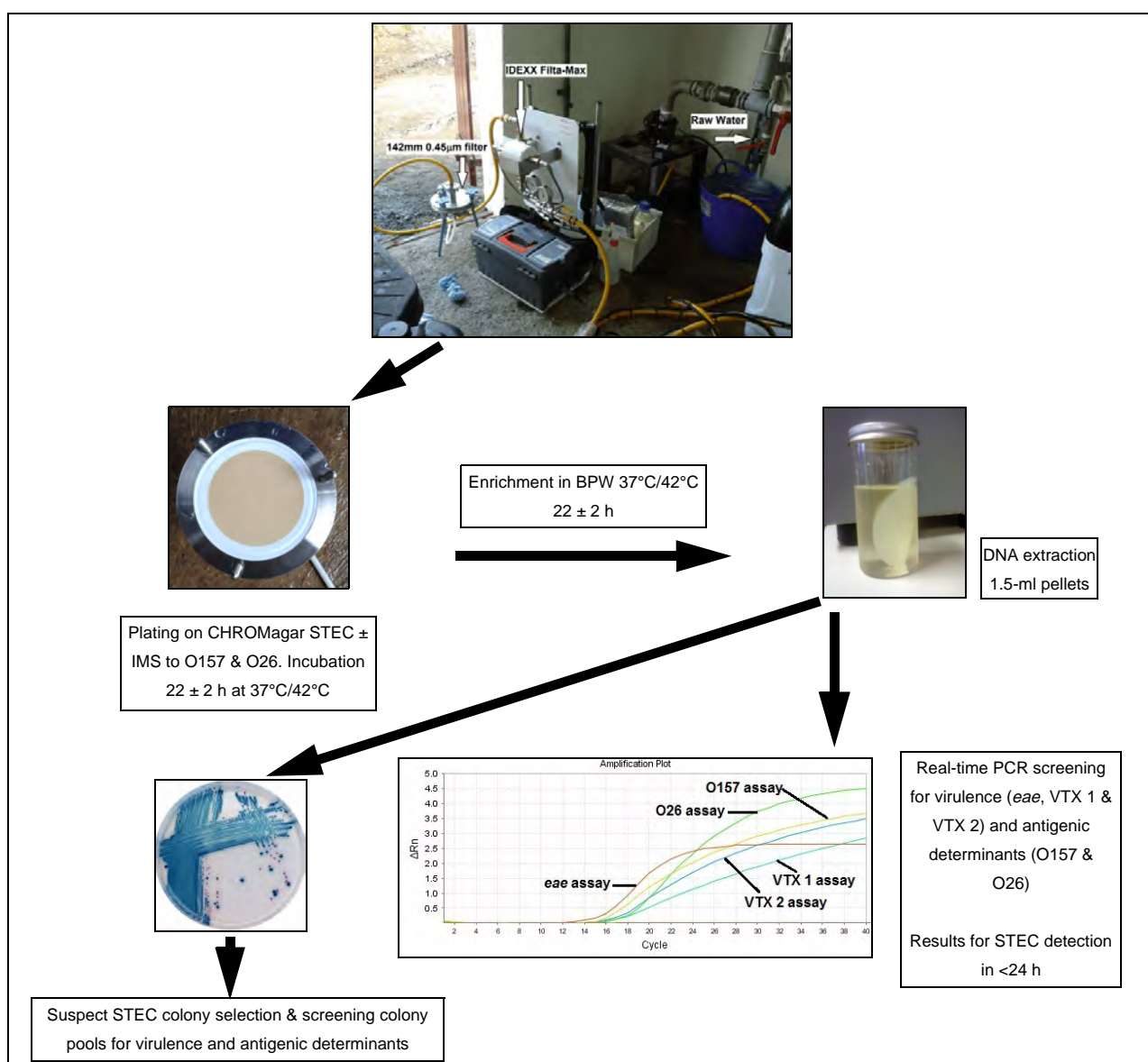

Figure 2.2. Workflow schematic of the CapE protocol. BPW, buffered peptone water; STEC, Shiga toxin-producing *Escherichia coli*; IMS, immunomagnetic separation; PCR, polymerase chain reaction.

Table 2.1. Validation experiments carried out using sterile waters spiked with *Escherichia coli* O157:H7 NCTC 12900 ± other bacteria in the laboratory.

	Water	Concentration of organism(s) spiked into sample
1	Autoclaved tap	*E. coli* O157:H7 NCTC 12900 10^3 CFU/l
2	Autoclaved tap	*E. coli* O157:H7 NCTC 12900 10^3–1 CFU/l
3	Autoclaved tap	*E. coli* O157:H7 NCTC 12900 10^3–1 CFU/l + 10^3 CFU/l *E. coli* ATCC 25922
4	Autoclaved river water	*E. coli* O157:H7 NCTC 12900 10^3–10 CFU/l
5	Autoclaved river water	*E. coli* O157:H7 NCTC 12900 10^3–10 CFU/l + mixed microbial background[1] of 10^3 CFU/ml

[1]The mixed microbial population contained two different strains of *Pseudomonas aeruginosa* and *Serratia marcescens*, and individual isolates of *Enterobacter cloacae*, *Acinetobacter ursingii* and *Escherichia coli* ATCC 25922, which were mixed to generate a suspension equal to a 0.5 McFarland standard.

2.2.2 Assessment of the CapE method for detection of VTEC in spiked sterile water

Ten litres of sterile water spiked with 10^3 CFU/l–1 CFU/l of *E. coli* O157:H7 NCTC 12900 alone and in combination with 10^3 CFU/l *E. coli* ATCC 25922 were filtered using the modified filtration system. The filtrate escaping the system was captured in a sterile collection vessel, and the Filta-Max® filter wash waters were examined for the presence of *E. coli* using the Colilert®-18 Quanti Tray 2000 method in accordance with the manufacturer's instructions. The 0.45-µm filters were enriched overnight in BPW at 37°C and screened for the presence of the *eae* gene by real-time PCR as outlined in Section 2.3. One hundred millilitres of enrichments were cultured on CHROMagar™ STEC (CHROMagar Microbiology, Paris, France) for isolation of *E. coli* O157. VTEC strains are reported to form mauve colonies on this agar; therefore, mauve colonies were isolated and screened for the presence of the *eae* gene by real-time PCR.

2.2.3 Assessment of the CapE method for detection of VTEC in spiked sterile river water

To examine the impact of particulate matter and other material in river water on the modified system, 10 l of river water were collected. Prior to sterilisation and spiking, total coliforms and *E. coli* in the raw river water were estimated using the Colilert®-18 Quanti Tray 2000 method and the presence of the *eae* gene was determined by real-time PCR.

River water (10 l) was sterilised by autoclaving, spiked with 10^3 CFU/l–10 CFU/l of *E. coli* O157:H7 NCTC

12900 alone and in combination with a mixed microbial population (Table 2.1) to a final concentration of 10^3 CFU/l and filtered using the modified filtration system. The 0.45-µm filters were divided into two and enriched overnight in BPW at 37°C and 42°C and screened for the presence of the *eae* gene by real-time PCR and cultured on CHROMagar™ STEC as outlined above.

2.2.4 Assessment of the CapE method for detection of VTEC in unmodified river water

On five sampling dates between January and June 2013, river waters (20–33 l) were screened for the presence of VTEC using the CapE method. Rainfall levels (mm) were recorded on each sampling date. The volume of water filtered on each sampling occasion was determined by the point at which the 0.45-µm filter became blocked, resulting in a decrease in pressure in the sampling rig pressure gauge. On each sampling occasion, a 1-l discrete water sample was also collected to test for total coliform/*E. coli* levels using the Colilert®-18 Quanti Tray 2000 method in accordance with the manufacturer's instructions.

2.2.5 Parallel assessment of the CapE method vs standard methods for detection of VTEC in raw and treated waters of rural GWSs

Six GWSs identified as vulnerable to contamination with *E. coli* were screened for the presence of VTEC in both source and piped waters using the CapE method and standard methods in parallel. The GWS raw water sources and the treatment method for raw water are listed in Table 2.2. Rainfall levels (mm) were recorded on each sampling date between August and October

Table 2.2. Group Water Scheme (GWS) raw water sources and treatment method for raw water.

Location	Raw water source[1]	Treatment	Sample number (raw & treated water)	Sampling location treated water
GWS 1	Borehole 91.44 m Static water level 34 m	UV & chlorination	3	Outside tap at house on scheme
GWS 2	Borehole 121.92 m Static water level 16.2 m	UV & chlorination	3	Pump house
GWS 3	Borehole 30 m Static water level 24.5 m	UV & chlorination	1	Pump house
GWS 4	Borehole 64 m Depth to water is 18.28 m	UV & chlorination	3	Outside tap at house on scheme
GWS 5	Borehole 54.86 m Depth to water 39.62 m	Chlorination	3	Outside tap at house on scheme
GWS 6	Borehole 6.5 m Static water level 4.8 m	UV & chlorination	3	Pump house

[1]All raw water originated from groundwater sources.
UV, ultraviolet.

2013. Raw and treated water was sampled on three separate dates for five of the GWSs. One GWS withdrew from the study after the first sampling date. At each scheme, treated water was filtered before raw water to avoid potential cross-contamination of samples. For both raw and treated water, 30-l volumes were collected aseptically for filtration, in a flexi-tub disinfected with 70% EtOH and deionised water. After filtration, 0.45-µm filters were removed on-site to sterile pots containing 100 ml BPW and transported back to the lab for enrichment at 37°C and 42°C within 2 h of sampling (Fig. 2.2).

After 22 ± 2 h, DNA extracts of the enrichments were screened by real-time PCR for virulence genes (*eae*, *vtx1* and *vtx2*) and antigenic determinants for *E. coli* O157 and O26. Enrichments were subcultured on CHROMagar™ STEC ± immunomagnetic separation (IMS) using the Automated Dynal BeadRetriever™ System and Dynabeads® MAX *E. coli* O157 and EPEC/VTEC O26 kits (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. For positive VTEC determination, characteristic colonies (mauve) were pooled (10 colonies in 200-µl volumes of molecular biology grade water) and DNA was extracted by boiling. Colony pool DNA extracts were screened with the O157, O26, *vtx1* and *vtx2* real-time PCR assays. Colonies for pooling

were preserved at –80°C for further testing. Discrete raw and treated water samples (2 l) were also collected at each sampling location in sterile bottles (Schott Duran) to test for total coliform/*E. coli* levels using the Colilert®-18 Quanti Tray 2000 method in accordance with the manufacturer's instructions, and to perform the standard protocol for the detection of VTEC in water. Briefly, this method involves the retention of bacteria from small-volume samples (1 l) on 0.45-µm membrane filters. The filters are incubated in modified tryptone soya broth (mTSB) plus novobiocin (16 mg/l) at 41.5 ± 1°C for 22 ± 2 h followed by IMS for *E. coli* O157 and *E. coli* O26 as described previously.

Following IMS, the eluates are subcultured on a non-selective agar, for *E. coli* O26 MacConkey Agar without Salt (MCWOS) and for *E. coli* O157 Sorbitol MacConkey Cefixime/Tellurite Agar (SM-CT). The agars are examined for characteristic colonies (red/pink on MCWOS and straw coloured on SM-CT), which are confirmed by serological and biochemical testing. For the purpose of this study, confirmation of the presence of *E. coli* O157 or *E. coli* O26 and the possession of *vtx1* and *vtx2* genes was carried out by real-time PCR assay testing of colony pool DNA extracts. The standard protocol in place at the time of this study did not include screening of the enrichments

for virulence genes and antigenic determinants for *E. coli* O157 and *E. coli* O26.

2.3 Real-Time PCR

DNA was extracted from 1.5 ml of BPW enrichments by boiling or using the MagMAX™ total nucleic acid isolation kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's instructions. For boiled extraction, 1.5-ml volumes of enrichments were concentrated by centrifugation at 14,000 rpm for 10 min, resuspended in 200 µl of molecular biology grade water and heated at 95°C for 20 min. The boiled extracts were centrifuged at 14,000 rpm for 2 min and the supernatants transferred to clean microcentrifuge tubes. DNA yields for all extract types were determined using the Qubit™ Fluorometer nucleic acid quantification system and the Quant-iT™ dsDNA High Sensitivity assay kit (Molecular Probes Inc., Invitrogen, Eugene, OR, USA) in accordance with the manufacturer's instructions. The sequences of the primers and probes used to target virulence (*eae, vtx1* and *vtx2*) and serotype-specific antigen genes (O157 and O26) are listed in Table 2.3. For all validation experiments, the *eae* assay was used to detect the presence of *E. coli* O157. River water and GWS enrichment extracts were also screened using the *vtx*

and antigen-specific assays. Appropriate positive controls were included in each assay.

2.4 Assessment of an Alternative Pre-Filter

To examine the viability of an alternative pre-filter to the IDEXX Filta-Max® filters, an aquarium sponge, Juwel® Aquarium fine filter sponge, Standard/ Standard H/Bioflow® 6.0, was divided into portions (5 cm thickness, 12 cm length) and inserted into the IDEXX filter module. Filtration runs (in triplicate) were carried out on 10-l water volumes spiked with *E. coli* O157:H7 (10 CFU/l) and mixed microbial background. In-situ filtrations of the river waters and one filtration run were carried out at each GWS in parallel with a filtration run incorporating IDEXX Filta-Max® run.

2.5 Statistics

The Wilcoxon Signed-Rank test, a non-parametric test designed to evaluate the difference between two treatments or conditions where the samples are correlated, was used for the comparison of cycle threshold (Ct) values obtained for *eae* (spiked volumes and in-situ filtration runs) *vtx1*, *vtx2*, O157 and O26 antigenic determinants (in-situ filtration runs) with DNA preparations from enrichments at 37°C and 44°C.

Table 2.3. Primer and probe sequences.

Primer/Probe	Sequence (5'–3')[1]	Target gene	Reference
eae **forward**	CATTGATCAGGATTTTTCTGGTGATA	*eae*	Nielsen and Andersen (2003)
eae **reverse**	CTCATGCGGAAATAGCCGTTA		
eae **probe**	ATAGTCTCGCCAGTATTCGCCACCAATACC		
stx1 **forward**	TTTGTYACTGTSACAGCWGAAGCYTTACG	*stx1*	Perelle et al. (2004)
stx1 **reverse**	CCCCAGTTCARWGTRAGRTCMACRTC		
stx1 **probe**	CTGGATGATCTCAGTGGGCGTTTCTTATGTAA		
stx2 **forward**	TTTGTYACTGTSACAGCWGAAGCYTTACG	*stx2*	Perelle et al. (2004)
stx2 **reverse**	CCCCAGTTCARWGTRAGRTCMACRTC		
stx2 **probe**[2]	TCGTCAGGCACTGTCTGAAACTGCTCC		
O157 **forward**	TTTCACACTTATTGGATGGTCTCAA	*rfbE*	Perelle et al. (2004)
O157 **reverse**	CGATGAGTTTATCTGCAAGGTGAT		
O157 **probe**	AGGACCGCAGAGGAAAGAGAGGAATTAAGG		
O26 **forward**	CGCGACGGCAGAGAAAATT	*wzx*	Perelle et al. (2004)
O26 **reverse**	AGCAGGCTTTTATATTCTCCAACTTT		
O26 **probe**	CCCCGTTAAATCAATACTATTTCACGAGGTTGA		

[1]Y(C/T), S(C/G), W(A/T), R(A/G), M(A/C).
[2]This combination of primer/probe recognises all of the *vtx2* variants except *vtx2f*.

3   Results

3.1   Evaluation of the IDEXX Filta-Max® System for Capture of VTEC

Results of the estimation of the number of *E. coli* present were compared for the IDEXX Filta-Max® filter and the filtrate escaping the system and the spiked water pre-filtration. The *E. coli* O157 strain used in the spiking experiments (*E. coli* O157:H7 NCTC 12900) is β-glucuronidase-negative, and therefore does not metabolise 4-methylumbelliferyl-β-D-glucuronide (MUG) and produce fluorescence under ultraviolet (UV) light. In the absence of other coliforms in the water, a change from colourless to yellow wells was taken as a positive result for the presence of *E. coli* within the different fractions tested. Following the manual washing protocol for the retrieval of *Cryptosporidium* cells, no *E. coli* was detected in the wash waters (Most Probable Number (MPN) <1.0/100 ml), indicating that the IDEXX Filta-Max® filter did not capture the bacterial cells at low spiking concentrations. *Escherichia coli* was detected in the filtrate escaping the system, with the percentage recovery of cells in five repeat runs of the system ranging from 34.5% to 93.3%. It was therefore concluded that the IDEXX Filta-Max® system in its current format was not capable of effectively capturing bacteria.

3.2   Validation of the CapE Method for Capture of VTEC

Following modification of the system by the addition of an in-line 142-mm, 0.45-µm filter sterile waters spiked with known concentrations and populations of bacteria, *E. coli* was not detected in the *Cryptosporidium* filter wash waters on any occasion. *Escherichia coli* was not detected in the filtrate escaping the system, suggesting that cells were being retained on the 142-mm, 0.45-µm filter. The *eae* assay was capable of detecting down to 1 CE and 1 CFU/ml of the test *E. coli* O157 (with Ct results of 31.57 (ΔRn 3.9) and 37.82 (ΔRn 0.96), respectively). DNA yields for the BPW enrichment extracts were in the range of 6.9–19.1 ng/µl and *eae* was consistently detected to a concentration of

10 CFU/l with or without a background of *E. coli* ATCC 25922, with amplification curves exhibiting similar ΔRn to positive controls. DNA extraction by boiling and the MagMAX™ automated system produced comparable results in terms of DNA yields and Ct values for real-time PCR detection of *eae*. Mauve colonies consistent with growth of VTEC on CHROMagar STEC were isolated to a concentration of 10 CFU/l *E. coli* O157 ± 10^3 CFU/l *E. coli* ATCC 25922 and presence of *eae* was confirmed by real-time PCR.

River waters were assessed pre-spiking for the presence of total coliforms and *E. coli* with results ranging from 547.5 to 30.5 MPN/100 ml for coliforms and 68.9 to 2 MPN/100 ml for *E. coli*. The *eae*, *vtx1*, *vtx2*, O157 and O26 genes were detected in unspiked river waters. BPW enrichments of river water pre and post-filtration produced a high microbial background on CHROMagar™ STEC media (Fig. 3.1), therefore two incubation temperatures (37°C and 42°C) were investigated to optimise detection of VTEC. The Ct values obtained from extracts of enrichments (spiked water and river water) incubated at 42°C were consistently lower than those from enrichments incubated at 37°C, with ΔCt values ranging from 0.16 to 11.63. The microbial background was consistently reduced following incubation of enrichments at 42°C and incubation was carried out at the two temperatures for the remainder of the study. The microbial background was also reduced by incubating post-spiking enrichments and CHROMagar™ STEC plates at 42°C. One isolate cultured from unspiked river water harboured the *eae*, *vtx1*, *vtx2* and O126 genes and was subsequently serotyped as *E. coli* O26.

The *eae* gene was detected from BPW enrichments of the bacterial filter following filtration of spiked sterile river waters (to remove microbial background and retain particulates and humics). Earlier detection of the *eae* gene was consistently observed from incubations at 42°C, with ΔCt values ranging from 1.67 to 12.47 cycles. To determine if the incubation temperature had an effect on the detection of *eae* in spiked water (tap water, river water and artificial river water), the
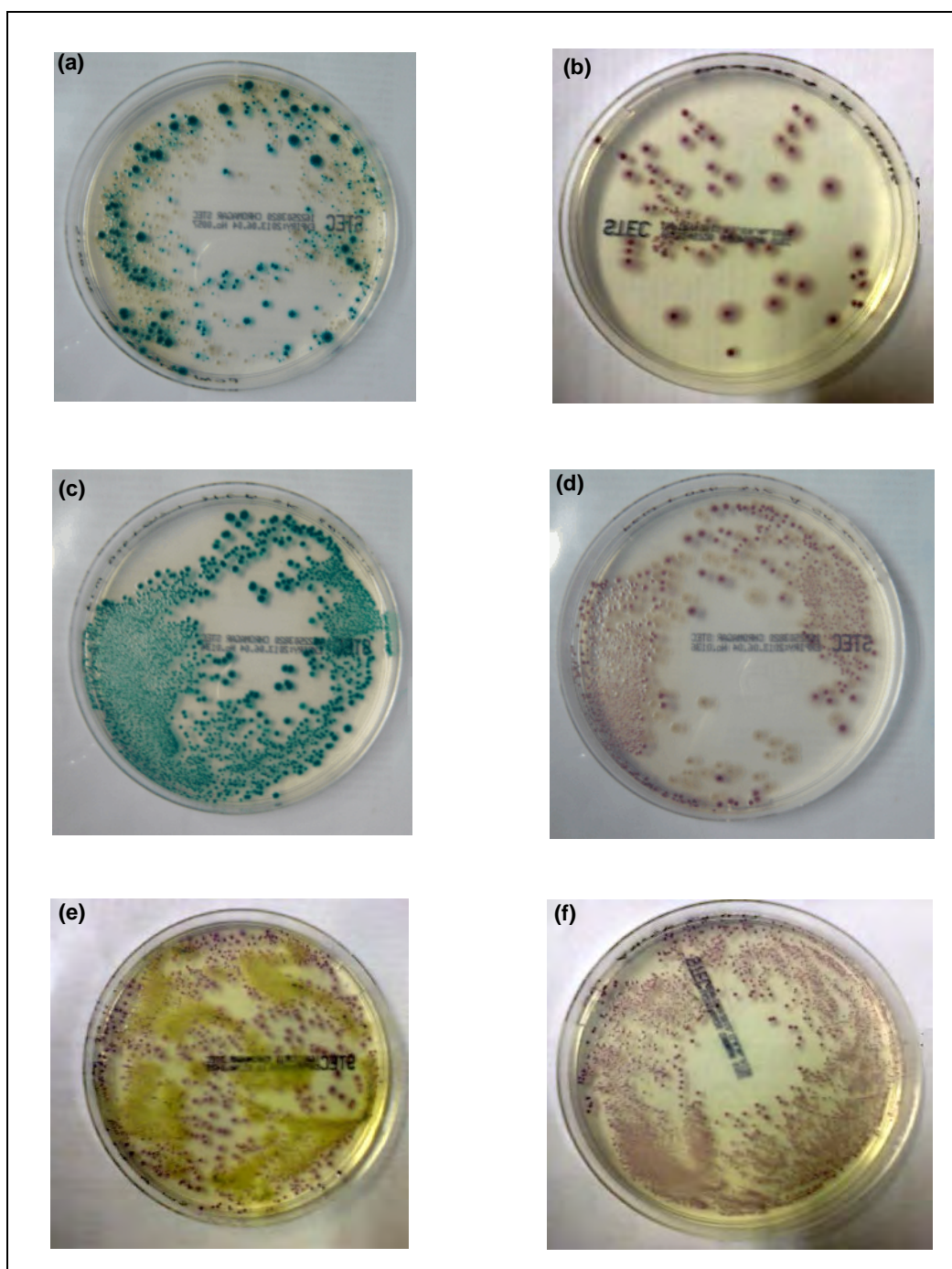
Figure 3.1. Culture of enrichment broths on CHROMagar^{TM} STEC agar plates. River water pre-spiking 5-l filtration incubated at (a) 37°C and (b) 42°C. Spiked river water (10^2 CFU/l) 10-l filtration incubated at (c) 37°C and (d) 42°C. Simulated river water (10 CFU/l with microbial background of 10^3 CFU/ml incubated at (e) 37°C and (f) 42°C.

Wilcoxon Signed-Rank Test was applied to paired Ct values for enrichments (37°C and 42°C) at 0.05 significance level. The test showed that the effect of temperature on the Ct value was significant at the 95% confidence level.

3.2.1 Assessment of the CapE method for detection of VTEC in unmodified river water

The *eae* gene was detected on all sampling occasions in BPW enrichments of the bacterial filter and was not impacted by rainfall levels (Table 3.1). The *vtx1* and

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Table 3.1. Detection of verocytotoxigenic *Escherichia coli* (VTEC) in unmodified river water using the CapE method.

Date	Volume (l)	Rainfall (mm)[1]	MPN/100 ml	Gene targets detected from BPW enrichments (37°C & 42°C)				
			Coliforms/*E. coli*	*eae*	O157	O26	*vtx1*	*vtx2*
25.01.13	20	12.7	210/35	37°C & 42°C	37°C	37°C & 42°C	37°C	37°C & 42°C
13.02.12	33	12.5	186/18	37°C & 42°C	37°C & 42°C	37°C & 42°C	42°C	37°C & 42°C
04.03.13	20	0	61/15	37°C & 42°C	nd	nd	nd	nd
23.04.13	28	0	16/2	37°C & 42°C	nd	nd	nd	nd
25.06.13	20	0.3	285/2	37°C & 42°C	nd	nd	nd	nd

[1]Rainfall measurements (mm) Athenry weather station. Data from Met Éireann website http://www.met.ie/climate/daily-data.asp.
MPN, Most Probable Number; BPW, buffered peptone water; ND, not detected.

vtx2, and antigenic determinants for O26 and O157 were detected in bacterial filters in January and February only. In January, *vtx*2 and O26 were detected in 37°C and 42°C enrichment extracts, with the remaining two targets only detected in 37°C extracts. In February, all targets, with the exception of *vtx1*, were detected in both 37°C and 42°C extracts. The *vtx1* gene was detected in the 42°C enrichment extract only.

3.2.2 Parallel assessment of the CapE method vs standard methods for detection of VTEC in raw and treated waters of rural GWSs

Coliforms and *E. coli* were detected in raw water from five out of six rural GWSs (Table 3.2), with one scheme (GWS 5) having low levels of coliforms on two occasions but no *E. coli*. The treated water tested negative for coliforms and *E. coli* at four of the six schemes. One scheme had a coliform count of 2 and an *E. coli* count of 1 for the treated water; this scheme withdrew from the study after the first sampling date. Treated water at another scheme was found to be grossly contaminated with coliforms (817/100 ml) and *E. coli* (144/100 ml) on one occasion. For this scheme, the treated water was sampled from an outside tap at a house on the scheme. Levels of coliforms and *E. coli* were higher than in the raw water, suggesting that the contamination was occurring between the pump house (where chlorination was applied to treat the water) and the house. Coliform/*E. coli* levels diminished to acceptable levels (0/100 ml) on repeated sampling.

VTEC was detected using the CapE method in raw water from five of the six schemes. The *eae* gene was detected in raw water from all of the schemes, with the exception of GWS 5. The *vtx1* gene was detected from five of the six schemes on each sampling occasion and the *vtx2* gene was detected in every replicate from four of the six schemes and in two of three replicates for one scheme. The O157 target was detected at four schemes on every occasion and at two schemes in one of three replicates. Detection of the O26 target was observed at two schemes in all replicates, at three schemes twice, and was not detected from GWS 5. In agreement with spiking experiments carried out in the lab, earlier detection of *eae* was observed from enrichments incubated at 42°C. Ct values were lower for 42°C enrichment extracts on the majority of

occasions (81.25%), with ∆Ct values ranging from 0.2 to 6.53 cycles. Application of the Wilcoxon Signed-Rank Test showed that the effect of temperature on the Ct value was significant at the 95% confidence level in detection of *eae* from in-situ sampling, river water and GWS. The W-value was 41 and the critical value of W (n = 20) at $p \le 0.05$ is 52. Therefore, the result is significant at $p \le 0.05$. W-values generated for the other gene targets (O157, O26, *vtx1*, *vtx2*) revealed that the effect of temperature on Ct value was not significant at the 95% confidence level ($p \le 0.05$).

The detection of virulence genes and antigenic determinants for O157 and O26 in GWS raw water from the standard method is outlined in Table 3.3. The targets *eae* and *vtx*2 were detected in all replicates from three schemes. For the remaining schemes, *eae* was detected from a single replicate in two other schemes and was not detected from GWS 5 and *vtx2* was detected for a single replicate from one scheme and was not detected in the remaining two schemes. The *vtx1* gene was only detected in one replicate of one scheme and the antigenic determinants for O157 and O26 were only detected at one scheme, O26 was detected in all replicates and O157 in two of three replicates. VTEC was not detected in treated water using either protocol.

Culturing on CHROMagar™ STEC was carried out from large-volume filtration enrichments (37°C and 42°C) ± IMS for *E. coli* O157 and *E. coli* O26. Mauve colonies were selected for screening with virulence and antigenic determinant assays, predominantly from enrichments and plates incubated at 42°C. Mauve colonies were more prominent on 42°C plates with less microbial background. Even after IMS for O26 and O157 37°C enrichments produced background growth. Mauve colony pools (n = 10) selected from plates with or without IMS were screened with the *vtx1* and *vtx2* and antigenic determinant assays. The results of colony pool screening are summarised in Table 3.3. Seventy-five per cent of colony pools that tested VTEC-positive (n = 20) were isolated after application of IMS. A higher proportion of 42°C pools tested VTEC-positive, 44%, compared with 21% of 37°C pools. A greater variety of VTEC types were isolated from 42°C enrichments, *vtx*2-positive and *vtx1*/*vtx2*-positive *E. coli* O157, and *vxt1*-positive, *vtx1*/*vtx2*-

Table 3.2. Detection of verocytotoxigenic *E. coli* (VTEC) in raw and treated water of six Group Water Schemes (GWSs) using the CapE method.

GWS	Sampling date	Rainfall (mm)[1]	Raw water (MPN/100 ml)		Treated water (MPN/100 ml)	
			Coliforms	*E. coli*	Coliforms	*E. coli*
GWS 1	21.08.13	0.4	187	36	816	144
	28.08.13	0.2	79	15	1	<1.0
	02.09.13	3.1	162	10	<1.0	<1.0
GWS 2	21.08.13	0.4	387	192	<1.0	<1.0
	16.09.13	2.2	219	57	<1.0	<1.0
	09.10.13	0.1	276	45	<1.0	<1.0
GWS 3	05.09.13	3.1	152	7	2	1
GWS 4	18.09.13	0.1	34	2	<1.0	<1.0
	30.09.13	0.3	26	1	<1.0	<1.0
	21.10.13	16.1	55	12	<1.0	<1.0
GWS 5	25.09.13	0	<1.0	<1.0	<1.0	<1.0
	16.10.13	16.5	1	<1.0	<1.0	<1.0
	30.10.13	2.6	5	<1.0	<1.0	<1.0
GWS 6	02.10.13	1.6	68	1	<1.0	<1.0
	14.10.13	0	50	5	<1.0	<1.0
	23.10.13	0.6	411	238	<1.0	<1.0

[1]Rainfall measurements (mm) Athenry weather station. Data from Met Éireann website http://www.met.ie/climate/daily-data.asp.
MPN, Most Probable Number.

Table 3.3. Comparison of the CapE and standard methods for detection of verocytotoxigenic *E. coli* (VTEC) in raw and treated waters of six Group Water Schemes (GWSs).

GWS	Large-volume (30 l) filtration protocol						Standard VTEC (1 l) filtration protocol					
	Real-time PCR detection of VTEC targets[1]					Mauve colony selection and VTEC confirmation[2]	Real-time PCR detection of VTEC targets					Colony selection and VTEC confirmation[3]
	eae	O157	O26	*vtx1*	*vtx2*		*eae*	O157	O26	*vtx1*	*vtx2*	
GWS 1	3/3	3/3	2/3	3/3	3/3	2/3 (n = 150) 1 pool positive O26, *vtx1* + *vtx2* 2 pools positive O157, *vtx1* + *vtx2* 1 pool positive *vtx2* serotype ? 1 pool O26-positive, *vtx*-negative	3/3	2/3	3/3	0/3	3/3	2/3 (n[4] = 140) 4 pools positive *vtx2*, serotype unknown
GWS 2	3/3	3/3	3/3	3/3	3/3	2/3 (n = 120) 2 pools positive O157, *vtx1* + *vtx2* 4 pools positive O26, *vtx1* 1 pool positive O26, *vtx1* 1 pool positive O26, *vtx*-negative 1 pool positive O157, *vtx2*	3/3	0/3	0/3	1/3	3/3	0/3 (n = 130) All pools negative
GWS 3	1/1	1/1	1/1	1/1	1/1	1/1 (n = 45) 2 pools positive O26, *vtx1* + *vtx2* 1 pool positive O26, *vtx1* 1 pool positive O157, *vtx2* 1 pool *vtx2*-positive serotype ?	1/1	0/1	0/1	0/1	1/1	0/3 (n = 120) All pools negative
GWS 4	3/3	1/3	2/3	3/3	2/3	0/3 (n = 185) All pools negative	1/3	0/3	0/3	0/3	1/3	0/3 (n = 125) All pools negative
GWS 5	0/3	1/3	0/3	0/3	0/3	0/3 (n = 40) All pools negative	0/3	0/3	0/3	0/3	0/3	0/3 (n = 60) All pools negative
GWS 6	3/3	3/3	2/3	3/3	3/3	1/3 (n = 74) 1 pool positive O26, *vtx1*	1/3	0/3	0/3	0/3	0/3	0/3 (n = 100) All pools negative

[1]Raw and treated water (results not shown) sampled at six GWSs on three occasions, with the exception of GWS 3, which was sampled once.
[2]All colonies tested were mauve on CHROMagar™ STEC.
[3]All colonies tested were pink–red on MacConkey Agar without Salt (MCWOS) or straw-coloured on Sorbitol MacConkey Cefixime/Tellurite Agar (SM-CT).
[4] n refers to total number of isolates tested. Each colony pool consisted of 10 isolates subjected to boiling DNA extraction.
PCR, polymerase chain reaction

positive and *vtx*-negative *E. coli* O26. Two 37°C pools testing positive for O26 and *vtx1* were isolated from a sample where *vtx1* and *vtx1*/*vtx2*-positive *E. coli* O26 had also been isolated from the 42°C enrichment.

Screening of colony pools from the standard protocol was also carried out. From the standard protocol, characteristic colonies were isolated more frequently on MCWOS (red–pink) after IMS for O26 than on SM-CT after IMS for O157, although the majority of pools tested VTEC-negative after real-time PCR. Using the standard protocol, VTEC was only confirmed in raw water from one GWS (*vtx2* detection from four colony pools, GWS 1 on two occasions) although real-time

PCR screening of enrichments detected VTEC at four of the six GWSs.

3.3 Preliminary Assessment of Alternative Pre-Filter

Results suggest that the Juwel® Aquarium fine filter sponge, Standard/Standard H/Bioflow® 6.0 was as effective a pre-filter as the IDEXX Filta-Max® filter. The alternative pre-filter allowed for comparable volumes of water to be filtered (30–50 l), without the 0.45-µm filter becoming blocked, and had a similar limit of detection (10 CFU/l) as the IDEXX Filta-Max® filter.

4   Discussion

This programme of work has achieved its objective of developing and validating a novel, rapid, efficient, sensitive and specific method for detection of VTEC from large water volumes.

Contamination of drinking water sources with microbial pathogens is a major global public health concern (Craun et al., 2010; Brinkman et al., 2012; Pall et al., 2013). Ireland has consistently had the highest incidence of human infection with VTEC across Europe since 2008, with rates continuing to increase. As water is recognised as a major transmission route for infection (HPSC, 2012b), the relevance of a rapid, sensitive detection method for pathogens in water is clear. In outbreak situations, rapid confirmation of VTEC contamination of waters is vital to protect the public, facilitate decision making on the imposition of water restrictions, e.g. boil water notices, and to improve treatment of water supplies (Sen et al., 2011). Traditional cultivation-based methods currently employed for the detection of VTEC in waters are laborious and time consuming (Mauro et al., 2013). The optimal method for detection of any pathogen should be sensitive and capable of detecting low numbers, which is particularly pertinent to VTEC given the infective low dose (Thompson et al., 2006). Screening methods should also be capable of detecting stressed cells in a viable but non-culturable (VBNC) state that retain the potential for virulence and to recover pathogenicity (Dinu and Bach, 2011). VBNC *E. coli* O157:H7 has been shown to retain expression of both *vtx1* and *vtx2* genes and there is evidence to suggest that expression of these genes may be higher in VBNC cells than in culturable cells (Liu et al., 2010). Therefore, the usefulness of molecular methods in the rapid detection of markers for water-borne pathogens is apparent (Fratamico et al., 2011; Brinkman et al., 2012).

A primary consideration in VTEC detection from water is the successful capture of cells, even when present at low concentrations. As commercially available filtration systems for the capture of *Cryptosporidium* in water are currently in use in Ireland, the IDEXX Filta-Max®, which is capable of filtering up to 1,000 l of finished water, was evaluated for capture of VTEC, potentially allowing for simultaneous monitoring of both pathogens. This study has demonstrated that the IDEXX Filta-Max® system was not capable of capturing VTEC without modification. The addition of an in-line 0.45-µm filter was optimal for the capture of VTEC to a level of 10 CFU/l, thereby meeting the detection limits required under EU drinking water legislation for monitoring of *E. coli* and *Enterococci.* The test *E. coli* O157 strain was detected in the *Cryptosporidium* filter after filtration of water with high background microflora but the target was still detectable in the bacterial filter. Some capture of cells by the *Cryptosporidium* filter is expected at higher microbial concentrations, specifically when particulates are present. Experiments carried out using portions of an alternative pre-filter demonstrate that monitoring for VTEC may be carried out independently of *Cryptosporidium* monitoring. This alternative pre-filter displayed the same capacity for large-volume filtration, similar limits of detection and is more cost-effective.

In developing the method, direct detection of VTEC from filters was evaluated in addition to detection after filter enrichment. At low spiking concentrations, DNA extraction directly from portions of filters resulted in low DNA yields, inconsistent and unreliable real-time PCR detection and, due to the large size of the bacterial filter (142 mm), considerable challenges were encountered in the retrieval of cells from the whole filter. In monitoring for low-level VTEC contamination, it is considerably more convenient to incorporate an enrichment step and detect VTEC from the enrichment buffer. Enrichment of filters is widely used prior to real-time PCR detection of VTEC (Heijnen and Medema, 2006; Bonetta et al., 2011; Fratamico et al., 2011; Sen et al., 2011), allowing for cell recovery and proliferation, therefore increasing the sensitivity of the method. After enrichment, VTEC was detectable within 24 h by real-time PCR. In agreement with a previous study (Heijnen and Medema, 2006), a crude DNA

extraction protocol, boiling and centrifugation to remove cell debris was sufficient for the successful detection of targets due to the high concentrations of bacteria after enrichment.

The CapE protocol developed in this study is based on an existing protocol (CEN/ISO TS 13136), with some modifications, for the detection of VTEC in water samples. This protocol describes filtration and enrichment, nucleic acid extraction and detection of virulence and serogroup-associated genes followed by isolation of VTEC from positive samples. For the purpose of this study only *E. coli* O157 and *E. coli* O26 were targeted using serogroup-specific real-time PCR as they are currently the most relevant serotypes associated with human infection in Ireland (HPSC, 2012b). Once VTEC is detected in enrichments, the protocol describes the isolation of VTEC by plating onto Tryptone-Bile-Glucuronic (TBX) medium or a specific selective medium. The medium chosen for VTEC isolation in this study was CHROMagar™ STEC, a chromogenic medium intended for detection of all VTEC serotypes, and widely used in clinical laboratories. Recent studies evaluating this medium for VTEC isolation found that it was suitable for the selection of strains belonging to major enterohaemorrhagic *E. coli* (EHEC) groups (Tzschoppe et al., 2012) and reported good performance for the selection of isolates belonging to the major VTEC types (Hirvonen et al., 2012). The CEN/ISO TS 13136 protocol recommends incubation of enrichments and plates at 37°C and, although initial experiments were carried out in the laboratory at this incubation temperature, difficulties were encountered with colony isolation from spiked river water. Incubation of enrichments at 37°C resulted in a high microbial background on CHROMagar™ STEC plates. In environmental samples, consideration must be given to the high and diverse bacterial background flora present (Fratamico et al., 2011) and detection of pathogens in water is often difficult due to high levels of non-pathogenic bacteria (Muniesa et al., 2006). Jinneman et al. (2012), in an evaluation of a method for the detection of VTEC O104 strains from sprouts, found that the presence of background microflora made identification of typical colonies challenging on a range of media. In line with the findings of the current study, Drysdale et al. (2004) found that enrichment at

42°C minimises background flora and incubation of CHROMagar™ STEC plates at 42°C further reduces microbial background, making it easier to recognise and isolate presumptive VTEC. The authors of the current study also observed that a higher incubation temperature resulted in the earlier detection of the *eae* gene in real-time PCR assays.

Despite the reduction in microbial background, selection testing of presumptive VTEC (mauve colonies) for *vtx1* and *vtx2* revealed a high percentage of non-VTEC. In contrast, Hirvonen et al. (2012) reported that only 1.6% (3/186) of common *E. coli* strains of non-faecal origin grew on CHROMagar™ STEC. Several studies have confirmed extensive genotypic diversity in *E. coli* populations from environmental sources (Gordon and Cowling, 2003; Ishii et al., 2006; Walk et al., 2007; Lyautey et al., 2010). The current study's findings suggest that it can be challenging to identify non-O157 VTEC without the inclusion of IMS to select for particular serotypes (Fratamico et al., 2011). In the current study, by the incorporation of IMS, *E. coli* O26 was isolated from an unspiked river water sample (100% of isolates tested) in which the enrichment tested positive for O26 and *vtx1* by real-time PCR. The application of IMS also resulted in improved isolation of *E. coli* O157 and *E. coli* O26 from raw water during GWS sampling, although both *E. coli* O157 and *E. coli* O26 were isolated from enrichments without the use of IMS on a number of occasions. Isolation of VTEC from enrichment broths testing positive for virulence genes and antigenic determinants by real-time PCR is a challenge that can be addressed by the incubation of enrichment broths at 42°C to reduce non-*E. coli* background and application of IMS.

However, in screening for VTEC, the isolation of particular serotypes does not guarantee the isolation of *vtx*-positive strains. Verotoxin-negative *E. coli* O157 and *E. coli* O26 have been isolated from cattle, sheep, food and clinical samples (Nielsen and Scheutz, 2002; Pearce et al., 2004, 2006; Breum and Boel, 2010; Evans et al., 2011; Joris et al., 2011; Madic et al., 2011) along with their *vtx*-positive counterparts. Isolation of *vtx*-negative strains from *vtx*-positive samples may raise some questions about molecular diagnostic results but non-O157 VTEC strains form a

very diverse group whose pathogenic characteristics and clinical significance are not as yet well established (Jinneman et al., 2012). Environmental phages can carry *vtx* genes, which studies have shown can survive better than their bacterial hosts (Imamovic and Muniesa, 2011) and can infect commensal *E. coli*, converting non-pathogenic strains into verotoxin producers (Langsrud et al., 2013). Madic et al. (2011) detected VTEC in raw-milk cheeses by real-time PCR and speculated that *vtx*-negative isolates were derivatives of VTEC strains that had lost their *vtx* genes during enrichment or isolation and that genes detected by real-time PCR might be carried by other bacterial strains. The direct detection of virulence genes and serogroup-specific genetic determinants from water shows great promise for a more comprehensive characterisation of water to determine the prevalence of non-O157 VTEC (Fratamico et al., 2011).

The application of the CapE protocol to river water and GWS samples resulted in frequent and widespread detection of VTEC. VTEC was detected in water filtered in situ from river water in January and February 2013, corresponding with periods of high rainfall. It was not detected in corresponding small-volume samples (500 ml) collected and processed in parallel using standard methods, indicating that smaller-volume samples fail to detect VTEC when present in the water. Previous studies have shown that structures of *E. coli* populations in water are not stable, and are affected by hydrological conditions and contamination events of different origin and intensity (Ratajczak et al., 2010). In March, *E. coli* levels in the river water tested were similar to those in February but VTEC was not detected. Links between high rainfall and contamination of water with VTEC have been reported (EPA, 2012, 2013) and a previous EPA-funded project found that increased rainfall resulted in increased levels of *E. coli* in source waters (Cormican et al., 2012). The study reported that rainfall close to the time of sampling was closely correlated with levels of *E. coli* in surface waters and that groundwater sources showed greater correlation with rainfall over a longer time period, which is thought to reflect a more gradual transfer of rainwater through soil and rock into groundwater. Each GWS sampled in this study is supplied by groundwater sources but no link between

rainfall and occurrence of VTEC in raw water samples could be established.

Approximately 18% of the Irish population receives its water from private rural group water supplies or private wells that may be particularly vulnerable to contamination with VTEC (EPA, 2013) and there is evidence to suggest that people using private water supplies in Ireland have a higher risk of acquiring water-borne infections (HPSC, 2012b). The current study has confirmed the widespread contamination of groundwaters with VTEC through the application of the CapE method at six GWSs between August and October 2013. The absence of VTEC in the treated water indicates that treatment systems in place were effective at eliminating *E. coli*, including VTEC. The presence of VTEC was further confirmed by isolation of *vtx*-positive *E. coli* O26 and *E. coli* O157 from four out of six GWSs. Analysis of 1-l samples, collected in parallel for examination by the standard IMS methods (ISO 16654:2001), resulted in the isolation of a number of presumptive *E. coli* O26 and presumptive *E. coli* O157 but none were confirmed as *E. coli* O26 or *E. coli* O157 using molecular methods. Isolates of an unknown *vtx2*-positive serotype were detected on two occasions from one scheme. The standard protocol at the time of this study did not include screening of enrichments using real-time PCR. This was performed for the purpose of this study and resulted in detection of VTEC from four out of six schemes, indicating that the application of molecular methods improved VTEC detection in low-volume samples.

This report describes a rapid sensitive method for the detection of low-level VTEC contamination of waters, which may be used for VTEC monitoring, alone or in combination with monitoring for *Cryptosporidium*. The authors have clearly demonstrated that a high-volume approach, with rapid detection of gene targets (under 24 h), provides a more complete representation of the occurrence of VTEC in water than traditional methods.

The CapE protocol is a rapid and sensitive method for the detection of VTEC in water in 24 h. The method could be further developed for the detection of other water-borne pathogens of public health concern, e.g. *Salmonella*, *Listeria*, *Vibrio cholera* and norovirus. As the limit of detection of the CapE method is in line with

EU drinking water regulations, the method could also be further developed to replace current MPN culture-based methods, with the advantages that results can be obtained within 24 h and that the method has the ability to detect not only faecal indicator organisms but can concurrently detect pathogens of public health concern. This study also demonstrates the widespread contamination with VTEC of groundwaters in one region of Ireland and highlights the importance of assuring that appropriate source protection measures and water treatment facilities are in place at vulnerable water supplies. A comprehensive study to identify and understand further the factors influencing these findings is warranted to protect public health and the environment.

5   Conclusions

- This programme of work has achieved its objective of developing and validating a novel, rapid, efficient, sensitive and specific method for the detection of VTEC from large water volumes.

- Isolation of VTEC from enrichment broths testing positive for virulence genes and antigenic determinants by real-time PCR is a challenge that can be addressed by incubation of enrichment broths at 42°C to reduce the non-*E. coli* background and by application of IMS.

- This study clearly demonstrated that a high-volume approach with rapid detection of gene targets (under 24 h) provides a more complete representation of the occurrence of VTEC in water than traditional methods.

- This study also demonstrates the widespread contamination with VTEC of groundwaters in one region of Ireland and highlights the importance of assuring that appropriate source protection measures and water treatment facilities are in place at vulnerable water supplies.

6   Recommendations

The focus of this study was to develop and validate a novel, rapid, efficient, sensitive and specific method for the detection of VTEC in large-water volumes. The recommendations presented here are based on the research and conclusions outlined in this report.

- Consideration should be given by relevant stakeholders to the implementation of the CapE method as a supplement to or a replacement for standard methods for the detection of VTEC in water.

- Further research should be performed to determine the full extent of contamination of drinking and recreational waters with VTEC in order to develop appropriate risk assessment and risk management policies and guidelines, particularly in the context of *Food Harvest 2020*

and the extension of this to 2025, which is currently under development.

- Further research is required to make the CapE method more portable and thus more readily applicable in a remote setting.

- Further research is required to assess the CapE method of detection of VTEC in other settings, e.g. water used in sprouting seeds, and to assess commercial potential.

- Further studies should be performed to assess the applicability of the CapE method to the detection of other water-borne pathogens of concern to human and animal health, e.g. norovirus, antimicrobial agents, antimicrobial-resistant bacteria, etc.

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Acronyms and Annotations

ATCC   American Type Tissue Culture Collection

BPW   Buffered peptone water

CapE   Capture, Extract and Amplify for the detection of *Escherichia coli*

CE   Cell equivalents

CFU   Colony forming unit

Ct   Cycle threshold

DNA   Deoxyribonucleic acid

EHEC   Enterohaemorrhagic *Escherichia coli*

EPA   Environmental Protection Agency

EtOH   Ethanol

FSAI   Food Safety Authority of Ireland

GWS   Group water scheme

HPSC   Health Protection Surveillance Centre

HSE   Health Service Executive

HUS   Haemolytic uraemic syndrome

IMS   Immunomagnetic separation

MCWOS   MacConkey agar without Salt

MPN   Most Probable Number

mTSB   Modified tryptone soy broth

MUG   4-Methylumbelliferyl-β-D-glucuronide

NAAT   Nucleic acid amplification test

NCTC   National Collection of Type Cultures

PBS   Phosphate-buffered saline

PCR   Polymerase chain reaction

PWS   Public water supply

qPCR   Quantitative polymerase chain reaction

Rn   The fluorescent intensity of the probe–PCR product complex

SM-CT   Sorbitol MacConkey cefixime/tellurite agar

STEC   Shiga toxin-producing *Escherichia coli*

TBX	Tryptone-Bile-Glucuronic
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VBNC	Viable but non-culturable
VTEC	Verocytotoxigenic *E. coli*

EPA Research Report 151

CapE-Capture, Extract, Amplify:
A Rapid Method for Monitoring Large Water Volumes for Pathogenic Contaminants

Authors: Dearbháile Morris, Siobhán Kavanagh and Martin Cormican

Ireland consistently has the highest incidence of human infection with Verocytotoxigenic E. coli (VTEC) across Europe with water recognised as a major transmission route. In this report, a two year study was undertaken to 1) evaluate the effectiveness of filters used for the concentration of Cryptosporidium from large volumes (up to 1000L) of water for capture of E. coli; 2) examine modifications which may enhance the performance of these systems to optimise the capture of E. coli; 3) evaluate and optimise methods for extraction of nucleic acid from a) filter membranes and b) concentrate generated post filtration; 4) evaluate nucleic acid amplification tests (NAATs) for detection of verotoxigenic E. coli (VTEC) in extracted material, and 5) use the developed (CapE) method to monitor selected drinking water and recreational water sources and supplies for the presence of VTEC over a period of time.

Identifying Pressures

This report demonstrates the widespread contamination with VTEC of ground waters in one region of Ireland and highlights the importance of assuring appropriate source protection measures and water treatment facilities are in place at vulnerable water supplies. The report also identifies that research is urgently required to determine the full extent of contamination of drinking and recreational waters with VTEC in order to develop appropriate risk assessment and risk management policies and guidelines, particularly in the context of Food Harvest 2020.

Informing Policy

This research informs current policies on protection of public health from water borne VTEC and indicates the necessity for development of additional indicators or monitoring strategies. This research builds capacity in the area of Environment and Health The research is relevant in the context of the EU Water Framework Directive (e.g. large-scale monitoring of vulnerable environments) and to the 7th EU Environment Action Programme which aims "to safeguard the Union's citizens from environment-related pressures and risks to health and wellbeing"

Developing Solutions

The research has resulted in the development, validation and field evaluation of a rapid, convenient method for evaluation of large volumes of water for low level or intermittent contamination with VTEC. The study provides valuable evidence on the extent of contamination of ground water in at least one region of Ireland with VTEC, and the potential threat this represents should water treatment processes fail.