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Note

Impact of beef extract used for sample concentration on the detection of *Escherichia coli* DNA in water samples via qPCR

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A B S T R A C T

There is increasing interest in methodologies for the simultaneous concentration and detection of multiple targets in individual samples. The aim of this study was to investigate the potential presence of *E. coli* DNA in beef extract powder used as part of a procedure to concentrate water samples for the simultaneous detection of bacteria, viruses and protozoa. DNA from *E. coli* was detected in five out of six beef extract lots tested, demonstrating the limitations of its inclusion when being used in assays that will be used for the detection of *E. coli* in water samples. Further work is required to clarify if this phenomenon also occurs for other microorganisms of interest in water.

Water used in the production and preparation of vegetables can be sourced from public supply systems, groundwater, recovered rain water, surface water from lakes, rivers or artificial ponds, or even treated wastewater in some countries (Allende and Monaghan, 2015; Jongman and Korsten, 2018; Uyttendaele et al., 2015). The microbial quality of different water sources, especially surface and treated wastewaters is of crucial importance, given the potential for pathogenic microorganisms present in the water to persist and be transferred to plant material. Several fresh produce related microbial disease outbreaks have been attributed to the use of contaminated water at different steps of the farm-to-fork chain, as exemplified by an outbreak in the United States caused by the consumption of raspberries contaminated with *Cyclospora* present in the water used for pesticide application (Herwaldt et al., 1997), an outbreak in Finland caused by the consumption of frozen raspberries contaminated with Norovirus during irrigation or water spraying before cooling (Ponka et al., 1999), an outbreak attributed to the consumption of spinach leaves contaminated with *Escherichia coli* O157, in the United States, caused by the use of water contaminated by cattle (Parker et al., 2012; Sharapov et al., 2016), or an outbreak of *Salmonella* Saintpaul infections in the United States related with the consumption of jalapeno or serrano peppers contaminated with farm water (Barton Behravesh et al., 2011). Ensuring the microbiological quality of the water used in the production of fresh crops is therefore of vital importance for both consumers and food business operators.

Guidelines and regulations for the microbiological quality of water used for the production of crops focus on indicator microorganisms, typically referring to the presence and/or quantification of *E. coli* and faecal or total coliforms as criteria for microbial quality of the irrigation water (Uyttendaele et al., 2015). In Europe, the quality of water used for crop production is regulated by Regulation (EC) 852/2004 on the hygiene of foodstuffs, stating that food business operators producing or harvesting plant products should use potable water or clean water whenever necessary to prevent contamination (European Commission, 2004). The definition of potable water is outlined in EC Regulation 98/83/EC on the quality of water intended for human consumption, that states that water that is in contact with food should be of good microbiological quality (European Commission, 1998), with the criteria being established as the absence of *E. coli* and enterococci in 100 mL of water. The methods specified for the analysis of these two parameters are presented in ISO 9308-1 and ISO 7899-2, respectively. Both standards outline culture methods based on membrane filtration of the water samples, providing results in up to 48 h after sample processing. Other methodologies, such as the detection of target microorganisms via PCR, have been developed and implemented for several matrices, with the advantages of more rapid results and testing of multiple

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microorganisms from the same sample. A method has been developed to allow for the analysis of large volumes of water for the presence of different microorganisms (Gunnarsdottir et al., 2020). Briefly, a large volume of water undergoes two concentrations steps: primary concentration via dead-end ultrafiltration, and secondary concentration via precipitation of the eluate with PEG and beef extract followed by ultracentrifugation. After sample concentration, DNA and/or RNA are extracted, allowing for the detection of multiple pathogens via qPCR. The primary concentration step is based on particle size exclusion, using a hollow fibre dead-end ultrafiltration setup. This filtration technique has been used as a cost effective alternative for the recovery of different bacteria, virus and protozoa from large water samples (Cashdollar and Wymer, 2013; Hunter et al., 2011; Smith and Hill, 2009). Precipitation of microorganisms from the eluate obtained after the filtration with PEG and beef extract has been documented previously (Lambertini et al., 2008). PEG precipitation is based on the reduction of the solubility of proteins in solution, explained by the excluded volume mechanism (Ingham, 1984). This mechanism is particularly used for the precipitation of viruses from solution. The viral particles in the eluate bind to beef extract, aiding their precipitation (Hill et al., 2005; Hurst et al., 1984; Lewis and Metcalf, 1988; Schwab et al., 1995). The combination of these concentration steps allows for simultaneous detection of bacteria, viruses and protozoa from water samples, providing time and consumable savings (Gunnarsdottir et al., 2020).

A method validation using irrigation and wash water samples collected from different vegetable producers in a number of European countries indicated a high degree of discordance with culture based assays for *E. coli*. Analysis of internal process controls (i.e. negative controls using sterile distilled water) showed that the utilisation of beef extract during the secondary concentration procedure could result in the detection of *E. coli* DNA in water samples obtained from fresh crop production sites (unpublished data). In order to investigate this, and the associated potential of this technique to generate false positive test results, this study focused on the impact of the utilisation of beef extract during the secondary concentration on the detection of *E. coli* DNA. The secondary concentration step was carried out with mock samples of sterile distilled water (SDW), precipitated with PEG with or without beef extract, followed by the analysis of the precipitates for the presence of *E. coli* DNA.

Samples of beef extract were obtained from different producers in different countries. A total of six samples were collected from five different suppliers across the EU (samples 1 to 6). For each sample, 4 g of beef extract and 100 mL of 5× PEG 8000 (Sigma Aldrich, Ireland) and NaCl (Sigma Aldrich, Ireland) solution were added to 300 mL of sterile distilled water (SDW). The mixture was allowed to precipitate overnight at 4 °C. It was then centrifuged at 10,000*g* at 4 °C for 30 min. The supernatant was discarded, and the precipitate was washed and resuspended in Phosphate Buffered Saline (PBS - Oxoid – ThermoFisher Scientific, Ireland) with 0.001% Antifoam A (Sigma Aldrich, Ireland) and 0.01% Tween 80 (Sigma Aldrich, Ireland). DNA from 1 mL of each sample was extracted with a QIAGEN DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and preserved at −80 °C for later use. A control sample with no added beef extract was processed as described above.

The presence of *E. coli* DNA in each sample was analysed via qPCR, using four different *E. coli* targets. Detection of target sequences was performed using a Roche 480 LightCycler II (Roche, Penzberg, Germany). For the detection of the *uidA* gene, the primers uidAF (5′-CAACGAACTGAACTGGCAG-3′) and uidAR (5′-CATTACGCTGCGATGGAT-3′) were used (Abberton et al., 2016; Chern et al., 2009). The *rodA* gene was detected using the primers rodA984F (5′- GCAAACCACCTTTGGT-3′) and rodA984R (5′- CTGTGGGTGTGGATTGACAT-3′) (Abberton et al., 2016). The *tuf* gene was detected using the primers TEcol553F (5′- TGGGAAGCGAAAATCCTG-3′) and TEcol754R (5′-CAGTACAGGTAGACTTCTG-3′) (Abberton et al., 2016). qPCR reactions were performed using the Lightcycler 480 SYBR Green I Master Kit (Roche, Penzberg, Germany), following the qPCR kit manufacturer's instructions: one initial activation step of 5 min at 95 °C, followed by 45 cycles of 10 s of denaturation at 95 °C, 10s of annealing at 60 °C and 10 s of elongation at 72 °C. The GPS™ EscCol dtec-qPCR kit (GPS™, Alicante, Spain) was also used for detection of *E. coli* DNA in the tested samples. The primers and probe used in the assay were undisclosed. qPCR reactions were performed according to the manufacturer's instructions: one initial activation step of 15 min at 95 °C, followed by 40 cycles of a 15 s denaturation step at 95 °C and an extension step of 60 s at 60 °C. The results were recorded as amplification threshold crossing point values (Ct) of each qPCR reaction. For confirmatory purposes, the qPCR products of each reaction were run on agarose gels for DNA visualisation. The qPCR products obtained from the reactions targeting the *uidA* gene were sequenced at Eurofins Genomics (Ebersberg, Germany) using the primer uidAF. The obtained sequences were compared to the non-redundant database of sequences at the National Center for Biotechnology Information (NCBI), using BLASTN (basic local alignment search tool) algorithm (Zhang et al., 2000).

Table 1
Detection of *E. coli* DNA in sterile distilled water precipitated with PEG and beef extract from different suppliers. Results are presented as the number of amplification cycles (Ct) required for detection of target DNA. Confirmatory gel electrophoresis results are presented in brackets (positive/negative). The SYBR Green qPCR protocol was run with 45 repetitions of the amplification cycle, and the GPS™ qPCR kit protocol was run with 40 repetitions of the amplification cycle. Late amplification results (in the last five amplification cycles of each kit protocol) are presented as > 40.00 or > 35.00 due to the probability of non-specific target amplification, leading to a higher uncertainty in the result.

Sample	Supplier	SYBR Green qPCR Ct			GPS™ EscCol qPCR kit Ct
		uidA	*rodA*	*tuf*	
1	1	27.05 (+)	30.21 (+)	34.81 (+)	27.49 (+)
2	1	35.52 (+)	39.47 (+)	> 40.00 (+)	> 35.00 (+)
3	2	32.21 (+)	34.51 (+)	35.52 (+)	31.99 (+)
4	3	35.59 (+)	> 40.00 (−)	> 40.00 (+)	> 35.00 (+)
5	4	25.59 (+)	28.65 (+)	29.22 (+)	27.75 (+)
6	5	nd (−)	nd (−)	nd (−)	nd (−)

E. coli DNA was detected in five out of the six tested samples (Table 1). In samples BE1-BE5, the qPCR reactions were positive for the *uidA*, *rodA* and *tuf* genes, and the five samples also tested positive for *E. coli* DNA using the GPS™ EscCol dtec-qPCR kit. All qPCR positive results were confirmed by gel electrophoresis, with the exception of the *rodA* gene fragment amplified from sample BE4, whose qPCR result corresponded to a later amplification signal, suggesting a lower target DNA concentration (Table 1). Further confirmation of the presence of *E. coli* DNA in samples 1–5 was obtained by sequencing the DNA fragments obtained in the reaction targeting the *uidA* gene (Table 2). No *E. coli* DNA was detected in sample 6, in any of the tested qPCR reactions. Additionally, qPCR reactions for the control sample were negative for all tested targets.

Table 2
Phylogenetic affiliations of the gene fragments obtained from the qPCR reaction targeting the *uidA* gene of *E. coli*. DNA sequences were compared to those available from the non-redundant database of sequences at the National Center for Biotechnology Information (NCBI) as of the 26th of August of 2019, using BLASTN (basic local alignment search tool) algorithm.

Sample	Closest match (accession no.)	Similarity (%)
1	*E. coli* (CP042934)	98.78
2	*E. coli* (CP019213)	94.03
3	*E. coli* (CP042953)	97.94
4	*E. coli* (CP019213)	98.72
5	*E. coli* (CP019213)	98.67

Beef extract is generally prepared by dehydration of beef tissue infusions, obtaining a paste or dried powder. It is used as a nutritive ingredient in different microbiological culture media, as an undefined source of amino acids, nitrogen, vitamins and carbon (Cote, 2009; Vanderzant and Splittstroesser, 2001). Beef extract has also been extensively used in the elution and precipitation of viruses from filters and water samples (Lambertini et al., 2008; Hill et al., 2005; Hurst et al., 1984; Lewis and Metcalf, 1988; Schwab et al., 1995; Ikner et al., 2012; Rhodes et al., 2016; Staggemeier et al., 2015). Other products used for the same purpose, such as meat peptones, are usually obtained from animal protein sources such as muscle, bone or skin tissues, which are hydrolysed with enzymes isolated from animal sources, including pepsin or trypsin. In order to preserve nutrients lost during the protein hydrolysis treatments used in production of these products, beef extract is not submitted to harsh hydrolysis treatments (BBL™ Beef Extract Powder, Bacto™ Beef Extract, Desiccated, n.d). If animal gut tissues are used for the production of beef extract, *E. coli* cells present in the tissue may persist during the manufacturing process, leading to the presence of this strain or its DNA in the final product.

The presence of *E. coli* DNA in beef extract which is used in a method designed to concentrate water samples for detection of different microorganisms raises important limitations. Given the importance of *E. coli* as an indicator microorganism used to monitor water quality, the utilisation of the proposed method for the detection of this species may be of limited utility, due to the potential of the technique to generate false positive results. In a food and water testing context, false positive results can lead to economic and reputational damage to food business operators, due to possible rejection of products or product recalls. It is therefore imperative to ensure that the method of choice is capable of detecting the microorganisms of interest with high sensitivity, avoiding false positive results. Alternatives based on centrifugation and membrane filtration for the secondary concentration of water samples are available (Gunnarsdottir et al., 2020), but those are designed specifically for detection of bacteria. Nonetheless, the method evaluated in this study remains a useful alternative for simultaneous detection of other pathogenic bacteria, viruses and protozoa from water samples, and further research on a potential alternative for replacement of beef extract for viral precipitation would improve the applicability of the developed method.

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Author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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