Aquavalens Project

"Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation."

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Deliverable D2.4

Establish molecular methods to evaluate the efficacy of virucidal treatments on viruses.

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D 2.4. Establish molecular methods to evaluate the efficacy of virucidal treatments on viruses.

Background.

It has been hypothesized that if virus inactivation by some type of disinfectant is based on viral genomic damage, molecular biological techniques may be able to effectively evaluate the disinfection effect since these techniques depend strictly on the integrity of the target nucleic acid as template. However this is not so obvious. Nevertheless, if specific regions of the genome harbour sequences sensitive to some disinfectants, as suggested in the literature (Li et al., 2004) it should be feasible to evaluate the disinfection effect by using molecular biological techniques targeting these specific sequences. In Task 2.3 of WP2, the specific genomic damage caused by free chlorine (FC) treatment to hepatitis A virus (HAV) and human norovirus (NoV) has been investigated.

Effect of free chlorine treatment on the hepatitis A virus genome.

It has been reported in the literature (Li et al., 2002) that chlorine, besides causing capsid protein oxidation, specifically affects a region of the 5' NCR of HAV. In the present project, suspensions of the HAV cytopathogenic strain 43c have been subjected to inactivation by 12 mg/L of FC for 30 min at room temperature in the dark. The effect of FC was monitored by determination of infectivity (TCID50/mL) in FRhK-4

cells as described elsewhere (Aragonès et al., 2010). Additionally, different target regions have been employed for end-point dilution RT-PCR detection of the HAV genome after FC treatment (Figure 1).

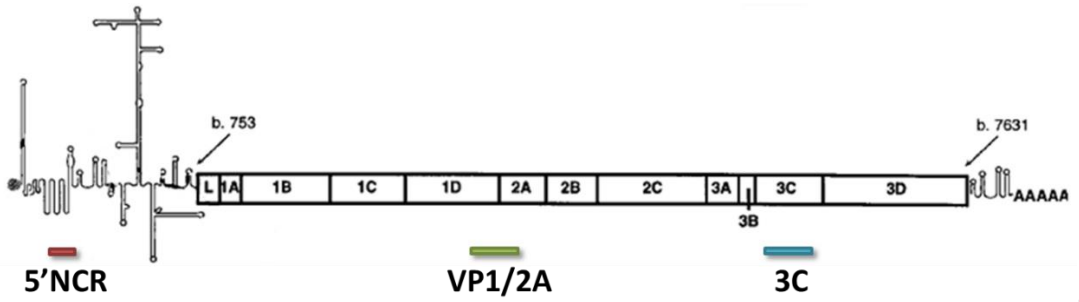

Figure 1. Target regions of the HAV genome employed to monitor FC inactivation.

Table 1 shows the validity of the different primer regions to evaluate FC disinfection of HAV. Although the 5'NCR primers seem to provide a better indication of HAV FC inactivation than primers from the other two target regions, only one-log decay was observed by end-point RT-PCR while a reduction higher than 5.29 logs was observed by infectivity assays (Table 2). The 5'NCR primers are those described in the ISO-15216 standard that were developed in the UB laboratory (Costafreda et al., 2006).

Table 1. Reciprocal dilution of the HAV suspension detected after FC treatment by end-point dilution RT-PCR with the different target regions.

	5' NCR	**VP1/2A**	**3C**
Untreated	-2	-1	-1
Chlorine-treated	-1	ND	-1

Table 2. HAV inactivation after FC treatment measured by infectivity.

	Untreated	Chlorine-Treated	Reduction
Log_{10} TCID50/ml	7.99	2.70 *	> 5.29

* One positive replica out of 4.

Effect of free chlorine treatment on the human norovirus genome.

Despite reports in the literature (Straub et al., 2007; Jones et al., 2014) on cell culture systems for human NoV, there is no possibility to conduct infectivity assays with these important human pathogens. In Aquavalens WP2, studies on the integrity of human NoV GII.4 genome regions after FC treatments have been conducted.

Stocks of human NoV GII.4 were prepared from 20% suspensions of a stool sample positive for Hu/GII.4/New Orleans1805/2009/USA. NoV contains a single-stranded positive-sense 7.6 kb RNA genome (Figure 2) encoding three open reading frames (ORFs). ORF1 encodes a nonstructural polyprotein, and ORF2 and ORF3 encode the major capsid protein VP1 and minor capsid protein VP2, respectively (Jiang et al., 1993).

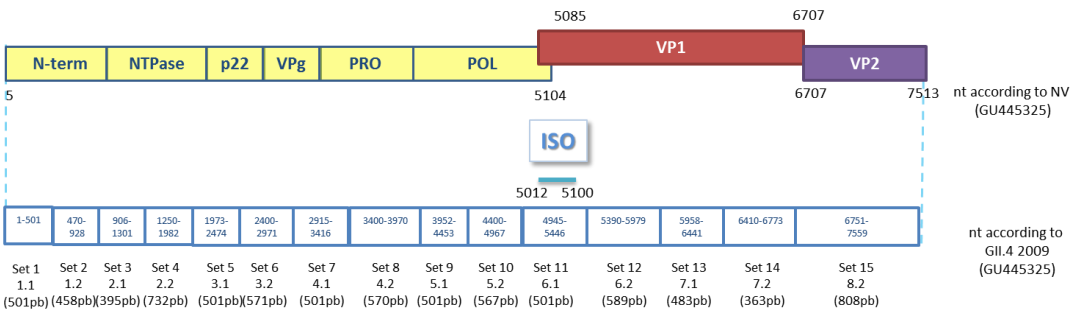

Figure 2. Human NoV genome organization and segments covering its full length. The ISO standard primers are included in segment 11.

Primers were designed in order to cover the full-length NoV genome in 15 fragments (Figures 2 and 3).

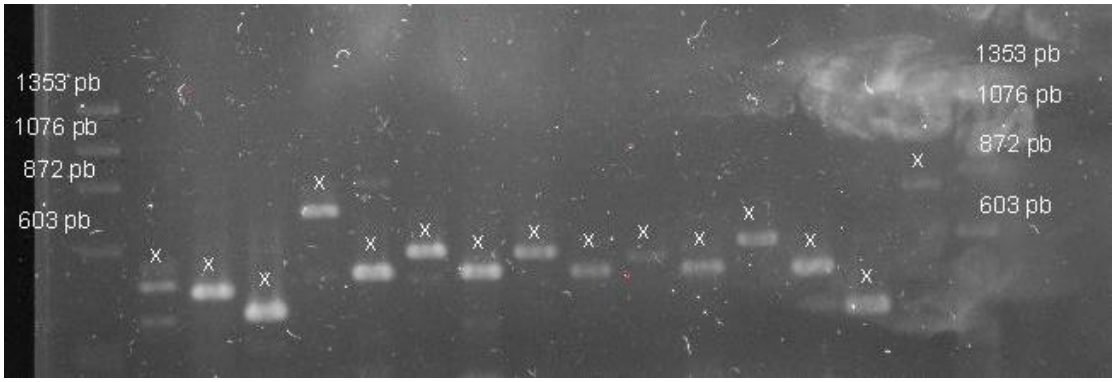

Figure 3. PCR bands of the 15 segments from the genome of human NoV GII.4.

The characteristics of the different genome segments are depicted in **Table 3**. The structure of the different genome segments is presently under study.

The experimental design was to apply to the NoV suspension increasing concentration of FC (from 0 to more than 9 mg/l) for 30 min at room temperature in the dark. After neutralization of FC with the addition of 1% sodium thiosulfate, RNA was extracted by the use of the NucliSENS® easyMAG® (Biomerieux) and subjected to standard RT-PCR.

Our data point to a differential degradation of the NoV genome segments (**Table 4**). Some segments were found to be more resistant than others to the effect of FC.

These findings open the possibility to employ specific genome fragments for different purposes; e.g., a resistant fragment may be appropriate to trace the presence of contaminant NoV in a sample (NoV-tracking), or a sensitive fragment is the best target option to evaluate the inactivation of NoV after FC treatment. However, more data are required to ascertain the consistency of this approach with other virucidal agents.

Table 3. Characteristics of the different segments of the human NoV genome.

Genome segments	Segment length (bases)	% GC	ΔG	Comments
1	479	51,67	-178.80	
2	458	46,63	-144.10	
3	395	49,75	-125.90	
4	732	52,52	-242.90	
5	501	48,61	-153.30	
6	571	48,95	-169.20	
7	501	51,79	-158.10	
8	570	53,42	-203	Putative CRE element
9	501	50,66	-193.30	
10	542	48,80	-154.80	
11	501	50,60	-177.70	ISO RT-qPCR target region (SG promoter)
12	589	46,27	-152.10	
13	483	38,02	-145.40	
14	363	49,73	-111.60	
15	808	48,95	-241.90	3'UTR stem loop

Table 4. Behavior of the different segments of the human NoV GII.4 in front of FC disinfection.

	Free chlorine concentration (mg/L)								
Genome segments	**0**							**>9**	**Behavior**
1	+	+	+				-	-	
2	+	+	+	+	+	+	+	-	**Resistant**
3	+	+	+				-	-	
4	+	+	+				-	-	
5	+	+	+	+	+	-	-	-	
6	+	+	+				-	-	
7	+	+	+				-	-	
8	+	+	+	+	+	+	+	-	**Resistant**
9	+	+	+	+	+	+	-	-	
10	+	+	+	-	-	-	-	-	**Sensitive**
11	+	+	+	-	-	-	-	-	**Sensitive**
12	+	+	+				-	-	
13	+	+	+	+	+	+	+	-	**Resistant**
14	+	+	+				-	-	
15	+	+	+	+	-	-	-	-	

Conclusions

Although FC action has been reported to oxidize the virus capsid, the viral genome may also be affected.

Despite what is described in the literature, it is not clear whether there is a specific damage caused in selected regions of the HAV genome.

Since cytopathogenic HAV strains do exist, it is advisable to employ infectivity assays for the validation of HAV inactivation by FC.

Some parts of the human NoV genome appear to be more sensitive than others to the effect of FC.

Depending on the purpose of the study, a given genome region may be more appropriate than another to detect NoV after FC treatment: a resistant fragment may be appropriate to trace the presence of contaminant NoV in a sample (NoV-tracking), or a sensitive fragment is the best target option to evaluate the inactivation of NoV after FC treatment.

Segment 11 corresponds to the target region of the ISO standard,

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