Specific interactions of rotavirus HAL1166 with *Enterobacter cloacae* SENG-6 and their contribution on rotavirus HAL1166 removal

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ABSTRACT

Contribution of specific interactions between human enteric viruses and wastewater suspended solids on human enteric virus removal by microfiltration was studied. A cross-flow microfiltration system was used with rotavirus HAL1166 and *Enterobacter cloacae* SENG-6 as the model virus and wastewater suspended solid. Cleavage of rotavirus HAL1166 protein VP4 by trypsin produces the VP8* subunit, which specifically interacts with histo-blood group antigen (HBGA). In the presence of *Enterobacter cloacae* SENG-6, the trypsin-treated rotavirus concentration reduced with time ($R^2 > 0.6$) compared to the reduction of non-trypsin treated rotavirus. Calculation of the gel/cake layer deposited on the membrane, consisting of *Enterobacter cloacae* SENG-6 and either trypsin-treated or non-trypsin treated rotavirus HAL1166, revealed that the microflocs consisting of trypsin-treated rotavirus and *Enterobacter cloacae* SENG-6 have lower porosity and permeability, displaying higher resistance to virus passage through the membrane. The results provide evidence that specific wastewater suspended solids–human enteric virus interaction can contribute to increasing the removal of human enteric viruses by microfiltration.

Key words | enteric viruses, HBGA-positive bacteria, specific interactions, wastewater solids

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INTRODUCTION

Adsorption to wastewater solids plays a major role in the enteric virus life cycle in aquatic environments. Previous studies have shown that non-specific interactions between enteric viruses and wastewater solids can contribute to either enteric virus removal or protection from inactivation stresses. Norovirus GII and sapovirus have shown higher log removals in the membrane bioreactor (MBR) process compared to enterovirus, and this is attributed to the lesser association of enterovirus with mixed liquor suspended solids (Miura *et al.* 2015). Microorganisms associated with wastewater solids are more resistant to free chlorine treatment than non-associated microorganisms (Winward *et al.* 2008). Compared to free or secondarily adsorbed poliovirus, a fourfold increase of combined chlorine was necessary to achieve the same degree of inactivation in faecal particle-associated or occluded poliovirus (Hejkal *et al.* 1979).

Adsorption of human enteric viruses to wastewater solids by non-specific interactions are explained using Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory (Gerba 1984) and attachment of viral particles to solids such as settleable, suspended, colloidal particles and MLSS has been already studied (Sano *et al.* 2004; Da Silva *et al.* 2008; Imai *et al.* 2011; Miura *et al.* 2015). Da Silva *et al.* (2011) reported that the presence of Ca^{2+} and Mg^{2+} dramatically improves the attachment of norovirus GI.1 and GII.4 into silica, because the addition of cationic salts decreases the layer thickness as less volume is needed to contain enough counter-ions, and facilitates the approach of the two surfaces allowing van der Waals forces to have an effect (Gerba 1984; Da Silva *et al.* 2011).

On the other hand, studies regarding the specific interaction between human enteric viruses and wastewater solids are limited. Enteric virus binding proteins from the

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bacterial culture derived from activated sludge have been discovered (Sano *et al.* 2004; Sano & Omura 2005; Imai *et al.* 2011). Miura *et al.* (2013) reported on specific interactions between human enteric viruses and enteric bacteria; *Enterobacter cloacae* SENG-6 isolated from a faecal sample of a healthy individual interacts with human norovirus-like particles (NoVLPs) through extracellular polymeric substances (EPS) where histo-blood group antigen (HBGA)-like substances were localized (Miura *et al.* 2013). A consequent study showed that HBGA-like substances excreted in the EPS of *E. cloacae* SENG-6 displayed the strain-dependent recognition and removal of human NoVLPs (Amarasiri *et al.* 2016). The presence of HBGA-expressing *Escherichia coli* protected NoVLPs from heat inactivation, while HBGA-positive *E. coli*-bound Tulane virus was inactivated by heat (Li *et al.* 2015; Li *et al.* 2017). A virus-HBGA positive bacteria system therefore provides an excellent platform to study the contribution of specific interactions on human enteric virus removal and survival.

Conclusions on this topic are drawn based on the experiments performed at microlitre scale using HBGA-positive bacteria and human NoVLPs or other surrogate viruses (Amarasiri *et al.* 2016). Since protocols of cultivating human noroviruses under laboratory conditions involve large costs, time and labour (Ettayebi *et al.* 2016), scaling up using human norovirus is not feasible and a suitable surrogate is necessary to replace human norovirus that can be cultivated at a larger scale and specifically interacts with wastewater solids. In this study, a human rotavirus strain (HAL1166) is used as the surrogate for human norovirus because the VP8* protein of rotavirus HAL1166 specifically interacts with A-type histo-blood group antigens (Böhm *et al.* 2015). In addition, rotavirus HAL1166 strain can be cultivated in large quantities in-vitro using host cells (MA104).

Removal of rotavirus HAL1166 in the presence of HBGA-positive *E. cloacae* SENG-6 is analysed using a bench scale cross-flow membrane system. Human rotavirus HAL1166 removal with time was evaluated and gel/cake layer formation on the membrane surface by deposition of microflocs generated by interactions between *E. cloacae* SENG-6 and rotavirus HAL1166 is observed for both trypsin-treated and non-trypsin treated conditions. Porosity, permeability and the thickness of the gel/cake layer formed on the membrane surface is calculated. Changes in the gel/cake layer properties based on specific or non-specific interactions of HAL1166 with *E. cloacae* SENG-6 and the possible consequences in virus reduction are also discussed.

MATERIAL AND METHODS

Enterobacter cloacae SENG-6 is used as the HBGA-positive bacterial strain (Miura *et al.* 2013). Rotavirus HAL1166 (G8P [14]) strain which recognizes A-type HBGA is used as the model virus in the study.

Specific interactions between *E. cloacae* SENG-6 and rotavirus HAL1166 were confirmed using enzyme-linked immunosorbent assay (ELISA). Mouse monoclonal antibody to rotavirus (Group A) (0.1 mg/ml, AMO1341PU-N, Acris Antibodies Inc., USA) and goat anti-mouse IgG H&L HRP-conjugated antibody (ab6789, abcam, Tokyo, Japan) were used and the ELISA was performed according to Amarasiri *et al.* (2016).

A laboratory scale cross-flow membrane system is developed as depicted in Figure 1. The cross-flow membrane device had a surface area of 2,000 mm^2 and equipped with a 0.2 μm nominal pore size PTFE membrane (Advantec, Japan). *E. cloacae* SENG-6 was cultivated overnight in LB–medium and the OD_{600} value was adjusted to 1 ($\approx 5 \times 10^8$ cells/ml). Rotavirus HAL1166 was treated with 1.0 mg/ml trypsin from porcine pancreas (Type IX-S, Sigma Aldrich, USA) for 30 mins at 37 °C or used without trypsin treatment. One hundred and eighty millilitres of *E. cloacae* SENG-6 were mixed with 1.8 ml of either trypsin treated or non-trypsin treated rotavirus HAL1166. The suspension is then mixed for 50 mins at 4 °C using a magnetic stirrer. The container including the mixture is then connected to the system. The permeate pump had an initial flow rate of 0.8 ml/min. Paired samples were collected from permeate and the reactor at different time intervals. Reactor bacteria concentrations were quantified at 600 nm using a spectrophotometer at each sampling event (Bio-Rad Laboratories, USA).

RNA extraction was performed using the spin protocol of the QIAamp Viral RNA mini kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions. After the extraction of RNA, samples were heated at 95 °C for 5 mins followed by cooling on ice to relax the secondary structures. Extracted RNA was then stored at −80 °C until further use. Synthesis of cDNA from extracted RNA was performed using PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa Bio. Inc., Japan) as per the instruction manual. Prepared cDNA was stored at −20 °C until further processing (Amarasiri *et al.* 2018).

Quantitative polymerase chain reaction (qPCR) was performed using primers NVP3-F (ACCATCTACACATGACCCTC), NVP3-R (GGTCACATAACGCCCC) and Taqman probe (5′-/56-FAM/ATGAGC ACA/ZEN/ATAGTTAAAAG CTAACACTGTCAA/3IABkFQ/-3′) as recommended by

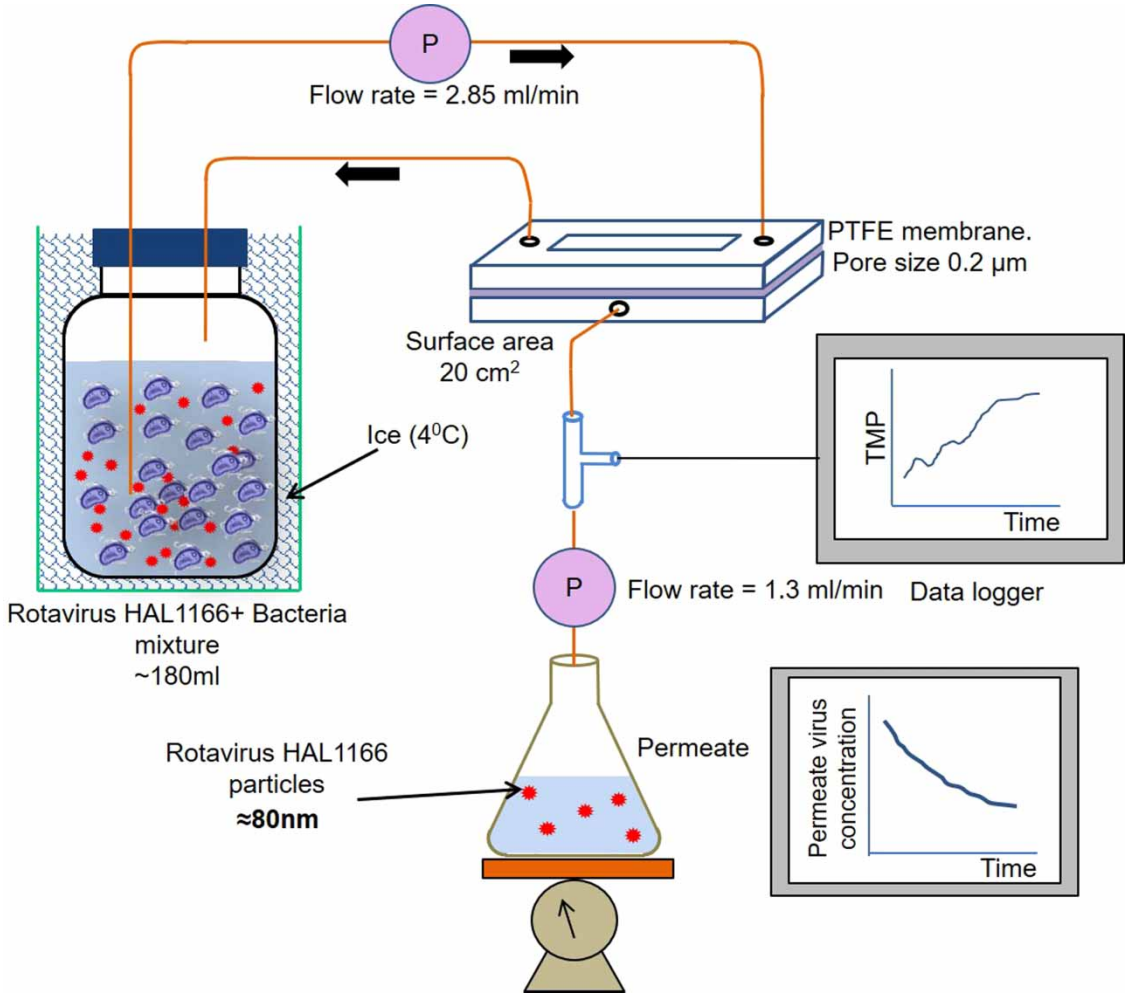

Figure 1 | Cross-flow membrane filtration setup used in the current study.

Pang *et al.* (2004). In a 96-well PCR plate, 25 µl of the reaction mixture was prepared as mentioned elsewhere (Amarasiri *et al.* 2018). All the reactions were performed in duplicate in a 7500-Fast Real Time PCR system (Applied Biosystems Inc., CA, USA). Reaction conditions were set as follows: initial denaturation at 95 °C for 5 mins followed by 45 cycles of amplification with denaturation at 94 °C for 20 seconds and annealing and extension at 60 °C for 1 min and final extension at 72 °C for 5 mins. Inhibition of the qPCR reaction was confirmed by the quantification of HAL1166 copy numbers in the 10-times dilutions of randomly selected samples.

To calculate thickness (δ_c), porosity (ε) and permeability (κ) of the gel/cake layer formed on the microfiltration membrane, specific cake resistance was calculated from Darcy's law (Equation (1)) which relates the permeate flux (J) to the total resistance to permeate flow. Here, ΔP is the transmembrane pressure (TMP), μ is the liquid viscosity, R_m is the membrane resistance and R_c is the cake resistance (Faibish *et al.* 1998). TMP data obtained in each trial were modelled with the combined cake filtration-intermediate blocking model as shown in Equation (2) (Bolton *et al.* 2006) where P and P_0 are the TMPs at time t and 0 respectively; K_i (/m) and K_c (s/m^2) are fitted parameters using Microsoft Excel solver package.

$$J = \frac{\Delta P}{\mu(R_m + R_c)} \tag{1}$$

$$\frac{P}{P_0} = \exp(K_i J_0 t)\left(1 + \frac{K_c J_0}{K_i}(\exp(K_i J_0 t) - 1)\right) \tag{2}$$

Cake resistance can alternatively be written as $R_c = \alpha M_d$ where M_d represents the accumulated mass per unit area on the membrane surface. The Carmen-Koezny equation can be

used to relate the specific cake resistance to the cake porosity (ε) (Equation (3)) (Faibish *et al.* 1998). In the Carmen-Koezny equation, ρ is the particle density and a_p is the particle radius. Thickness of the cake layer δ_c can be calculated from the mass balance on the cake layer using the following equation with M_c; the total number of particles deposited per unit area of the cake layer and previously calculated porosity value (Equation (4)) (Faibish *et al.* 1998). Average permeate flux was calculated using the permeate volume data recorded. For the calculation of the porosity and the boundary layer thickness at each condition, ρ is taken as 1,105 kg/m^3 (Martinez-Salas *et al.* 1981); mass of a bacterial cell is considered as 1×10^{-12} g, radius of *E. cloacae* SENG-6 is taken as 0.4 μm, viscosity of the LB medium is taken as 0.0014 kg/ms and modified using the Einstein equation where $(\mu_{eff}/\mu_0) = 1 + 2.5 \times$ volume fraction.

$$\alpha = \frac{45(1-\varepsilon)}{\rho a_p^2 \varepsilon^3} \tag{3}$$

$$\delta_c = \left(\frac{\frac{4}{3}\pi a_p^3}{1-\varepsilon}\right) M_c \tag{4}$$

$$\kappa = \frac{d^2}{18}\left(\frac{3 - 4.5\gamma + 4.5\gamma^5 - 3\gamma^6}{\gamma^3(3 + 2\gamma^5)}\right) \tag{5}$$

μ_{eff} is the effective viscosity calculated from the volume fraction of bacterial cells in the suspension. The volume fraction of rotavirus HAL1166 was neglected, since it is at least 10^9 orders smaller compared to the bacterial volume fraction. Pristine membrane resistance is measured by dead-end filtration and R_m was calculated as 1.82×10^{10}/m. Considering the time as 200 mins and substitution of these data in Equations (1)–(4) yielded the porosities and the thicknesses of the cake layers in three different conditions. Permeability (κ) of the membrane gel layers was calculated using the Happel model considering the fractal geometry using Equation (5) (Li & Logan 2001).

RESULTS AND DISCUSSION

Specific interaction between *enterobacter cloacae* SENG-6 and human rotavirus HAL1166

ELISA results showed a significant difference ($p < 0.001$, t-test) in the signal/noise (S/N) ratio for the interaction between *E. cloacae* SENG-6 and trypsin-treated rotavirus HAL1166 compared with non-trypsin treated rotavirus HAL1166 (Figure 2). This difference is attributed to the specific interaction between VP8* protein and *E. cloacae* SENG-6 because trypsin-treatment of rotavirus HAL 1166 cleaves the VP4 protein and produces VP8*, which specifically interacts with A-type HBGA-like substances of *E. cloacae* SENG-6.

Variation of rotavirus HAL1166 concentration in the permeate

In the presence of *E. cloacae* SENG-6, rotavirus HAL1166 concentration in the permeate reduced with time (Figure 3). Rotavirus HAL1166 removal performance of the cross-flow membrane system at a given time (t) was evaluated using Rejection $(R) = (1 - (C_p/C_f))$ where C_p and C_b correspond to the rotavirus HAL1166 concentration in permeate and the feed at any given time, respectively (Figure 4). Percentage reduction of trypsin treated rotavirus HAL 1166 displayed an increasing trend with time, while the percentage reduction of non-trypsin treated rotavirus HAL 1166 displayed abrupt variations with time. The rejection of trypsin-treated rotavirus HAL 1166 in the presence of *E. cloacae* SENG-6 varied between 10–82%, whereas the non-trypsin treated rotavirus HAL 1166 rejection varied between 3–69%. Calculation of deposited *E. cloacae*

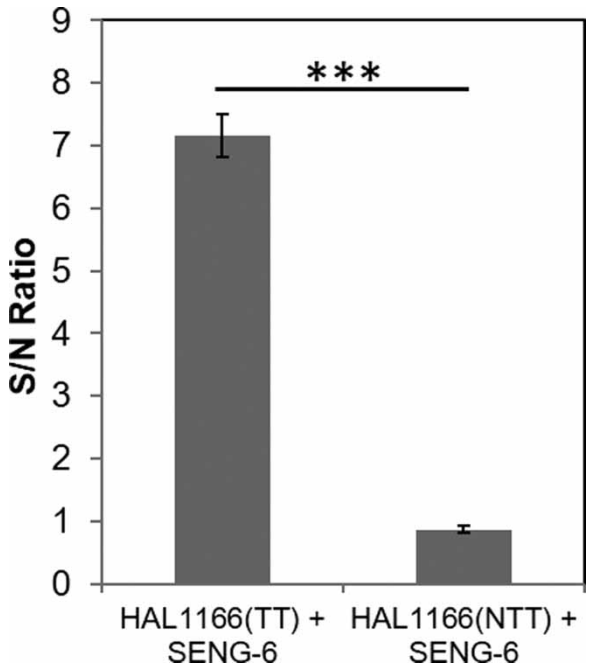

Figure 2 | Specificity of interaction between *Enterobacter cloacae* SENG-6 and rotavirus HAL1166. *** - ($p < 0.001$, t-test).

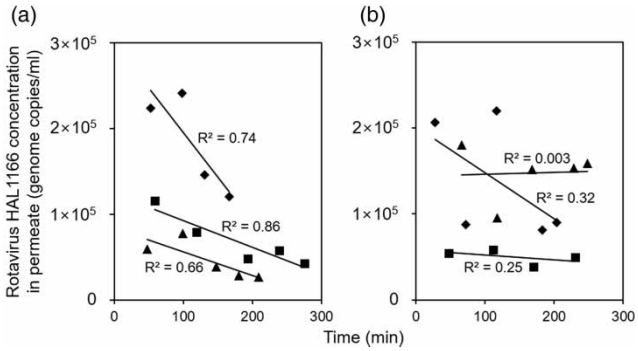

Figure 3 | Rotavirus HAL1166 concentration in permeate in the presence of *E. cloacae* SENG-6. (a) Trypsin-treated rotavirus HAL1166; (b) non-trypsin treated rotavirus HAL1166. Different trials are shown in different symbols.

quantity on the membrane surface with time showed similar deposition rates in the presence of both trypsin-treated and non-trypsin treated rotavirus HAL 1166.

Similar percentage reductions with trypsin-treated rotavirus HAL 1166 were observed for the non-trypsin treated rotavirus HAL1166 at several sampling points (Figure 4(b)). This may be due to non-specific interactions between *E. cloacae* SENG-6 and rotavirus HAL1166 and the consequent deposition of viruses on the gel/cake layer (Zhao *et al.* 2014). According to a previous study, microfiltration of a mixture of gold particles and latex beads resulted in the attachment of gold particles to the beads and retention on the membrane side (Madaeni 2001). Therefore, even without specific interactions, the presence of larger particles in the suspension can contribute to the virus rejection by attachment to the particle surfaces (Madaeni *et al.* 1995). Non-specific interactions are based on surface properties like zeta potential and hydrophobicity (Busscher & Weerkamp 1987). The possibility for non-specific interactions with higher affinity than the specific interactions to occur may explain the observation of higher reductions of non-trypsin treated rotavirus HAL1166.

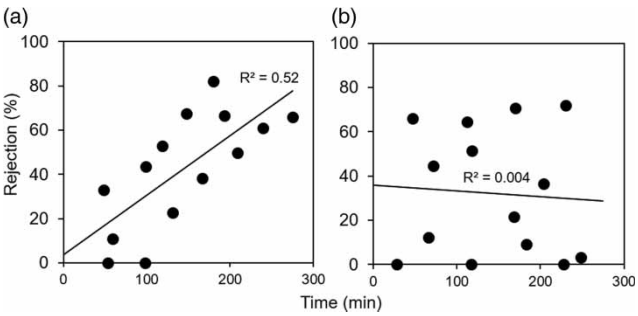

Figure 4 | Rejection of (a) trypsin-treated rotavirus HAL1166 and (b) non-trypsin treated rotavirus HAL1166 in the presence of *E. cloacae* SENG-6.

Gel layer formation and their contribution to the flux reduction and virus rejection

During the cross-flow filtration, permeate flux reduces with time due to many reasons including concentration polarization and gel/cake layer formation. Aggregates formed between *E. cloacae* SENG-6 (>0.2 μm) and rotavirus HAL1166 (~80 nm) can accumulate on the membrane surface. By analysing the properties of different gel/cake layers it is possible to understand how these properties contributed to the membrane flux reduction and ultimately virus rejection (Table 1).

The gel/cake layer of *E. cloacae* SENG-6 and non-trypsin treated rotavirus HAL1166 is 1.45 times thicker than the gel/cake layer developed when trypsin-treated rotavirus HAL1166 is present. However, the bacterial cell mass deposited over a unit area is similar in both conditions, making the gel/cake layer with trypsin-treated HAL1166 more compact. The lower porosity and permeability values further confirm the compact nature of the gel/cake layer formed by *E. cloacae* SENG-6 and trypsin-treated rotavirus HAL1166. The gel/cake layer made with non-trypsin treated rotavirus HAL1166 and *E. cloacae* SENG-6 provides lesser resistance to the flow of solutes and in turn can let the unattached rotavirus HAL1166 particles pass through the gel/cake layer and membrane with less resistance. In the case of trypsin-treated rotavirus HAL1166, there is a possibility that unbound virus particles pass through the membrane with less obstruction due to the lower thickness of the gel/cake layer.

Implications and limitations

Several studies have reported the presence of human enteric viruses in wastewater effluents, and it is a necessity to address this concern in order to provide microbiologically-safe reclaimed wastewater for beneficial purposes (Myrmel *et al.* 2006; Da Silva *et al.* 2007). Higher log reduction of human enteric viruses during the biological treatment can positively affect the subsequent treatment processes, particularly disinfection. Chemicals used in the disinfection process generate disinfection by products (DBPs) (Hua & Reckhow 2007) associated with adverse health effects (Villanueva *et al.* 2004). The improved pathogen removal performance in biological treatment unit processes can reduce the usage of disinfection chemicals, as the credit value assigned on disinfection can be lowered (Sano *et al.* 2016). Usage of a lower quantity of disinfectants will consequently lead to a lower quantity of generated DBPs.

Table 1 | Properties of gel/cake layers developed under different conditions

Gel/cake layer	Bacteria mass (g/m^2)	Porosity (ε)	Gel layer thickness/(μm)	Permeability (κ)/mD
Enterobacter cloacae SENG – 6 only	33.28 ± 6.05	0.66 ± 0.06	27.42 ± 9.70	9.12 ± 5.45
Enterobacter cloacae SENG – 6 + Trypsin-treated rotavirus HAL1166	28.13 ± 4.05	0.63 ± 0.03	20.24 ± 2.43	6.15 ± 1.40
Enterobacter cloacae SENG – 6 + Non-trypsin treated rotavirus HAL1166	29.64 ± 2.16	0.72 ± 0.07	29.40 ± 6.22	16.80 ± 11.98

The calculation of the gel/cake layer thickness and the related parameters was performed based on a model developed for inert molecules (Faibish *et al.* 1998). Even though it is rare to find models describing the interactions between biological colloids that include the specific interactions (Berg & Purcell 1977), current models can still be utilized because the attachment of biological molecules on surfaces is shown to follow the DLVO theory (Gerba 1984).

In this study, quantification of rotavirus HAL1166 was done using genome copy numbers. A further study on evaluating infectious virus particle numbers will provide very important insights on rotavirus HAL1166 removal and deposition on the membrane gel/cake layer. The design of the current study was relatively ideal because the major objective was to isolate the fraction of specific interactions and eliminate other wastewater solids particles that may contribute to non-specific interactions with rotavirus HAL1166. Usage of wastewater consisting of many types of solids that can specifically or non-specifically interact with human enteric viruses may provide further information on the importance of virus-solids interaction on virus removal.

HBGA-positive bacteria are ubiquitously present in wastewater (Amarasiri *et al.*, submitted) and therefore the prevalence of specific wastewater solids–human enteric virus interactions can be expected in wastewater. Concentration of different genotypes of human noroviruses varies in the same wastewater sample (Amarasiri *et al.* 2018). It has also been shown that different genotypes of human norovirus are removed up to different degrees by microfiltration in the presence of *Enterobacter cloacae* SENG-6 (Amarasiri *et al.* 2016). A further analysis of the environmental factors that can be manipulated in order to achieve improved specific interactions can contribute to increased human enteric virus removal.

CONCLUSIONS

The current study analysed the importance of specific interactions between wastewater solids–human enteric viruses on virus removal using a cross-flow microfiltration system. In the presence of HBGA-positive *E. cloacae* SENG-6, the concentration of trypsin-treated rotavirus HAL1166 in the permeate displayed a decreasing trend with time, while the non-trypsin treated rotavirus HAL1166 concentration in the permeate changed abruptly. The properties of the gel/cake layer developed on the membrane surface were different depending on the trypsin treatment of rotavirus HAL1166. Trypsin-treated rotavirus HAL1166 contributed to the development of a gel/cake layer with lower porosity, permeability and thickness and ultimately provided increased reductions of rotavirus HAL1166. The results reinforce the idea that specific wastewater solids–human enteric virus interactions can contribute to increased removal of human enteric viruses by microfiltration.

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