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Characterization of the efficiency and uncertainty of skimmed milk flocculation for the simultaneous concentration and quantification of water-borne viruses, bacteria and protozoa

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ABSTRACT

In this study, the use of skimmed milk flocculation (SMF) to simultaneously concentrate viruses, bacteria and protozoa was evaluated. We selected strains of faecal indicator bacteria and pathogens, such as *Escherichia coli* and *Helicobacter pylori*. The viruses selected were adenovirus (HAdV 35), rotavirus (RoV SA-11), the bacteriophage MS2 and bovine viral diarrhoea virus (BVDV). The protozoa tested were *Acanthamoeba*, *Giardia* and *Cryptosporidium*. The mean recoveries with q(RT)PCR were 66% (HAdV 35), 24% (MS2), 28% (RoV SA-11), 15% (BVDV), 60% (*E. coli*), 30% (*H. pylori*) and 21% (*Acanthamoeba castellanii*). When testing the infectivity, the mean recoveries were 59% (HAdV 35), 12% (MS2), 26% (RoV SA-11) and 0.7% (BVDV). The protozoa *Giardia lamblia* and *Cryptosporidium parvum* were studied by immunofluorescence with recoveries of 18% and 13%, respectively. Although q(RT)PCR consistently showed higher quantification values (as expected), q(RT)PCR and the infectivity assays showed similar recoveries for HAdV 35 and RoV SA-11. Additionally, we investigated modelling the variability and uncertainty of the recovery with this method to extrapolate the quantification obtained by q(RT)PCR and estimate the real concentration. The 95% prediction intervals of the real concentration of the microorganisms inoculated were calculated using a general non-parametric bootstrap procedure adapted in our context to estimate the technical error of the measurements. SMF shows recoveries with a low variability that permits the use of a mathematical approximation to predict the concentration of the pathogen and indicator with acceptable low intervals. The values of uncertainty may be used for a quantitative microbial risk analysis or diagnostic purposes.

1. Introduction

Diseases related to water contamination constitute a major human health issue. Inadequate drinking water and poor sanitation are estimated to cause 842,000 diarrhoeal disease-related deaths per year (World Health Organization, 2014). They are related to a broad range of health problems and cause impacts on productivity due to water-borne diseases (Amini and Kraatz, 2014). Moreover, the creation of protocols to measure water quality, considering the diversity of pathogens that may be present, is one of the major problems that must be solved for improving the control of water quality and Quantitative Microbial Risk Assessment (QMRA) studies.

The following four main critical steps in the process of evaluating the microbiological quality of water need to be considered: (1) which pathogens may be present; (2) which microorganisms are used as indicators of contamination; (3) which method is used to concentrate the particular indicator or indicators; and (4) which technique is used to detect them.

Indicator organisms are used for a range of purposes as follows: indicators of faecal pollution and to evaluate the effectiveness of processes such as filtration or disinfection. The most popular indicator organisms are thermotolerant coliforms, *E. coli* and intestinal enterococci. However, the suitability of *E. coli* as an indicator has been questioned, because its survival in water and sensitivity to treatment and disinfection processes differ substantially from those of excreted viruses and protozoa.

Abbreviations: BVDV, Bovine viral diarrhoea virus; HAdV, Human adenovirus; IFA, Immunofluorescence assays; JCPyV, JC polyomavirus; NoV, Norovirus; PI, Prediction intervals; QMRA, Quantitative microbial risk assessment; q(RT)PCR, Quantitative (reverse transcriptase) PCR; RoV, Rotavirus; SMF, Skimmed milk flocculation; TCID50, 50% Tissue culture infective dose.

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E. coli is far more sensitive, and the consequence is a low correlation with the presence of other pathogens (Amini and Kraatz, 2014; Bofill-Mas et al., 2013; Gorchev and Ozolins, 2011).

Coliphages share many properties with human viruses and are used as models to assess the behaviour of excreted viruses in the water environment. In this regard, they are superior to faecal bacteria. However, there is no direct correlation between the numbers of coliphages and the numbers of excreted viruses (Gorchev and Ozolins, 2011). The use of excreted viruses as microbial indicators is based on the shortcomings of the existing choices. Human adenovirus (HAdV) has been proposed as a viral indicator of contamination (Gorchev and Ozolins, 2011; Pina et al., 1998) and has been used in various studies as a viral indicator of human faecal contamination and a microbial source tracking tool (Bofill-Mas et al., 2011, 2013; Rusiñol et al., 2014).

Most HAdVs are associated with respiratory disease, but types 40 and 41 are responsible for gastroenteritis outbreaks in children (Wold and Horwitz, 2013). Rotavirus (RoV) is also associated with gastroenteritis; RoV-A is the most common cause of severe vomiting and diarrhoea among children up to 30 months old (Estes and Greenberg, 2013). The coliphage MS2 is commonly used as a surrogate and process control in microbiological food and water analyses (van Duin and Olsthoorn, 2012). BVDV is an important cause of morbidity, mortality, and economic loss in dairy and beef cattle worldwide (MacLachlan and Dubovi, 2011).

E. coli is commonly found in the lower intestine of warm-blooded organisms. Most strains are harmless, but others can cause serious food poisoning and are responsible for product recalls due to food contamination (Madigan et al., 2014). *H. pylori* is an acid-tolerant bacterium usually found in the stomach and is related to gastric cancer (Johnson et al., 1997). *H. pylori* has been detected in wastewater (Moreno and Ferrús, 2012), surface water and other environmental samples all over the world (Eusebi et al., 2014) and has even demonstrated the capacity to survive in chlorinated water when the enumeration of coliforms indicates that the water is potable (Santiago et al., 2015). *G. lamblia* and *C. parvum* are responsible for outbreaks of gastroenteritis related to the consumption of contaminated water (Gascón, 2006). *Acanthamoeba* spp., free-living protozoa, are considered to be opportunistic pathogens (Marciano-Cabral and Cabral, 2003) and are known to have a role in the persistence of some bacterial pathogens, such as *Legionella*, in water environments (Lambrecht et al., 2015).

The direct examination of water is difficult due to low and fluctuating concentrations of microorganisms and because concentration procedures are usually organism and/or matrix-specific and most techniques have high or unknown variability parameters. One-step skimmed milk flocculation (SMF) has been proposed as an efficient low-cost method to concentrate viruses in all types of water samples. This method has been used in environmental water matrices such as river water (Calgua et al., 2013a), seawater (Calgua et al., 2008), ground water (Bofill-Mas et al., 2011) and wastewater (Calgua et al., 2013b). However, the efficacy of the recovery in controlled conditions has not been properly described until now.

Quantitative Microbial Risk Analysis (QMRA) is a scientific tool used to assess the microbial safety of water and is needed for developing a strategy of risk management models. QMRA models each variable using a probability distribution. The advantage is that the result is represented by a probability distribution function instead of a single value. The objective of QMRA is the ability to calculate the combined impact of the uncertainty in the model's parameters to determine an uncertainty distribution of the possible model outcomes (Vose, 2008).

The aim of the present study was to determine the efficacy of the SMF recovery to simultaneously concentrate viruses, bacteria and protozoa and then compare q(RT)PCR and infectivity assays to detect and quantify the number of viruses recovered. Finally, an extrapolation method was evaluated with the q(RT)PCR quantification using the prediction interval (PI) based on the known recoveries to correctly achieve the actual concentration of the spiked microorganisms and define the uncertainty values of the method.

2. Materials and methods

2.1. Microorganism stocks and cell lines

The following viruses were analysed and spiked into the water samples: HAdV-35 (ATCC, LGC Standards AB, Borås, Sweden) cultured in cell line A549 (ATCC CCL-185), MS2 (ATCC 23631) cultured in *Salmonella typhimurium* strain WG49 (NCTC 12484), RoV SA-11 (ATCC VR-1565) cultured in MA104 (ATCC CRL-2378) and Bovine viral diarrhoea virus (BVDV) strain NADL kindly donated by the EU and OIE Reference Laboratory for Classical Swine Fever, Institute of Virology, University of Veterinary Medicine, Hannover, Germany, and cultured in NDBK (ATCC CCL-22). The analysed bacteria were *E. coli* (ATCC 23725) and *H. pylori* (NCTC11637). The protozoa tested in the study were *A. castellanii* (CCAP 1534/2), *G. lamblia* H3 isolate (Waterborne Inc., New Orleans, LA) and a *C. parvum* Iowa isolate (Waterborne Inc., New Orleans, LA).

2.2. Water samples

This experiment was conducted with tap water from the metropolitan area of Barcelona; the volume of water evaluated in each bucket was 10 L. The number of buckets inoculated with each of the microorganisms and their respective inoculated concentration are specified in Table 1. The tap water was previously treated with 100 mL of sodium thiosulfate (10% (*w*/*v*)) to eliminate chloride residues. Four additional buckets with the same volume of water were analysed as negative control samples.

2.3. Skimmed milk flocculation concentration

The skimmed milk flocculation concentration protocol has been described in previous studies (Bofill-Mas et al., 2011; Calgua et al., 2008). In summary, a pre-flocculated skimmed milk solution (1% (*w*/*v*)) was prepared by dissolving 10 g of skimmed milk powder (Difco-France) in 1 L of artificial seawater and carefully adjusting the pH to 3.5 with 1 N HCl. One hundred millilitres of this solution was added to each of the previously acidified (pH 3.5) 10 L water samples (the final concentration of skimmed milk was 0.01% (*w*/*v*)). The conductivity was also measured and adjusted with artificial sea salt (Sigma, Aldrich Chemie GMBH, Steinheim, Germany) to achieve a minimum conductivity of 1.5 mS/cm^2. The samples were stirred for 8 h at room temperature, and the flocs were allowed to settle by gravity for another 8 h. The supernatants were removed, and the sediment was collected and transferred to a 500 mL centrifuge container and centrifuged at 8000 × *g* for 30 min at 4 °C. The obtained pellet was resuspended in 8 mL of

Table 1
The number of microorganisms inoculated in each of the ten litre water buckets used for the skimmed milk flocculation concentration process.

Microorganisms (number of samples)	Molecular quantification		Quantification by infectious assays	
HAdV (10)	2.88E + 07	GC	4.60E + 06	IFA
MS2 (13)	2.92E + 09	GC	2.07E + 09	PFU
	2.92E + 07	GC	5.03E + 06	
RoV (19)	6.31E + 08	GC		
	2.09E + 07	GC	4.08E + 05	TCID50
BVDV (3)	2.10E + 08	GC	6.31E + 05	TCID50
E. coli (10)	2.37E + 06	GC		
H. pylori (9)	1.97E + 08	GC		
A. castellanii (9)	7.27E + 04	GC		
C. parvum (8)	1.46E + 04	IFA		
G. lamblia (8)	1.56E + 04	IFA		

GC: genomic copies; IFA: immunofluorescence assay; PFU: plaque-forming units; TCID50: 50% tissue culture infective dose.