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ORIGINAL PAPER

Comparative Virucidal Efficacy of Seven Disinfectants Against Murine Norovirus and Feline Calicivirus, Surrogates of Human Norovirus

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Abstract Human noroviruses (HuNoV) are the leading cause of acute non-bacterial gastroenteritis in humans and can be transmitted either by person-to-person contact or by consumption of contaminated food. A knowledge of an efficient disinfection for both hands and food-contact surfaces is helpful for the food sector and provides precious information for public health. The aim of this study was to evaluate the effect of seven disinfectants belonging to different groups of biocides (alcohol, halogen, oxidizing agents, quaternary ammonium compounds, aldehyde and biguanide) on infectious viral titre and on genomic copy number. Due to the absence of a cell culture system for HuNoV, two HuNoV surrogates, such as murine norovirus and feline calicivirus, were used and the tests were performed in suspension, on gloves and on stainless steel discs. When, as criteria of efficacy, a log reduction >3 of the infectious viral titre on both surrogates and in the three tests is used, the most efficacious disinfectants in this study appear to be biocidal products B, C and D, representing the halogens, the oxidizing agents group and a mix of QAC, alcohol and aldehyde, respectively. In addition, these three disinfectants also elicited a significant effect on genomic copy number for both surrogate viruses and in all three tests. The results of this study demonstrate that a halogen compound, oxidizing agents and a mix of QAC, alcohol and aldehyde are advisable for HuNoV disinfection of either potentially contaminated surfaces or materials in contact with foodstuffs.

Keywords Norovirus · Murine norovirus · Feline calicivirus · Foodborne viruses · Disinfection · Food-contact surfaces

Introduction

Human noroviruses (HuNoV), small icosahedral non-enveloped enteric viruses, are members of the family *Caliciviridae* and are divided into six genogroups (Green 2013). They are considered to be one of the most common causes of acute non-bacterial gastroenteritis worldwide in humans and are detected in approximately 50 % of acute gastroenteritis outbreaks across Europe and the USA (De Wit et al. 2001; Green 2007; Patel et al. 2008; Scallan et al. 2011; Hall et al. 2013; Ramani et al. 2014). They are also considered to be the leading global cause of foodborne outbreaks, the main involved products being mixed food, seafood, buffet meals and commodities such as fruits and vegetables (EFSA and ECDC 2011, 2012, 2013; Hannah Gould et al. 2013; Hall et al. 2014).

The primary transmission route is the faecal–oral one, either via person-to-person contact or by consumption of contaminated food or water (Lopman et al. 2003; Siebenga et al. 2007; Kroneman et al. 2008; Scallan et al. 2011; Mathijs et al. 2012). Noroviruses are very resistant and can persist for several days in the environment (D'Souza et al. 2006). Due to this high persistence on various inanimate surfaces and on food, coupled with the prolonged shedding of high amounts of virus in faeces and the low infectious

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dose, person-to-person transmission is very efficient and noroviruses constitute a serious public health issue (Mathijs et al. 2012). Alternatively, food can be contaminated with HuNoV either at the source in the growing or harvesting areas by contaminated irrigation water or during handling or preparation of meals (Tuan Zainazor et al. 2010; Mathijs et al. 2012). Foodhandlers have been involved in almost half of all reported outbreaks, and this proportion is probably underestimated (Koopmans and Duizer 2004; Baert et al. 2009a; Tuan Zainazor et al. 2010; Rodriguez-Lazaro et al. 2012; Bellou et al. 2013). HuNoV are commonly identified in closed or semi-closed communities such as hospitals, nursing homes, cruise ships, military and holiday camps, restaurants and catered functions (Glass et al. 2009; Tuan Zainazor et al. 2010; EFSA and ECDC 2012).

The infective dose is not clearly determined, but studies have suggested that about 10 to 100 virus particles are sufficient to induce infection (De Wit et al. 2001; Seymour and Appleton 2001; Bresee et al. 2002; Green 2007; FAO/WHO 2008; Doré et al. 2010). More recently, the 50 % human infectious dose was estimated to lie between 18 and 1000 viral particles or between 1320 and 2800 genome equivalents for the HuNoV prototype Norwalk strain (Teunis et al. 2008; Glass et al. 2009; Atmar et al. 2014). This infectious dose could vary depending on both the involved strain and the experimental methodology.

No robust cell culture system is available for HuNoV although norovirus RNA is infectious in mammalian cells and a recent study showed the development of an in vitro infection model for human noroviruses in human B cells (Duizer et al. 2004b; Malik et al. 2005; Guix et al. 2007; Jones et al. 2014). Due to the complexity and the specific requirements for this new in vitro cell culture system for HuNoV, the use of viral surrogates is still required. The murine norovirus (MNV) and the feline calicivirus (FCV) are good candidates as HuNoV surrogates as they can grow in cell culture, are genetically related and share similar physiochemical characteristics (Jiang et al. 1993; Wobus et al. 2006; Kniel 2014). Tulane virus could be considered as an alternative candidate as HuNoV surrogate. However, Tulane virus does not belong to the genus *Norovirus* and is less resistant than MNV which is currently considered to be a better surrogate for HuNoV (Hirneisen and Kniel 2013). First isolated in 2003, MNV belongs to genogroup V and infects mice (Karst et al. 2003; Wobus et al. 2006). Although, FCV is a respiratory virus and is more susceptible to low pH and high temperature than MNV, it is also used as HuNoV surrogate (Slomka and Appleton 1998; Doultree et al. 1999; Clarke and Lambden 2000; Green et al. 2000; Bidawid et al. 2003; Nuanualsuwan and Cliver 2003; Malik et al. 2006). Despite the degree of resistance of HuNoV being unknown, the effect of a biocide on HuNoV was inferred from the results obtained on the most resistant HuNoV surrogates.

Preventive methods to control outbreaks of HuNoV are non-specific. They consist of appropriate hygienic measures among foodhandlers and in environmental decontamination (Baert et al. 2009a; Glass et al. 2009; Atmar 2010; Hirneisen et al. 2010). Disinfectants are biocide substances that destroy microorganisms or inhibit their activity on inanimate objects or surfaces (McDonnell and Russell 1999). Previous studies (Doultree et al. 1999; Duizer et al. 2004a; Radford et al. 2007) concluded that ethanol and quaternary ammonium-based products were ineffective disinfectants of FCV and the inactivation of FCV required high concentrations of sodium hypochlorite. MNV is sensitive both to alcohols and to bleach, and its resistance to basic and acidic pH conditions as well as its long-term resistance at room temperature is higher than that of FCV (Cannon et al. 2006; Belliot et al. 2008; Park and Sobsey 2011).

The aim of this study was to infer the efficacy of biocides on HuNoV surrogates from the screening of seven products as virucidal agents by measuring their effect on infectious viral titre and genomic copy number of MNV and FCV in suspension, on gloves and on stainless steel discs. These biocides (six disinfectants and one hand sanitiser) belong to different major biocide groups such as alcohols, halogens, oxidizing agents, quaternary ammonium compounds (QAC), aldehydes and biguanides (McDonnell and Russell 1999; Maillard 2001).

Materials and Methods

Viruses and Cells

CW1 strain of MNV-1 was propagated in RAW 264.7 cells (ATCC TIB-71) maintained in Dulbecco's modified Eagle's medium (Invitrogen) complemented (DMEMc) with 10 % heat-inactivated foetal calf serum (FCS) (BioWhittaker), 2 % penicillin (5000 U ml^{-1}) and streptomycin (5000 mg ml^{-1}) (PS; Invitrogen), 1 % of a non-essential amino acids preparation (NEAA) (Invitrogen) and 1 % HEPES buffer (1 M; Invitrogen).

F9 strain of FCV was propagated in Crandell's feline kidney (CRFK) cells (ATCC CCL-94) (Crandell et al. 1973) maintained in Eagle's minimal essential medium (Invitrogen) complemented (MEMc) with 10 % heat-inactivated FCS (BioWhittaker), 2 % PS (Invitrogen) and 1 % NEAA (Invitrogen).

MNV-1 (CW1) and FCV (F9) were grown for 72 h at 37 °C and 5 % CO_2 until cytopathic effect was observed. Viruses were harvested after three freezing/thawing cycles

of the cells and the suspensions were centrifuged at 1000×*g* for 20 min to remove cell debris. Supernatants were collected and purified by ultracentrifugation on a 30 % sucrose cushion in a SW28 rotor (Beckman Coulter) at 11,2000×*g* for 4 h at 4 °C. Pellets were suspended in phosphate-buffered saline (PBS) overnight at 4 °C, aliquoted and stored at - 80 °C.

Biocides

Group, substance, final concentration used in the biocide testing and contact time, for each biocidal product, are listed in Table 1. The contact times used were those recommended by the manufacturer's instructions for achievement of a virucidal effect, namely 5 and 15 min., in order to better mimic field conditions.

Cytotoxicity Tests

Cytotoxicity of Biocidal Products was Tested by Two Different Ways

First, a quantitative evaluation of cell viability and metabolism after exposure to biocidal products was performed using thiazolyl blue (MTT). In this MTT test, the initial biocide concentration was either the undiluted biocide for biocides A, F and G (initial concentration of 100 %) or a 1:1 (vol/vol) dilution in cell culture medium for biocides B, C, D and E (initial concentration of 50 %). Thus, for the MTT tests, initial concentrations differed from concentrations recommended by the manufacturer in that they were always higher. However, the concentrations used during later biocide testing corresponded to those of manufacturer's instructions (Table 1). Consequently, during the MTT test, cell cultures were exposed to the initial concentration and the nine 10-fold factor dilutions of the biocides. The optical density of the wells was determined at a test wavelength of 570 nm with background subtraction at 630 nm.

For the second evaluation of cytotoxicity, monolayers of RAW 264.7 or CRFK cells were inoculated with three ten-fold dilutions starting from biocide concentrations corresponding to those recommended in manufacturers' instructions (Table 1). After 1 h at 37 °C, cell monolayers were overlayed with agarose for 72 h and incubation continued at the same temperature. Thereby, the biocide dilutions used in this second cytotoxicity test were the same as those used during the following biocide testing. Alteration of cell monolayers was checked after fixation and staining as described in the "infectivity assay" section.

Biocide Interaction with Virus Infectivity

A test of cell sensitivity to biocidal products was performed. To check if exposure to a biocide decreases the cell sensitivity to a virus, virus titrations were performed on either untreated cells or cells treated with biocides. The same dilutions as used in the biocide testing were added to cell monolayers for each biocidal product. After washing twice with PBS, cells were inoculated with dilutions of viral suspensions and the viral titre was determined by

Table 1 Biocides (substances and commercial products) and their conditions of use during the biocide testing

	Product	Biocide group	Active ingredient	Final concentration (%) used in this study	Contact time[a] (min)
A	Ethanol	Alcohol	Ethanol	70	5
B	Kenochlore®	Halogen	Sodium hypochlorite	0.5	5
C	Kenocid 2100-S®	Oxidizing agents	Peracetic acid and hydrogen peroxide	0.05	5
				0.2	
D	Virocid®	Quaternary ammonium compounds	Benzylammoniumchloride	0.5	15
		Alcohol	Didecyldimethyl-ammoniumchloride		
		Aldehyde	Isopropanol		
			Glutaraldehyde		
E	Kenocid 210®	Quaternary ammonium compound	Didecyldimethyl-ammoniumchloride	0.25	15
		Alcohol	Isopropanol		
		Aldehyde	Glutaraldehyde		
F	Alcocid®	Alcohol	Isopropanol	70	To dryness
		Biguanide	Chlorhexidine	0.1–1	
G	Kenosept G®	Alcohol	Isopropanol	70	To dryness
		Biguanide	Chlorhexidine	0.5	

Products A–F are intended for the disinfection of surfaces and product G for hand disinfection

[a] Contact time recommended by manufacturer's instructions

plaque assay. In parallel, cell monolayers without contact with biocides were inoculated with dilutions of viral suspension. A difference of less than one $\log_{10}$ between the viral titre obtained from infected cells in contact with biocides and from those without contact with biocides indicates the absence of cell sensitivity to biocidal products.

Infectivity Assay

Titres of each virus were determined by plaque assay (Hyde et al. 2009). Briefly, cells were split into six-well plates at a density of 10^6 cells per well. On the following day, cells were inoculated with tenfold dilutions of virus in DMEM (MNV) or MEM (FCV). After 1 h at 37 °C, the inoculum was removed and the cells were overlaid with 2 ml DMEMc (or MEMc) with 0.5 % SeaPlaque agarose. Plates were stored at room temperature for 20 min and then incubated at 37 °C and 5 % CO_2 for 72 h. To count the plaques, the overlaying medium was removed and cells were fixed by adding 1 ml of 4 % formaldehyde/well for 30 min and then stained with 2 ml of 0.3 % crystal violet at room temperature.

The same protocol was used to determine the viral titre after biocide exposure.

Molecular Detection by RT-qPCR

RNA was extracted from 100 µl of each sample, using the QIAamp viral RNA kit (Qiagen) and following manufacturer's instruction. Total RNA was diluted in 60 µl of elution buffer and stored at −80 °C before use. One-step RT-qPCR was performed with a C1000 Touch thermocycler (Biorad) on a final volume mix of 25 µl following manufacturing instructions. The RT-qPCR reaction for MNV or FCV consisted of 12.5 µl of 2× RT-qPCR reaction mix (iScriptTM One-Step RT-PCR Kit for Probes, Biorad), MNV primers and probe (at the final concentrations of 100 and 200 nM, respectively) or FCV primers and probe (at the final concentrations of 500 and 200 nM respectively), 0.5 µl of iScript reverse transcriptase, 5 µl of RNA template and water to 25 µl.

Cycle conditions were as follows: 10 min at 50 °C, 5 min at 95 °C and 45 cycles of 10 s at 95 °C and 30 s at 60 °C. The primers and probes used to detect MNV and FCV are detailed in Table 2.

For quantification, each amplicon (FCV and MNV) was first cloned into pGEM-T easy (Promega®). Cloning reactions were analysed by sequencing after plasmid purification with the high pure plasmid isolation kit (Roche). Plasmid DNA was quantified via nanodrop and converted into genomic copies. Serial dilutions were then prepared (from 10^{-5} to 10^{-9}) and tested for the determination of standard curves for MNV-1 and FCV quantification.

Biocide Testing

In this experiment, we used two different surrogate viruses, MNV and FCV, and three different tests, i.e. suspension, glove and stainless steel disc tests. The method was adapted from the AFNOR norm EN 14476 and is illustrated in Fig. 1 (AFNOR 2007). Clean conditions were simulated by addition of 0.3 % bovine serum albumin (BSA). BSA mimicked the presence of organic load that could be present in field conditions on surfaces and could interfere with the activity of the disinfectant.

For each experiment, a sample without biocidal product was used as a control sample, following the same steps as described for the other samples, and was used for comparison with the samples containing one of the seven biocidal products.

For suspension tests, the average initial virus titre for MNV and FCV was 1.4×10^7 and 1.1×10^8 pfu ml^{-1}, respectively, and the inoculum volume was 10 or 15 µl. For suspension tests, a mixture containing 10 % of the virus (either MNV or FCV), 10 % of BSA (to reach a final concentration of 0.3 %) and 80 % of the biocidal product at the specific concentration, recommended by the manufacturer's instructions (Table 1), was prepared. After the determination of a contact time for each biocidal product (Table 1), the tested suspension was diluted three times by a ten-fold factor in cell culture media (at 4 °C) in order to reduce both the activity of the biocide and the cytotoxic effect. From these three dilutions of virus-biocide suspensions, a first aliquot was used for an infectivity assay (500 µl as inoculum in a 6-well plate) and a second aliquot was used for molecular detection (100 µl for the RNA extraction followed by the one-step RT-qPCR).

A biocidal product with a $\log_{10}$ reduction equal to or higher than three was equivalent to a 99.9 % decrease of infectious titre and was considered effective following the Guidelines on the application of general principles of food hygiene to the control of viruses in food (Codex Alimentarius 2012). Each biocidal product was tested in three independent experiments.

The efficiency of a disinfectant could vary depending on the test. Suspension tests for virucidal activity do not exactly reflect field conditions. To better mimic these field conditions, two other experiments were performed using gloves and stainless steel discs as tested surfaces. For the gloves and stainless steel discs tests, a mixture containing 10 % of the virus (either MNV or FCV) and 10 % of BSA (to reach a final concentration of 0.3 %) was deposited at the centre of the surface (either glove or stainless steel disc) and allowed to dry for 30 min under a laminar flow.

Table 2 Primer and probe sets and concentrations used to detect murine norovirus (MNV) and feline calicivirus (FCV) by RT-qPCR; both probes labelled 5′ 6-carboxyfluorescein (FAM) and 3′ MGB (minor groove binder)

Virus	Primer and probe	Final concentration (nM)	Sequence (5′-3′)	Location (bp)[a]	Ref.
MNV	FW-ORF1/ORF2	100	CACGCCACCGATCTGTTCTG	4972–4991	Hannah Gould et al. (2013)
	RV-ORF1/ORF2	100	GCGCTGCGCCATCACTC	5064–5080	
	MGB-ORF1/ORF2	200	CGCTTTGGAACAATG	5001–5015	
FCV	p30F	500	TGGATGAACTACCCGCCA	2415–2432	Hirneisen et al. (2010)
	p30R	500	CATATGCGGCTCTGATGGCTTGAAACTG	2507–2534	Hirneisen and Kniel (2013)
	P30P	200	TCGGTGTTTGATTTGGCCTG	2456–2475	[b]

[a] Nucleotide positions based on MNV (GenBank access NC_008311.1) and FCV sequences (GenBank access NC_001481.2)

[b] Zicola, personal communication

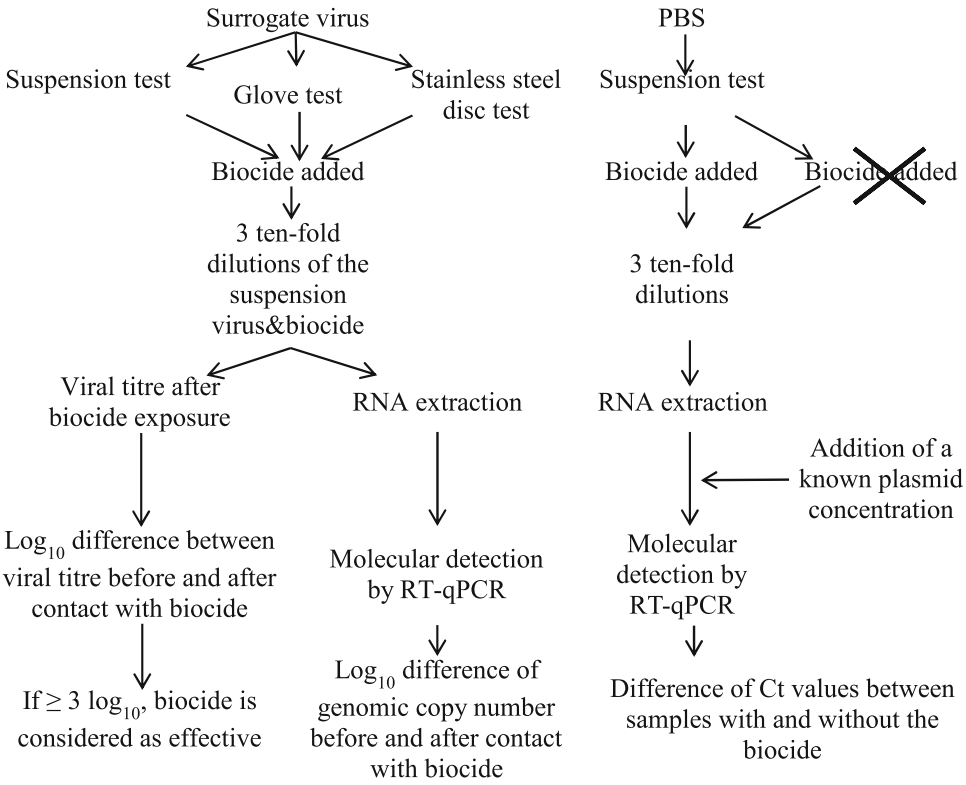

Fig. 1 Protocol and steps of the study

Stainless steel discs were washed and sterilized by autoclaving before use. Fingers of sterile latex gloves (KimtechPure, Kimberly-Clark) were used to form a tube and were inverted to mix virus and biocide on the external surface of the glove. Then the biocide was deposited on the same area during a biocide-dependent contact time (Table 1). In the glove test, cell culture media was directly added on the gloves and pipetted several times to recover the virus. In the stainless steel disc test, the discs (Stainless steel disc, AISI 304 cold rolled, Laserflash sa) were placed into 6-well plates and DMEMc (for MNV) or MEMc (for FCV) was added to dilute the biocide and to stop its effect. The plate was placed on a rocking platform for 15 min at 4 °C. From this first dilution, two further ten-fold dilutions were performed and finally, all dilutions were used for the infectivity assay and for the quantification of genomic copies as previously described for the suspension tests.

To evaluate the putative inhibitory effect of each biocide on the RT-qPCR, all the steps of the biocide test were also performed, starting with a suspension of one of the seven biocidal products, the BSA and PBS instead of the surrogate virus. After the extraction process on the three dilutions, a RT-qPCR was performed on these samples, containing a part of the extraction and a part of the DNA

plasmid corresponding to the surrogate virus. The Ct values were compared with the Ct values of RT-qPCR performed only with the DNA plasmid without any remaining part of the biocides.

Data Analysis

Each assay was performed in three independent experiments and from these, three replicates were tested by RT-qPCR. For the same test, samples treated with a disinfectant and untreated samples were compared and the results were expressed as log reduction. Thus, the virus elution efficiency should not interfere with our results and statistical analyses. Log reduction was expressed as mean ± standard deviation. Statistical analyses were performed using SAS software, version 9.3. Genomic copy number and infectivity were modelled using a linear model involving two factors—namely the biocide, the virus and their interaction—for each condition. Differences between biocides were assessed using least square means and their standard errors, using a Tukey correction for multiple comparisons. Results were considered significant when the associated p-values were lower than 0.05 or 0.01.

Results

Evaluation of Biocide Cytotoxicity

The highest non-cytotoxic concentrations of biocides after quantitative evaluation of cell viability and metabolism with MTT were 10^{-1} (biocide F), 10^{-2} (biocides A, B, C and G) and 10^{-4} (biocides D and E) on RAW 264.7 cells and 10^{-1} (biocide G), 10^{-2} (biocide A) and 10^{-3} (for biocides B, C, D, E and F) on CRFK cells (Table 3).

The biocide dilutions used in the following biocide testing were the 100- and 1000-fold dilutions of biocides which had already been diluted to concentrations recommended for use by the manufacturer (presented in Table 1).

Since these 100- and 1000-fold dilutions corresponded to concentrations lower than (or equal to) the non-cytotoxic ones previously evaluated by MTT test (Table 3), they were logically also not cytotoxic.

The microscopic examination of the cell monolayers confirms the results obtained with the MTT test.

Biocide Interaction with Virus Infectivity

When the residual effect of biocidal products was tested on the virus infectivity, the difference between infectious viral titres determined on cells in contact with biocides and cells without biocides was lower than 1 $\log_{10}$, showing that, in the infectivity assay, a reduction of infectious viral titre reflects biocide effect only on the virus.

Biocide Interaction with Molecular Detection

The Ct values were compared for samples containing either plasmids and biocide or only plasmids. Theoretically, delta Ct lower than 3.3 means a difference of genomic copy number lower than 1 $\log_{10}$. In our study, the mean delta C_t values were 1.5 ± 1.3 and 1.2 ± 0.7 for MNV and FCV, respectively. These findings showed that the presence of biocide residue within the experiment assay had non-significant effect on neither MNV nor FCV molecular detection.

Biocide Efficacy

The log reduction and standard deviation of both the infectious viral titre and the genomic copy number of MNV and FCV during the three different tests are given in Table 4. According to both Codex Alimentarius and Afnor Norm 14476, a biocide was considered effective when the log reduction of the infectious viral tire was ≥3 $\log_{10}$ (AFNOR 2007, Codex Alimentarius 2012).

Effect on MNV Infectivity

In suspension and in stainless steel disc tests, the $\log_{10}$ reduction of MNV infectious viral titre was between 3.32 and 3.85 for the biocidal products A, B, C, D, E, F and G (Table 4). In the glove tests, the $\log_{10}$ reduction of MNV infectious viral titre was higher than 4.36 $\log_{10}$ for the biocidal products A, B, C, D and E and lower than 2.95 $\log_{10}$ for the biocidal products F and G (Table 4). In both suspension tests and disc tests, no significant difference was observed between the biocides. For the glove tests, the $\log_{10}$ reduction of biocides A to E was significantly different ($P < 0.01$) from biocides F and G (Fig. 2).

Effect on MNV Genome Integrity

The average reduction of genomic copy number was higher than 2 $\log_{10}$ for biocidal products A and B, between 1 and 2 $\log_{10}$ for biocidal products D and G and lower than 1 $\log_{10}$ for biocidal products C and E. For the biocidal product F, there was no $\log_{10}$ reduction (Table 4). The effect of all biocidal products on MNV genomic copy number was significant ($P < 0.01$) in comparison to the control sample (Fig. 3). There was no significant difference between biocides C, D, E and G. On the contrary, the effect on MNV genomic copy number of biocides C, D, E and G was significantly different to biocides A and B on the one hand and to biocide F on the other hand ($P < 0.01$) (Fig. 3).

Table 3 Determination of the cytotoxicity of biocides in Raw and CrFK cells by MTT test

Biocide group	Biocide	Cells	O.D. for initial biocide concentration[c]	O.D. for ten-fold dilutions of the initial biocide concentration[ab]									O.D. for reference (untreated cells)	
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Alcohol	A	Raw	0.08	0.09	**0.27**	**0.60**	0.47	0.54	0.50	0.39	0.36	0.34	0.26	0.37
		CrFK	0.09	0.10	**0.20**	**0.55**	0.41	0.39	0.39	0.29	0.31	0.27	0.21	0.31
Halogen	B	Raw	0.08	0.13	1.88	1.76	**1.62**	**1.36**	1.12	1.87	1.41	1.41	1.34	1.02
		CrFK	0.04	0.13	0.52	0.94	**0.92**	**0.91**	0.93	0.98	0.92	0.72	0.66	0.63
Peracetic acid, hydrogen peroxide	C	Raw	0.05	0.10	1.73	2.01	**1.92**	**1.72**	1.80	1.40	1.70	1.70	1.19	0.96
		CrFK	0.05	0.10	0.36	0.92	**0.93**	**0.98**	0.92	0.87	0.95	0.92	1.02	0.59
Quaternary ammonium compounds, alcohol, aldehyde	D	Raw	0.26	0.11	0.07	0.08	**0.82**	**1.88**	1.49	1.43	1.88	2.23	1.82	0.85
		CrFK	0.26	0.11	0.19	0.90	**0.97**	**0.93**	0.98	0.96	1.01	1.00	0.87	0.88
	E	Raw	0.23	0.11	0.09	0.09	**1.97**	**2.38**	2.14	1.80	2.27	1.86	1.44	1.84
		CrFK	0.05	0.11	0.36	0.87	**0.90**	**0.89**	0.93	0.82	0.92	0.89	1.00	0.58
Alcohol, iguanide	F	Raw	0.07	1.76	**1.91**	**1.44**	1.63	1.80	1.30	1.68	1.55	1.57	1.19	1.16
		CrFK	0.06	0.06	0.11	**0.37**	0.47	0.58	0.63	0.57	0.61	0.52	0.47	0.37
	G	Raw	0.08	0.14	**1.98**	**1.88**	1.37	1.43	1.32	1.72	1.34	1.46	1.43	1.13
		CrFK	0.08	0.94	**1.05**	**1.01**	0.93	1.03	0.79	0.98	0.57	0.49	0.77	0.65

Results represent the average optical density (O.D.) measured in 8 wells at 570 nm with background subtraction at 630 nm

[a] The underlined O.D. correspond to the highest non-cytotoxic biocide concentration

[b] O.D. in bold correspond to the biocide concentration used in the biocide testing to determine the log reductions of both pfu and genomic copy number

[c] The initial concentration was either the undiluted biocide (the biocides A, F and G) or the biocide mixed 1:1 with cell culture media in 50 % (vol/vol) suspension (the biocides B, C, D and E)

Effect on FCV Infectivity

The $\log_{10}$ reduction with biocidal products B, C and D was higher than 4.09 $\log_{10}$ in the three different tests, except in the case of biocide B on disc tests with a 3.77 $\log_{10}$ reduction (Table 4). The $\log_{10}$ reduction with biocides A, E, and F ranged from 3.52 to 3.94 in suspension tests and was lower than 2.89 $\log_{10}$ in glove and disc tests. The $\log_{10}$ reduction with the biocidal product G was lower than 2.25 $\log_{10}$ in the three different tests. In suspension tests, the $\log_{10}$ reduction of biocides A, B, C, D, E and F was significantly different to the $\log_{10}$ reduction of biocide G ($P < 0.01$) and the $\log_{10}$ reduction of biocides B and C was also significantly different to the $\log_{10}$ reduction of biocides A ($P < 0.01$), E and F ($P < 0.05$) (Fig. 2). The $\log_{10}$ reduction of biocide D was significantly different to the $\log_{10}$ reduction of biocides A ($P < 0.05$) and G ($P < 0.01$). For the glove tests, the $\log_{10}$ reduction of biocides B, C and D was significantly different to the $\log_{10}$ reduction of biocides A, E, F and G ($P < 0.01$). For the disc tests, the $\log_{10}$ reduction of biocides B ($P < 0.05$), C ($P < 0.01$) and D ($P < 0.01$ with A and G; $P < 0.05$ with F) was significantly different to the $\log_{10}$ reduction of biocides A, F and G and the $\log_{10}$ reduction of biocide C was also significantly different to the $\log_{10}$ reduction of biocide E ($P < 0.05$) (Fig. 2).

Effect on FCV Genome Integrity

The $\log_{10}$ reductions of FCV genomic copy number with the biocide product B were highest, ranging from 2.39 to 3.88 depending on the different tests (Table 4). The $\log_{10}$ reductions of FCV genomic copy number with biocidal products C, D and E ranged from 1.04 to 2.30, 0.08 to 2.08 and 0.06 to 1.46, respectively (Table 4). The effects of biocidal products B, C, D and E on FCV genomic copy number were significant ($P < 0.01$) (Fig. 3). The $\log_{10}$ reductions of FCV genomic copy number with biocidal products A, F and G ranged from 0.03 to 0.42, from −0.11 to 0.07 and from 0.21 to 0.42, respectively (Table 4).

Biocidal products A, F and G had no significant effect on genomic copy number (Fig. 3).

Discussion

Two HuNoV surrogates, namely MNV and FCV, were used to evaluate the biocidal effect of seven fully formulated products, representing the major groups of disinfectants.

When, as criteria of efficacy, a log reduction >3 of the infectious viral titre on both surrogates and in the three tests is used, the most efficacious disinfectants in this study

Table 4 Results on log reduction and standard deviation of infectious viral titre and of genomic copy number of murine norovirus (MNV) and feline calicivirus (FCV) in suspension (s), on gloves (g) and on stainless steel discs (d)

Biocide group	Biocide	Test	Log_{10} reduction of			
			MNV		FCV	
			Infectious viral titre	Genomic copy number	Infectious viral titre	Genomic copy number
Alcohol	A	s	≥3.85 ± 0.0*	2.14 ± 1.4	≥3.52 ± 0.1*	0.03 ± 0.1
		g	≥5.18 ± 0.0*	2.94 ± 1.4	≤2.35 ± 0.2	0.42 ± 1.1
		d	≥3.69 ± 0.5*	2.08 ± 0.4	≤2.15 ± 0.4	0.04 ± 0.3
Halogen	B	s	≥3.85 ± 0.0*	6.07 ± 1.9	≥4.70 ± 0.0*	3.88 ± 2.4
		g	≥4.87 ± 0.5*	2.65 ± 1.4	≥4.47 ± 0.3*	2.68 ± 1.2
		d	≥3.69 ± 0.5*	2.3 ± 0.7	≥3.77 ± 0.8*	2.39 ± 1.1
Peracetic acid, hydrogen peroxide	C	s	≥3.85 ± 0.0*	0.68 ± 0.4	≥4.70 ± 0.0*	2.30 ± 0.3
		g	≥4.36 ± 0.1*	0.43 ± 0.3	≥4.72 ± 0.8*	1.18 ± 0.8
		d	≥3.69 ± 0.5*	1.63 ± 0.2	≥4.77 ± 0.8*	1.04 ± 0.1
Quaternary ammonium compounds, alcohol, aldehyde	D	s	≥3.85 ± 0.0*	0.62 ± 0.3	≥4.48 ± 0.4*	2.08 ± 0.3
		g	≥5.18 ± 0.0*	1.22 ± 0.3	≥4.09 ± 0.8*	0.63 ± 0.9
		d	≥3.69 ± 0.5*	1.19 ± 0.7	≥4.36 ± 1.1*	0.08 ± 0.2
	E	s	≥3.85 ± 0.0*	0.58 ± 0.3	≥3.70 ± 1.7*	1.46 ± 0.8
		g	≥4.54 ± 0.6*	0.81 ± 0.4	≤2.50 ± 0.3	0.38 ± 0.7
		d	≥3.32 ± 0.2*	0.79 ± 0.6	≤2.89 ± 1.6	0.06 ± 0.2
Alcohol, biguanide	F	s	≥3.85 ± 0.0*	−0.37 ± 0.4	≥3.94 ± 0.2*	0.07 ± 0.1
		g	≤2.95 ± 0.8	−0.02 ± 0.3	≤2.25 ± 0.3	−0.11 ± 0.6
		d	≥3.69 ± 0.5*	−0.92 ± 0.5	≤2.51 ± 0.8	0.02 ± 0.3
	G	s	≥3.85 ± 0.0*	1.29 ± 1.2	≤1.80 ± 0.2	0.42 ± 0.6
		g	≤2.85 ± 1.2	0.68 ± 0.3	≤2.25 ± 0.3	0.24 ± 0.5
		d	≥3.69 ± 0.5*	1.02 ± 0.8	≤2.15 ± 0.4	0.21 ± 0.6

* log_{10} reduction of infectious viral titre >3 log_{10}

≥ Indicates that the virus titre was below the detection limit

appeared to be biocidal products B, C and D, representing the halogens, the oxidizing agents group and a mix of QAC, alcohol and aldehyde, respectively. In addition, these three disinfectants also elicited a significant effect on genomic copy number for both surrogate viruses and in all three tests.

Biocide B, containing sodium hypochlorite, is often used to disinfect water and environmental surfaces. Its significant effect on infectious viral titre and genomic copy number confirms results of other studies which also revealed a significant effect on HuNoV genome integrity (Girard et al. 2010; Lim et al. 2010; Park and Sobsey 2011; Hirneisen and Kniel 2013; Tung et al. 2013).

Biocide C contains peracetic acid (PAA) and hydrogen peroxide (H_2O_2), representing the oxidizing agents, and reduced the MNV and the FCV viral titres by more than 3 and 4 log_{10}, respectively; these findings are consistent with several studies (Baert et al. 2009b; Fraisse et al. 2011; Vimont et al. 2015). The effect on the genomic copy number was significant but lower than that of other biocidal products such as sodium hypochlorite for example; again, this finding is in accordance with the results of Fraisse et al. (2011). FCV seems to be more sensitive to sodium hypochlorite and to PAA than MNV (Gulati et al. 2001; Baert et al. 2009b; D'Souza and Su 2010; Fraisse et al. 2011; Park and Sobsey 2011; Kim et al. 2012; Vimont et al. 2015); therefore, FCV could overestimate the effect of these two biocides on HuNoV.

Biocide D, composed of glutaraldehyde, QAC and isopropanol, significantly reduced the infectious viral titre and the genomic copy number of both surrogates. Previously published results may be controversial, reporting a significant effect either on MNV or FCV infectious titres (Doultree et al. 1999; Jimenez and Chiang 2006; Belliot et al. 2008; Girard et al. 2010; Whitehead and McCue 2010; Su and D'Souza 2012). These apparently contradictory results could be explained by the biocide concentrations, the kind of QAC, the contact time and the nature of the surfaces tested. The difference between biocide D (significant reduction of both MNV and FCV infectious titres) and biocide E (significant reduction of MVN infectious titre only) could be explained by the chemical

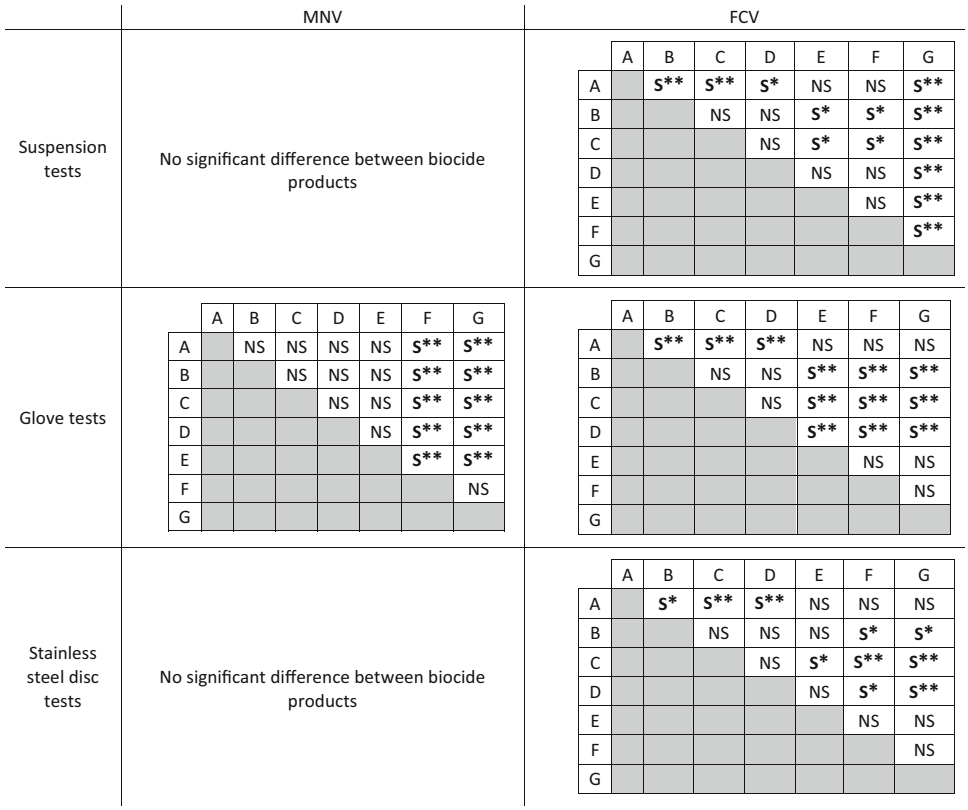

Fig. 2 Comparison of the effect of the biocidal products (*A–G*) on MNV and FCV titres. *S* significant difference with *P* values <0.01 (**) or <0.05 (*); *NS* no significant difference; *A* ethanol; *B* sodium hypochlorite; *C* peracetic acid and hydrogen peroxide; *D* benzylammoniumchloride, didecyldimethylammonium chloride, isopropanol and glutaraldehyde; *E* Didecyldimethylammonium chloride, isopropanol and glutaraldehyde; *F* isopropanol and chlorhexidine; *G* isopropanol and chlorhexidine

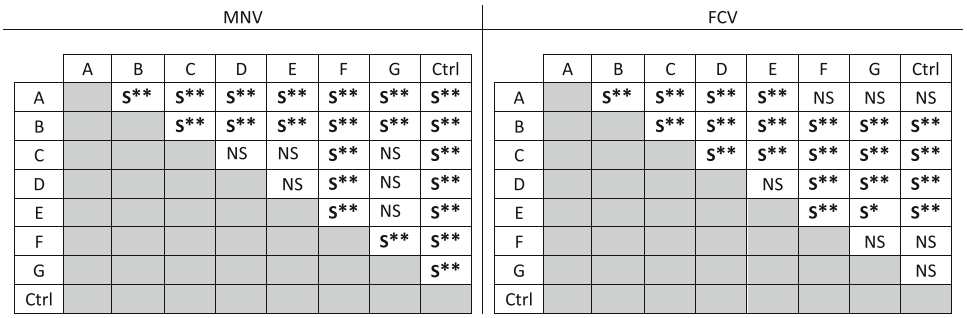

Fig. 3 Comparison of the effect of the biocidal products (*A–G*) on detection of MNV and FCV genome copies. *S* significant difference with *P* values <0.01 (**) or <0.05 (*); *NS* no significant difference; *Ctrl* controls; *A* ethanol; *B* sodium hypochlorite; *C* peracetic acid and hydrogen peroxide; *D* benzylammoniumchloride, didecyldimethylammonium chloride, isopropanol and glutaraldehyde; *E* didecyldimethylammonium chloride, isopropanol and glutaraldehyde; *F* isopropanol and chlorhexidine; *G* isopropanol and chlorhexidine

composition of the QAC used. Glutaraldehyde significantly reduced MNV and FCV titres by $\geq$6 $\log_{10}$ at a 2 % concentration (D'Souza and Su 2010), while the reduction in MNV and in FCV titres was $\leq$3 $\log_{10}$ after the use of QAC (Belliot et al. 2008; Girard et al. 2010; Whitehead and McCue 2010). In this study, biocide D, a mixture containing glutaraldehyde, QAC and isopropanol, showed a significant effect on the infectious titre with a log reduction $\geq$3 $\log_{10}$ on both surrogates and in the three tests, as well as a significant effect on the genome integrity ($P < 0.01$). Therefore, interactions between different substances, e.g. providing synergistic or antagonistic effects, are likely to exist and should be taken into account. Comparisons between studies on biocidal products are difficult due to the large number of disinfectants and the variety of conditions of use: concentration, contact time, test set-up and surrogate virus.

The mechanisms of action of these disinfectants showing a significant efficiency on both MNV and FCV surrogates are not fully clarified. Previous studies demonstrated a virucidal activity of halogens by a modification of capsid integrity as it is observed with PAA and glutaraldehyde (O'Brien and Newman 1979; McDonnell and Russell 1999). H_2O_2 acts as an oxidant on lipids, proteins and DNA

(McDonnell and Russell 1999; Wutzler and Sauerbrei 2000). After PAA and H_2O_2 treatments of HuNoV faecal samples, HuNoV binding was only reduced by less than 1 $\log_{10}$. These results suggest that binding was not affected and capsids were not altered by these oxidizing agents (Kingsley et al. 2014). The mechanisms of action of glutaraldehyde on viruses are still unknown but probably also involve cross-linking of proteins, RNA and DNA (Chambon et al. 1992; McDonnell and Russell 1999). QAC have an effect on lipids and enveloped viruses, and, in studies on bacteriophages, QAC had an effect on infectivity but did not affect the genomic DNA (Doultree et al. 1999; McDonnell and Russell 1999).

Alcohols and chlorhexidine associated with alcohol had differing effects on the two surrogates in the three tests. Biocide A (alcohol) had a significant effect on infectious viral titre and genomic copy number of MNV but no significant effect on these same parameters of FCV, thus confirming previous studies (Park et al. 2010; Tung et al. 2013). The biocide products F and G are an association of alcohol and chlorhexidine (which is a major member of the biguanide group). They produced a significant effect on MNV in suspensions and stainless steel disc tests but not on FCV (except biocide F in the suspension test). Several studies (Park et al. 2010; Iwasawa et al. 2012; Matsuhira et al. 2012) showed poor effects of chlorhexidine on both MNV and FCV viral titres. Our results are in accordance with this with regard to FCV. The short contact time used in previous studies (<5 min) could explain the absence of any significant effect of alcohol (D'Souza and Su 2010) and of chlorhexidine (Park et al. 2010; Iwasawa et al. 2012; Matsuhira et al. 2012) on the infectious titre of MNV and FCV. Our results as well as previous studies confirmed that MNV is more susceptible to alcohol and chlorhexidine than FCV (Park et al. 2010; Tung et al. 2013; Cromeans et al. 2014). These data also suggest differences between MNV and FCV regarding viral capsid integrity (Cannon et al. 2006; Park et al. 2010).

While the results above present useful information with regard to effects of disinfectants on HuNoV surrogates, it must be noted that this work has some limitations. Obviously, results extrapolated from HuNoV surrogates should always be carefully interpreted, as they are not directly obtained for HuNoV. However, since these surrogates are genetically related to HuNoV, they still remain the best approach for evaluation of the effect of disinfectants on HuNoV. Information obtained from other existing HuNoV surrogates (e.g. Tulane virus) could contribute to a better understanding of disinfectant effects against HuNoV. The second limitation is the use of only one parameter per test condition as a fixed temperature, contact time and disinfectant concentration were maintained per disinfectant. It could be interesting to comprehensively analyse a possible effect of different conditions of application on infectious viral titre and genomic copy number. Thus, determination of a shorter efficient contact time could be interesting for practical use.

In conclusion, halogen compounds, oxidizing agents and a combination of QAC, alcohol and aldehyde showed the best biocide activity for the disinfection of surfaces and materials. The information on the lowest efficient concentration and the shortest contact time of these three biocides on HuNoV surrogates will be useful during selection of the most appropriate disinfectant against HuNoV. Both harmonization and standardization of test conditions could be beneficial to compare biocide efficacy.

In addition, hand and surface disinfection are not the only targets to control and reduce HuNoV contamination in food industry and human healthcare centres. These measures should be combined with an optimal management of foodhandlers hygiene and with optimized detection of HuNoV at critical points of possible contamination.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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