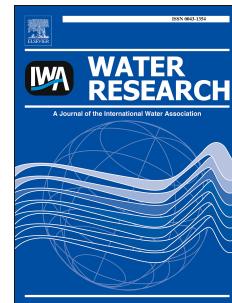


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Transmission of hepatitis E virus by water: an issue still pending in industrialized countries

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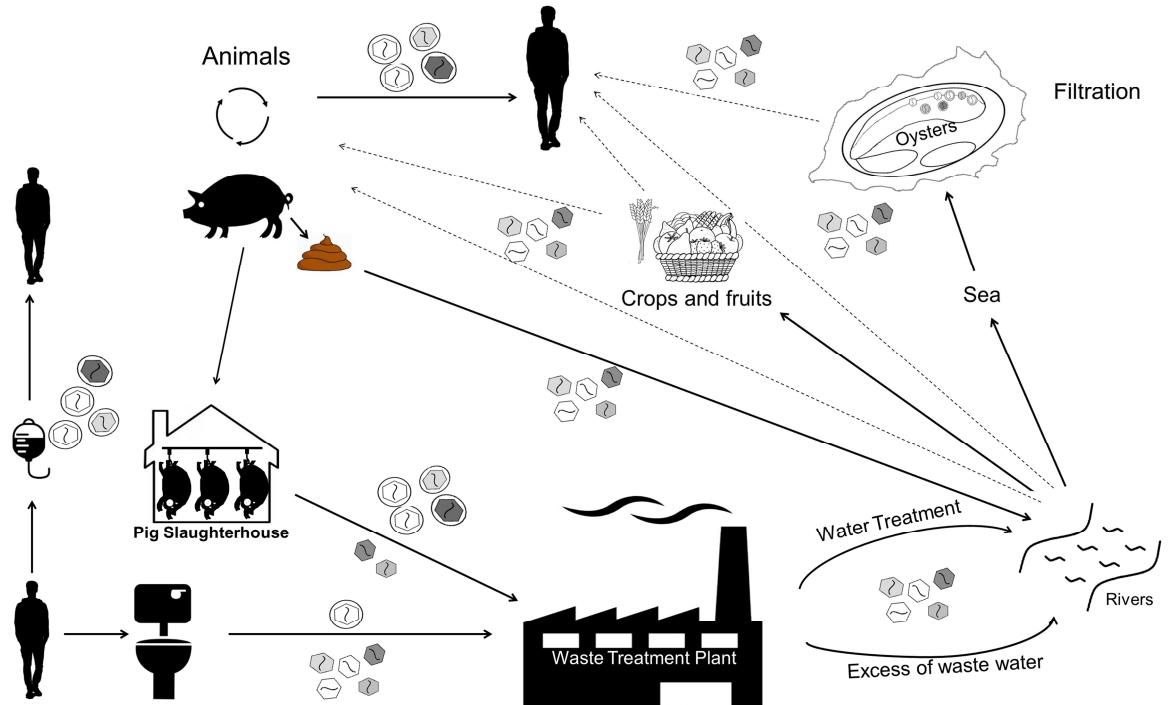
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Transmission of hepatitis E virus by water in industrialized countries

1    **Title**

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3    countries

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12   reading of the manuscript, as an engineer.

13

14   Keywords: hepatitis E virus (HEV), water, occurrence, variability, transmission,  
15   survival.

16

17   Highlights:

- 18   - Hepatitis E virus (HEV) is a highly variable virus, waterborne in some cases.  
19   - HEV seems sensitive to water treatments compared to other enteric viruses.  
20   - Cell culture methods should be improved especially for HEV detection in  
21   water.  
22   - Role of water for animal transmission of HEV should be investigated.

23 - Presence of enveloped HEV particles in the environment may be explored.

24

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26

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30

31 **Abstract**

32 Hepatitis E virus (HEV) is an enteric virus divided into eight genotypes.  
33 Genotype 1 (G1) and G2 are specific to humans; G3, G4 and G7 are zoonotic  
34 genotypes infecting humans and animals. Transmission to humans through water  
35 has been demonstrated for G1 and G2, mainly in developing countries, but is only  
36 suspected for the zoonotic genotypes. Thus, the water-related HEV hazard may be  
37 due to human and animal faeces. The high HEV genetic variability allows considering  
38 the presence in wastewater of not only different genotypes, but also quasispecies  
39 adding even greater diversity. Moreover, recent studies have demonstrated that HEV  
40 particles may be either quasi-enveloped or non-enveloped, potentially implying  
41 differential viral behaviours in the environment.

42 The presence of HEV has been demonstrated at the different stages of the  
43 water cycle all over the world, especially for HEV G3 in Europe and the USA.  
44 Concerning HEV survival in water, the virus does not have higher resistance to  
45 inactivating factors (heat, UV, chlorine, physical removal), compared to viral  
46 indicators (MS2 phage) or other highly resistant enteric viruses (Hepatitis A virus).  
47 But the studies did not take into account genetic (genogroups, quasispecies) or  
48 structural (quasi- or non-enveloped forms) HEV variability. Viral variability could  
49 indeed modify HEV persistence in water by influencing its interaction with the

50 environment, its infectivity and its pathogenicity, and subsequently its transmission by  
51 water. The cell culture methods used to study HEV survival still have drawbacks  
52 (challenging virus cultivation, time consuming, lack of sensitivity). As explained in the  
53 present review, the issue of HEV transmission to humans through water is similar to  
54 that of other enteric viruses because of their similar or lower survival. HEV  
55 transmission to animals through water and how the virus variability affects its survival  
56 and transmission remain to be investigated.

57 **1. Introduction**

58 HEV is an enteric virus that causes not only acute but also chronic hepatitis. It  
59 has been increasingly studied in the past decade and has been observed to also  
60 cause syndromes other than liver-related such as neurological or renal HEV-related  
61 diseases, underlying its highly variable pathogenesis (Pischke et al., 2017). This  
62 virus is widespread or endemic in large parts of the world. It has been largely  
63 demonstrated to be a waterborne virus in developing countries, and since 1997 it  
64 has also been demonstrated to be a zoonotic virus in developed countries (Meng et  
65 al., 1997; Pavio et al., 2015). This has led to numerous recent literature reviews  
66 focussing on the zoonotic origin of HEV (Pavio et al., 2015), its infection in humans  
67 and its treatments (Echevarría et al., 2013; Miyamura, 2011; Sridhar et al., 2015), its  
68 molecular epidemiology (Purdy and Khudyakov, 2011) and the water transmission of  
69 human-adapted HEV in developing countries (Khuroo et al., 2016). They all agree  
70 with the conclusion that a better understanding of the route of transmission is needed  
71 especially for the zoonotic HEV. However, the transmission paths are still unclear in  
72 developed countries where a waterborne transmission is still questioned. Indeed,  
73 some reports coming from these countries suggest shellfish-borne outbreaks (Said et  
74 al., 2009) or higher HEV seroprevalence in shellfish consumers or non-bottled water  
75 consumers (Mansuy et al., 2015, 2016). HEV is a variable virus, which makes it  
76 difficult to be studied as some different genotypes or forms might behave differently  
77 from one another especially through the water cycle. Indeed, the high HEV genetic  
78 variability can suggest a high potential for adaptation to various organs and tissues in  
79 humans and animals. This genetic feature implies distinct pathogenic mechanisms in  
80 different syndromes for the hosts and encourages us to consider the presence in

81 wastewater of different HEV genotypes as well as complex viral quasispecies with  
82 various transmission routes favouring HEV circulation in the environment.

83 The unravelling of these transmission paths would help prevent HEV infection  
84 and develop sanitary policies in order to control infections. Our aim was to provide an  
85 overview of the transmission of HEV through water with particular attention to  
86 variability linked to the source of pollution, occurrence, survival in water and removal  
87 by water treatment.

## 88 **2. Source of water pollution: host and viral particle variability**

89 HEV is an enteric virus, which infects its hosts through the faecal-oral route; in  
90 this review, transmission through ingestion of contaminated meat will not be  
91 discussed. There is only one serotype belonging to the *Hepevirus* genus and the  
92 *Hepeviridae* family (Geng et al., 2011). It is usually a non-enveloped icosaedric  
93 capsid measuring between 27 and 34 nm, which contains a single-strand, positive-  
94 sense RNA virus of 7.2 kb in length. RNA is capped and polyadenylated at the 5' and  
95 3' ends, respectively. It has two short untranslated regions (UTR) at the 5' and 3'  
96 ends and three open reading frames (ORF) - ORF1, ORF2 and ORF3 (Graff et al.,  
97 2006). ORF1 encodes non-structural proteins such as methyltransferase, papain-like  
98 cysteine protease, helicase and RNA-dependent RNA polymerase (Okamoto, 2007).  
99 The ORF2 and ORF3 proteins are encoded by a same subgenomic mRNA, which  
100 produces the capsid protein and phosphoryl protein, respectively (Graff et al., 2006).  
101 The size of quasi-enveloped viruses is between 50 and 110 nm (Dalton and Izopet,  
102 2018).

103 Among the eight HEV genotypes, five cause human infections. G1 and G2  
104 infect only humans and are mainly reported in regions with low levels of resources

105 and poor sanitation resulting in contamination of food and water supplies (Khuroo et  
106 al., 2016). They are mainly observed in Asia (Indian subcontinent, China,  
107 Bangladesh, Nepal, Pakistan and Afghanistan) and sub-Saharan Africa for G1, and  
108 Mexico and some African countries (Nigeria, Chad, Sudan and the Central African  
109 Republic) for G2 (Nelson et al., 2018). HEV G3 and G4 infect humans but also a  
110 wide range of mammals such as pigs, boars, deer and rabbits (Colson et al., 2010;  
111 Masuda et al., 2005; Matsuda et al., 2003; Meng, 2013). G3 infections are qualified  
112 as “emerging” diseases in European countries, including France, but they are just  
113 currently better identified and can be observed in North and Latin America, South  
114 Africa, Asia, Australia and New Zealand. Nevertheless, the seroprevalence seems to  
115 be lower in Oceania compared to Europe. G4 can be found in China, northern Japan,  
116 India and sporadically in Europe (Dalton and Izopet, 2018; Hofmeister et al., 2018;  
117 Nelson et al., 2018). The zoonotic transmission of G3 and G4 has been largely  
118 demonstrated for pigs, wild boars and deer with sometimes no modification in the  
119 sequence between the animal and human strains, meaning that there is no  
120 adaptation necessary to infect humans or animals. A zoonotic transmission is  
121 suspected for rabbits (Abranavel et al., 2017; Izopet et al., 2012; Pavio et al., 2015).  
122 The common ancestor of mammalian HEV was shown to be between HEV-1/2 and  
123 HEV-3/4: it has been estimated that an ancestor strain gave rise on the one hand to  
124 human HEV and on the other hand to zoonotic HEV more than 500 years ago. Then,  
125 both genotypes split again (Purdy and Khudyakov, 2011). Finally, only one human  
126 case has been described with HEV G7 primarily infecting dromedaries and initially  
127 named DcHEV (dromedary camel HEV) (Woo et al., 2014). Therefore, depending on  
128 the geographical area, genotypes found in water may differ but overall a large

129 number of hosts, not all of whom have been identified yet, may contaminate the  
130 water through their faeces.

131 HEV is a virus subject to a certain amount of genetic variability. Some authors  
132 took HEV sequences, some of which belonging to different genotypes, and measured  
133 distances between them: nucleotide similarity between genotypes is about 75% and  
134 within a genotype it is over 81% (Smith et al., 2013). Such genetic variability makes a  
135 single HEV genotype-based study insufficient for relevant conclusions to be drawn.

136 HEV infection of many host species could require the involvement of many  
137 viral structural sites to favour adaptation to highly heterogeneous biotic or abiotic  
138 environments. According to Purdy and Khudyakov (2010), HEV G3 and G4 would  
139 carry more polymorphic positions than G1 and G2. The high genetic diversity seen in  
140 G3 and G4 may reflect a strong adaptation to many hosts (Purdy and Khudyakov,  
141 2010). Under different selective pressures, many positive selection (i.e. mutations  
142 leading to an amino acid substitution) sites have been located in the overlapping  
143 region of ORF2 and ORF3. The ORF1 and non-overlapping ORF2 have many  
144 negative selection (i.e. silent mutations) sites which are proportionally in greater  
145 numbers in G1 than in G3 and G4 (Purdy and Khudyakov, 2010). We have made  
146 phylogenetic trees with two of the ORF2 domains: Middle (M) and Protruding (P) in  
147 amino acids in G1 and G3 reference strains (Figure 1). These two domains are  
148 involved in the viral immunogenicity and interactions between HEV and cellular  
149 membranes. The overall mean distance is greater for G3 than for G1 for both M and  
150 P domains. Moreover, this great difference between HEV genotype sequences can  
151 also lead to potential technical concern, failure of diagnostic tools (Reuter et al.,  
152 2009) and failure of methods used for environmental analysis. Indeed, the genetic  
153 variability can lead to physical variability: the virus surface properties might be

154 modified by the amino acid sequence modifications. We have tested 25 amino acid  
155 sequences of ORF2 of different HEV subgenotypes belonging to G1, G2, G3 and G4  
156 with the AnTheProt software. This software, developed by Parker et al. (1986), was  
157 principally used to analyse the viral antigenicity; it takes into consideration three  
158 parameters to determine the antigenicity of a position within a polypeptide:  
159 hydrophobicity, accessibility and flexibility of the amino acid, and the same  
160 parameters of the three amino acids before and after it (Parker et al., 1986). In this  
161 review, it was used to work on the hydrophobicity profile of the capsid protein which  
162 has 660 amino acids. Through comparison of the amino acid sequences of the  
163 different genotypes and subgenotypes, different profiles were observed.  
164 Subgenotypes 3 showed a large number of differences in their hydrophobicity profiles  
165 between residues 68 and 100. The hydrophobicity profiles obtained for G3f and G3h  
166 were compared (Figure 2) and significant differences could be observed. For  
167 subtypes of G4, differences in their hydrophobicity profiles were observed between  
168 residues 67 and 74 and residues 95 and 100 (data not shown).

169 HEV has a quasispecies distribution, with complex mixtures of genetically  
170 distinct but closely related viral populations (Lhomme et al., 2012). This means that  
171 HEV populations coexist at any time point and any change in the environment of the  
172 virus can modify the equilibrium and the quasispecies distribution. Such a  
173 phenomenon has been described for poliovirus (Vignuzzi et al., 2006) and hepatitis C  
174 virus (Farci et al., 1997). The variability of viruses with an RNA genome results first  
175 from errors occurring during genome replication steps with a lack of RNA-dependent  
176 RNA polymerase proofreading activity. There is about one mutation for a 10,000-  
177 nucleotide genome inducing a new virion sequence in each replication process, thus  
178 producing viral quasispecies (van Tong et al., 2016). These mutations can have a

179 high functional impact on viral fitness. Second, selective pressures from host-related  
180 immunity, cellular tissues or interactions with external environmental surfaces could  
181 also have a role in HEV variability. As an example, poliovirus virulence is determined  
182 by quasispecies diversity (Vignuzzi et al., 2006). There will be a cooperative  
183 interaction within a viral population in which some variants may facilitate the  
184 colonization of tissues; others will have an impact on the immune system, while some  
185 may even facilitate circulation of the virus. The hypothesis of highly dominant variants  
186 within quasispecies combining strong pathogenesis, immune evasion and effective  
187 viral circulation in the outside environment can be stated. Moreover, the complexity of  
188 the viral quasispecies could allow the virus to combine complementary functions of  
189 different viral subpopulations.

190 For HEV in the clinical setting, a study from Lhomme [et al.] (2012) has been  
191 conducted to correlate viral genetic variability with the progression towards a chronic  
192 infection and/or cirrhosis. Interestingly, the greater the variability of the M and P  
193 domains, the greater is the likelihood of developing a chronic HEV infection (Lhomme  
194 et al., 2012). As a consequence, long-term HEV faecal excretion from patients  
195 suffering from chronic hepatitis will favour extensive viral spread in the outside  
196 environment.

197 Review of the current literature seems to indicate that the quasispecies  
198 heterogeneity could favour HEV persistence in an immunocompromised host and  
199 that higher variability between HEV populations enhances a virus's ability to infect  
200 varied hosts. We can hypothesize that these genetic characteristics can also have an  
201 impact on the transmission of the virus through water. To gain further insight into  
202 these questions, studies on HEV genetic variability and quasispecies in different  
203 types of samples are needed: hosts with chronic evolution versus hosts with virus

204 clearance, different host species and different environmental samples (Bisseux et al.,  
205 2018).

206 HEV is more commonly defined as a non-enveloped virus. Nevertheless, when  
207 it leaves the hepatocytes, the virion has a membrane envelope, derived from the  
208 exosomes and composed of cholesterol, phospholipids, sphingomyelin,  
209 phosphatidylethanolamine, phosphoinositides and ceramides (Chapuy-Regaud et al.,  
210 2017). It is further exposed to intestinal detergents, which accounts for the  
211 destruction of the envelope (Yin et al., 2016a). Release from infected hepatocytes  
212 mostly consists of quasi-enveloped forms, whereas the non-enveloped form is more  
213 frequent within the infected cells. The quasi-enveloped particles bind to cells less  
214 effectively and more slowly than do non-enveloped particles. This could explain why  
215 HEV from a patient's blood sample seems to be less infectious than that of faecal  
216 origin (Yin et al., 2016b). HEV cellular entry mechanisms remain poorly understood  
217 but it is known that non-enveloped HEV binds to heparan sulfate proteoglycans as a  
218 receptor on the hepatocyte surface. The quasi-enveloped HEV enters the cells  
219 through a number of different steps; it needs two GTPases, Rab5 and Rab7, an  
220 endosomal acidification in the hepatic cells and the lipid degradation activity in the  
221 lysosome (Yin et al., 2016b) (Figure 3). Thus, non-enveloped HEV particles are  
222 excreted in the environment in the case of faecal pollution of animal or human origin,  
223 but quasi-enveloped HEV particles may also be released in the environment in the  
224 case of animal blood pollution from slaughterhouses (Figure 4). To our knowledge,  
225 the environmental excretion of quasi-enveloped HEV particles, compared to non-  
226 enveloped particles which are supposed to be the most prevalent, has not been  
227 taken into account.

228 To sum up, the sources of water pollution are diverse, having human, livestock  
229 and/or wild animal origins, and so are the viral particle types with, from a physical  
230 point of view, quasi-enveloped versus non-enveloped HEV particles and, from a  
231 genetic point of view, four major genotypes (G1 to G4), subgenotypes and  
232 quasispecies.

233

### 234 **3. Epidemiological proof of the water transmission of HEV**

235 G1 and G2 have been largely demonstrated to be transmitted by water in  
236 developing countries (Amany et al., 2017; Hakim et al., 2017; Haque et al., 2015;  
237 Maila et al., 2004; Singh et al., 2016). In India, for example, highly polluted water  
238 caused waves of G1 outbreaks (1978-1984, 1993-1995, 2007-2008, 2012-2013).  
239 Such a wave outbreak pattern has been explained by successive  
240 infection/immunization and immunity loss cycles whose period length is estimated to  
241 be about 10 years. Outbreaks start when immunity decreases to 4% of total  
242 population and stop when it increases to 20% of total population. In such situations  
243 transmission of the zoonotic genotypes seems insignificant. The improvement of  
244 sanitation practices is clearly the solution for G1 control in developing countries  
245 (Khuroo et al., 2016). Interestingly, as observed in China, industrialization of a  
246 country decreases HEV risk related to G1, but increases that related to G3 and G4  
247 (Sridhar et al., 2015).

248 While zoonotic transmission of G3 and G4 has been largely demonstrated  
249 mainly through food (Colson et al., 2010; Masuda et al., 2005; Matsuda et al., 2003;  
250 Meng, 2013) and exposure to pigs (Bouwknegt et al., 2008; Drobeniuc et al., 2001;  
251 Pérez-Gracia et al., 2007) in developed countries, the roadmap to control  
252 autochthonous HEV is not so clear apart from better control of food products. The

253 role of water in the transmission of zoonotic HEV has only been suspected (Sridhar  
254 et al., 2015). Detection of G3 in shellfish and seemingly shellfish-related outbreaks  
255 have recently raised the question (Sridhar et al., 2015). Sometimes no clear direct  
256 transmission from pigs to humans is observed; indirect contamination through the  
257 environment is then probable but difficult to prove (Purdy and Khudyakov, 2011).

258 Some studies conducted in developed countries seem to show higher HEV  
259 seroprevalence in people exposed to water or in shellfish consumers, which supports  
260 the idea of a hydric transmission of HEV G3 (Mansuy et al., 2015, 2016). As there is  
261 only one HEV serotype, it is not possible, in seroprevalence studies, to assess which  
262 genotype the individuals have been in contact with. However, when a study is  
263 conducted in a country where G3 is by far the most frequent HEV genotype, it can be  
264 assumed that it is this genotype that seropositive people have encountered. Thus,  
265 two studies about HEV seroprevalence in blood donors in France show that it is  
266 higher in mussel consumers (Mansuy et al., 2015) and oyster consumers (Mansuy et  
267 al., 2016), compared to non-consumers. In one of these studies, drinking bottled  
268 water seems to be a protective factor (Mansuy et al., 2016). An outbreak on a cruise  
269 ship seemed to be linked with shellfish consumption; G3 was determined in three  
270 patients (Said et al., 2009). A study conducted in Cornwall showed a higher risk of  
271 hepatitis E when living near the coast, whereas there was no difference between  
272 cases and controls for people living near a pig holding (Hunter et al., 2016). A G4-  
273 related outbreak has been suspected to be waterborne in China (Chen et al., 2016).

274 To conclude, HEV G1 and G2 are known to be transmitted through water and  
275 G3 and G4 through a zoonotic path. However, a number of arguments suggest that  
276 the latter genotypes could also be transmitted through water.

277

278 **4. Occurrence of HEV in the water cycle: from the source to the water use in**  
279 **the food chain.**

280       The first stage of the environmental cycle of HEV particles is their excretion  
281 (Figure 3). Of course, non-enveloped particles excreted in the faeces of  
282 contaminated humans (wastewater treatment plants) and animals (slaughterhouses,  
283 farming, faeces of wild animals) may be considered as the main part of HEV particles  
284 released in the environment. However, quasi-enveloped particles coming from blood  
285 might also be excreted in the environment, especially in wastewater originating from  
286 slaughterhouses.

287       Detection of viral HEV genome by molecular tools (e.g. retro-transcription  
288 quantitative polymerase chain reaction [RT-qPCR]) can be used to test for the  
289 occurrence and quantification of the total number of HEV particles (Baez et al., 2017;  
290 La Rosa et al., 2018). The advantages of this procedure include its fast running time,  
291 high sensitivity and specificity, and ease of use. The genome can be detected even if  
292 the capsid is no longer able to bind to its receptor, and free RNA molecules can be  
293 found in some environmental samples (Cook et al., 2016). The detection of the viral  
294 RNA only means that the virus is present but not necessarily that it is infectious  
295 (Gassilloud et al., 2003). In addition, no difference can be made between quasi-  
296 enveloped and non-enveloped HEV. Nevertheless, the molecular approach is useful  
297 in occurrence and epidemiological studies. There are pan-genotypic quantitative real-  
298 time PCR methods that allow the determination of whether a sample contains HEV  
299 genome or not (Jothikumar et al., 2006). For positive samples, a PCR followed by  
300 sequencing can then be performed in order to determine the virus genotype (Erker et  
301 al., 1999; Inoue et al., 2006; Takahashi et al., 2002).

302

303 Numerous studies have described the presence of HEV genome in untreated  
304 sewage over the last 15 years, especially in Europe (Table 1). Occurrences were  
305 very variable; indeed, they ranged from 0% to 93% (UK) and even 100% (India). But  
306 this has to be interpreted with caution since there is substantial variation between  
307 studies in terms of the volumes of samples, the methods used and the period of  
308 sampling. In Europe, the most prevalent HEV genotype seems to be G3, but  
309 sometimes G1 may also be present in wastewater. In other industrialized countries  
310 outside Europe, a lower number of studies have been published but G3 also seems  
311 to be the most frequent. Unsurprisingly, G1 is the most frequent in Asia, but G4 has  
312 been recently detected in China. Fewer analyses have been performed on treated  
313 than on untreated sewage, again mostly in Europe (Table 2). The samples are mainly  
314 negative for HEV genome and the only determined genotype is a G1 in India. To  
315 date, there have been several works on HEV detection and phylogeny but there is a  
316 lack of information on HEV viral loads before and after treatments applied in  
317 wastewater treatment plants (WWTPs). Nevertheless, a study performed in India  
318 showed a slow decrease in the prevalence of HEV from 15.2% (n=46) to 10.8%  
319 (n=46) during wastewater treatment consisting of primary screening, aeration,  
320 agitation, primary-secondary clarification, anaerobic digestion and chlorination  
321 (Vaidya et al., 2002). This shows at least that some HEV particles may cross the  
322 wastewater treatment plant, but quantitative molecular methods should be applied to  
323 raw and treated wastewater in order to better assess virus particle removal.

324 Unsurprisingly, the study of four articles showed that the highest values of  
325 prevalence of HEV in surface water are lower than those described in sewage and do  
326 not exceed 20%. Nevertheless, values ranging from 0.6% (1/154) in 1 L (Gentry-  
327 Shields et al., 2015) to 17% (2/12) in 230-260 L (Rutjes et al., 2009) are reported.

328 Genome concentrations of up to  $10^4$  genome copies/L have been reported in lake  
329 and river water in Italy; these two sites either were located in an urbanized area or  
330 had a history of enteric viral contamination (D'Ugo et al., 2016). A study conducted in  
331 the USA showed that in surface waters the occurrence and concentration of HEV  
332 increased significantly after manure application; the genotype could not be  
333 determined because of the shortness of the sequence and its location in a highly-  
334 conserved region. The authors suggested that runoff and tile input are important  
335 mechanisms of HEV transport to surface waters and that subsurface drainage and  
336 overland flow contribute to the transport of HEV to adjacent waters (Givens et al.,  
337 2016). In poor countries, humans defecate in backyards and open fields, the rain  
338 then washes away these faecally contaminated areas into other waterways, a  
339 phenomenon which can contaminate crops, waterways, groundwater and open  
340 waterways (Khuroo et al., 2016); the same phenomenon of HEV being transported by  
341 water could be hypothesized with G3 and G4 in developed countries with animal  
342 faeces and manure or bypass of untreated water in a wastewater treatment plant. As  
343 a result, HEV has the potential to contaminate surface waters used for drinking water  
344 production and recreational purposes. Thus, surface waters could be a source of  
345 contamination for animals, shellfish and humans.

346 Bivalve molluscan shellfish (BMS) are known transmitters of enteric viruses, in  
347 particular norovirus (NoV) and hepatitis A virus (HAV) (Bellou et al., 2013); an ISO  
348 method has been proposed for genome quantification of NoV and HAV in various  
349 foodstuffs including BMS (NF EN ISO 15216 2017). Studies have been led on  
350 different BMS in Europe and Asia with various results (Table 3), and again G3 is the  
351 most represented in Europe, whereas G3 and G4 are found in Asia. Elsewhere, as  
352 mentioned above, water can contaminate food products by irrigation of crops (Khuroo

353 et al., 2016): studies led in Canada and various countries in Europe have found HEV  
354 on field-grown strawberries, vegetables, frozen raspberries and salad (Brassard et  
355 al., 2012; Kokkinos et al., 2012; Maunula et al., 2013; Terio et al., 2017).

356

357 To conclude on this part, it appears that HEV particles may be found at each  
358 stage of the water cycle even if their infectivity cannot be confirmed by molecular  
359 methods. They may be found in large numbers in rivers in some cases resulting in a  
360 direct contamination risk for animals or indirect risk for humans through irrigation of  
361 crops, contamination of resources for drinking water production and in BMS  
362 producing areas. To our knowledge, the minimal HEV infectious dose is still unknown  
363 but by analogy with NoV (Hall, 2012) and HAV (Sánchez, 2013), we can assume that  
364 10 to 50 viral particles can be enough to make people ill and lead to an outbreak. To  
365 know if this HEV can really infect hosts and in what proportion, we need to assess its  
366 capacity to remain infectious in water, that is its survival.

367 **5. HEV survival in water**

368 Under natural conditions, temperature and sunlight (UV) are the two main  
369 factors that may inactivate HEV in water. During drinking water treatment, the  
370 physical treatment allows the removal of the viral particle, whereas the disinfection  
371 process mainly based on chlorine impacts virus survival.

372 **5.1. Method used to evaluate survival**

373 The reference method to evaluate viral infectivity is *in vitro* cell culture or  
374 assays which can be performed *in vivo*. The use of the term “inactivation” for a virus  
375 means that the infectivity has been assayed by a cell culture method. Many enteric  
376 viruses can be cultivated on cells but some are difficult to cultivate, and HEV is one

377 of them. That being said, some culture systems have been found. A G3 strain, JE03-  
378 1760F, and a G4 strain, HEV-JF5/15F, originating from human stool samples have  
379 been cultivated on PLC/PRF/5 cells (human hepatic carcinoma) and A549 cells  
380 (human lung cancer). The G4 strain originated from a patient with a fulminant  
381 hepatitis. This strain was propagated and passed more effectively than the G3 one  
382 (Tanaka et al., 2009). Moreover, HepG2 and Huh7 cells (human hepatic carcinoma)  
383 are not permissive for JE03-1760F and HEV-JF5/15F strains originating from the  
384 stool samples but can support propagation and passages of the cell culture-adapted  
385 JE03-1760F and HEV-JF5/15F strains (Okamoto, 2013). G1, G3 and G4 strains  
386 originating from blood samples can also be cultivated on PLC/PRF/5 and A549 cells  
387 (Okamoto, 2013). A G3 strain, Kernow-C1, isolated from a chronically infected  
388 patient's stool could effectively infect numerous cell lines from humans (hepatic  
389 carcinoma: HepG2/C3A and HUH7.5, hepatoma: PLC/PRF/5, lung carcinoma: A549  
390 and colorectal adenocarcinoma: Caco-2), pigs (kidney: LLC-PK1, LLC-PK1A and SK-  
391 RST) and deer (liver: OHH1.Li). After being passed six times on HepG2/C3A cells,  
392 the Kernow-C1 strain was able to grow more effectively in these cells (Shukla et al.,  
393 2011). Among these cell lines, LLC-PK1 seems to be the most permissible to Kernow  
394 C1 and Kernow C1/p6 infections (Shukla et al., 2011). Another strain has been  
395 described by other authors: LBPR-0379 strain, isolated from a chronically infected  
396 patient (G3), has a growth advantage in cell culture (HepG2/C3A cells) (Nguyen et  
397 al., 2012). Recently, a three-dimensional culture system has been developed for  
398 HEV; it seems to be a promising system that needs to be improved (Berto et al.,  
399 2013). However, the drawback of all these culture methods is that they are time  
400 consuming and difficult to apply. Another method has been developed by some  
401 authors in order to overcome the problems of culture and RT-PCR; it consists in a

402 pre-treatment of the sample with an RNase, prior to RNA extraction. Free RNA  
403 (damaged viruses) will be destroyed by the RNase and only encapsidated RNA will  
404 be taken into account. This method has been used on HEV in order to study its  
405 resistance to some temperatures (Schielke et al., 2011).

406 Another approach has been developed: the use of intercalating dyes such as  
407 propidium monoazide (PMA) and ethidium monoazide (EMA). This strategy allows  
408 the assessment of capsid integrity. These molecules will enter the cell if the  
409 membrane integrity is disrupted, covalently bind to DNA and inhibit PCR (Girones et  
410 al., 2010). Some authors have tested this approach on bacteria and recently others  
411 have attempted to develop it on RNA viruses (Leifels et al., 2015; Parshionikar et al.,  
412 2010) with various results. This method is not relevant if the virus is inactivated with a  
413 preserved capsid and the secondary RNA structure seems to interfere with the  
414 binding of the intercalating agent (Parshionikar et al., 2010). The results can also  
415 vary according to which virus is studied and which region of its genome is targeted by  
416 PCR (Leifels et al., 2015).

417 In order to properly draw conclusions with cell culture work, it is useful to know  
418 if we are working with non-enveloped or quasi-enveloped HEV. It seems that when  
419 working with liver cells such as Huh-7, PLC/PRF/5 and HepG2 or lung cells such as  
420 A549, the produced virions are predominantly quasi-enveloped. If the virus is then  
421 treated with a bile salt (e.g. deoxycholic acid) and a protease (e.g. trypsin), it can lose  
422 its envelope (Okamoto, 2013; Takahashi et al., 2010; Yin et al., 2016b, 2016a).

## 423 5.2. Thermal inactivation

424 A review has summarized all tested methods of inactivation for HEV in food  
425 matrices (Cook and van der Poel, 2015). As a consequence, it focused on high

426 temperatures used for food treatment or cooking which do not match environmental  
427 conditions. Nevertheless, some interesting data were described.

428 First, even if in the review no discussion was made about enveloped and non-  
429 enveloped virus we can extract some data concerning the survival of non-enveloped  
430 HEV, since stool-extracted strains were studied. Moreover, some of these data could  
431 be compared with those reported in a review focusing on the impact of temperature  
432 on the inactivation of enteric viruses in food and water (Bertrand et al., 2012). In the  
433 latter review, the authors determined the TFL value of each enteric virus, which was  
434 defined as the time needed (in days) to observe the first  $\log_{10}$  reduction. The TFL  
435 values of the different enteric viruses were compared as a function of the  
436 temperature range ( $<50^{\circ}\text{C}$  or  $\geq 50^{\circ}\text{C}$ ), of the matrix type (simple or complex) and the  
437 detection method (cell culture or PCR). The  $\log_{10}$ TFL was then used. A few studies  
438 described in Cook's review allow us to calculate some TFL values. Thus, a one log  
439 reduction was observed for HEV G1 strain in stool suspension after exposure to  $56^{\circ}\text{C}$   
440 for 15 min (Emerson et al., 2005). This means that the TFL value reached 0.01 day  
441 under these conditions, which is close to the TFL values observed for highly resistant  
442 enteric viruses such as PhiX174 phage and F-specific RNA phages of genogroup I.  
443 This also suggests that HEV could be more resistant than HAV under these particular  
444 conditions, but there is a huge difference in the number of data between these two  
445 hepatitis viruses with 38 data for HAV (Bertrand et al., 2012) and only one for HEV.  
446 From another study (Yunoki et al., 2008), the TFL of HEV at  $60^{\circ}\text{C}$  could be estimated  
447 at 0.02 day in a 25% albumin solution and 0.007 day in phosphate-buffered saline. In  
448 both cases, the comparison with data from the review (Bertrand et al., 2012)  
449 suggests that HEV has a resistance close to that observed for F-specific RNA  
450 phages and higher than that observed for HAV. When HEV was subjected to dry

451 heating, the TFL seemed to be of 0.4 day at 60°C and of 0.16 day at 80°C, which is  
452 much higher than under the previous heat conditions (Yunoki et al., 2008).

453 Johne et al. (2016) have evaluated the impact of high temperatures in the  
454 range of 60°C - 80°C on quasi-enveloped HEV in suspension in cell culture medium.  
455 The TFL value reached  $2.3 \times 10^{-4}$  day (20 sec) at 70°C. These authors worked with  
456 quasi-enveloped HEV particles; we can thus attempt a comparison with an  
457 enveloped virus. We have chosen hepatitis C virus (HCV) which is a well-  
458 documented enveloped virus; like HEV, its genome consists of a positive polarity  
459 single strand RNA. HCV in cell culture medium at a concentration of  $2.5 \times 10^3$  FFU  
460 (Focus Forming Unit) had a TFL of about  $6 \times 10^{-4}$  day (40 seconds) at 65°C (Song et  
461 al., 2010a). Thus, the resistance of quasi-enveloped HEV might be close to that  
462 observed for HCV at high temperatures. In the study by Schielke et al. (2011), the  
463 authors assayed the effect of short-term heating on RNase-protected HEV G3  
464 particles. A reduction of 74% was observed after incubation at 56°C for 15 min and  
465 reduction rates above 99% (-2.16 to -4.42 log<sub>10</sub>) were observed after incubation at  
466 56°C for 30 min or 60 min and at 60°C for 15 min, 30 min or 60 min. As these authors  
467 worked on a liver suspension, we can infer that they mostly worked on quasi-  
468 enveloped HEV; we can thus attempt a comparison with HCV as above. When in  
469 human serum, a 3.6 log<sub>10</sub> inactivation was observed at 56°C for 30 min (Song et al.,  
470 2010a). Caution is required when comparing these data since the assays were  
471 performed using a PCR-derived technique for the detection of RNase-protected HEV  
472 (Schielke et al., 2011) and by cell culture for HCV (Song et al., 2010a). Johne et al.  
473 (2016) and Schielke et al. (2011) also worked at low temperatures (i.e. ≤37°C) more  
474 frequently encountered under environmental conditions. TFL values could be  
475 calculated from the results of Johne et al. (2016). A TFL of about 3 days at 22°C and

476 about 2 days at 37°C in cell culture medium could be determined. If, again, we  
477 compare that to HCV, the TFL is of about 5 days at 25°C ± 2°C and about 9 hours at  
478 37°C (Song et al., 2010a). This would make quasi-enveloped HEV less resistant than  
479 HCV at room temperature and more resistant at 37°C, but these results need  
480 confirmation from other studies.

481 Second, the thermal resistance of HEV may depend on the virus strain as  
482 Emerson et al. (2005) suggested while working with two G1 strains and one G2  
483 strain. Third, the culture-based detection method directly or indirectly provides highly  
484 variable results, and sometimes only qualitative results.

485 The resistance of HEV to heat might depend on the genotype and on the  
486 origin of the virus, which makes it either quasi-enveloped or non-enveloped. All in all,  
487 on average, HEV seems about as resistant as F-specific RNA phages for non-  
488 enveloped HEV or HCV for quasi-enveloped HEV. However, other studies would be  
489 needed with, as much as possible, harmonized temperatures, durations, virus forms,  
490 matrices and measurement methods.

491 **5.3. UV inactivation**

492 A recent study has compared the impact of UV radiation on HEV G3 and MS2  
493 phage in terms of loss of infectivity and degradation of the genome (Guerrero-Latorre  
494 et al., 2016). The inactivation of HEV was estimated by cell culture combined with  
495 immunofluorescence. A viral suspension of  $5.93 \times 10^2$  FFU/mL was first irradiated  
496 with fluences of 10, 20, 40, 80, 100 and 140 mJ/cm<sup>2</sup>. Since a fluence of only 10  
497 mJ/cm<sup>2</sup> was sufficient to inactivate HEV, a second experiment was performed with a  
498 viral suspension of  $5.58 \times 10^3$  FFU/mL by using fluences of 0, 2, 5, 7 and 10 mJ/cm<sup>2</sup>.  
499 A regression curve was then constructed to predict fluences needed for viral

500 inactivation; it allowed the authors to determine that a fluence of  $23.19 \text{ mJ/cm}^2$  was  
501 needed for a  $4 \log_{10}$  (99.99%) inactivation. The viral suspensions were coming from  
502 culture; it can thus be assumed that the inactivation of HEV by UV was determined  
503 for quasi-enveloped HEV. A review paper has compared the UV radiation values  
504 needed to inactivate enteric pathogenic viruses such as poliovirus ( $n=6$ ), HAV ( $n=3$ ),  
505 coxsackievirus ( $n=2$ ) and adenovirus ( $n=7$ ), and bacteriophages such as MS2 ( $n=5$ )  
506 and Q $\beta$  ( $n=1$ ) F-specific RNA phages, and PhiX174 ( $n=4$ ) phages (Hijnen et al.,  
507 2006). The inactivation rate constants named k-values were determined for each  
508 virus; the higher it is, the more sensitive to UV the microorganism is. The lowest k-  
509 values were obtained for adenovirus and MS2 phage. The k-value of MS2 phage  
510 reached 0.08 in the experiments performed by Guerrero Latorre et al. (2016) and  
511 was in accordance with the review by Hijnen et al. (2006). For quasi-enveloped HEV,  
512 a k-value of 0.153 could be calculated; it was close to that of HAV, which was the  
513 least resistant of the viruses compared in the review published by Hijnen et al.  
514 (2006). For experiments performed with poliovirus and F-specific RNA phages it has  
515 been shown that inactivation by UV is correlated with genome size; the longer the  
516 genome is, the more sensitive it is to UV (Simonet and Gantzer, 2006). We should  
517 notice that HAV and HEV have about the same genome length with  $7.5$  and  $7.2 \times 10^3$   
518 nucleotides, respectively. Moreover, Simonet and Gantzer (2006) have also shown  
519 that genome degradation could explain UV-induced viral inactivation.

520 To summarize, quasi-enveloped HEV virus has a resistance to UV similar to  
521 that of other enteric viruses such as HAV, but lower than that of MS2 phage.  
522 However, other experiments would be needed in order to test non-enveloped HEV,  
523 which would be more representative of particles that can be found in environmental

524 waters; other studies on other genotypes also need to be conducted because, as  
525 with thermal inactivation, virus behaviour might vary from one genotype to another.

526 **5.4. Chlorine inactivation**

527 In a study reported by Cook and van der Poel (2015), HEV treated with  
528 sodium hypochlorite was inoculated into a culture of PLC/PRF/5 cells: no cytopathic  
529 effect was observed but replication could be inferred from detection of HEV RNA.  
530 However, HEV seems to be chlorine sensitive; indeed, in the same study it was  
531 reported that in cultures inoculated with untreated HEV RNA was detectable up to  
532 day 33, whereas it was detectable only for 7 days with treated HEV (results not  
533 shown). Another study, led by Girones et al. (2014), compared chlorine resistance of  
534 HEV G1 to that of Human Adenovirus 2 (HAdV2). In that study, HEV G1, originating  
535 both from culture and faeces, was treated with sodium hypochlorite at 1,000 ppm; a  
536 first phase with an initial decline in infectious units of approximately 2 log was  
537 reported, followed by a phase of further decline of less than 1 log and a still  
538 detectable RNA and infectious viruses at 30 minutes. HAdV2 was more resistant to  
539 chlorination than HEV: CT (concentration × time) values of 0.15 mg/L × min for quasi-  
540 enveloped HEV and 0.12 mg/L × min for non-enveloped HEV for 1-log reduction for  
541 HEV and 1.15 mg/L × min for 1-log reduction for HAdV2. The authors then compared  
542 chlorine activity on HEV with and without sewage in the water: CT values needed for  
543 a 2-log reduction of HEV infectivity was 20 times higher with 1% sewage in the water,  
544 compared to without sewage. We can attempt a comparison with HAV: Sobsey et al.  
545 (1988) have studied the resistance of HAV to chlorination. From their data, the CT  
546 value needed for 1-log reduction was estimated to be about 0.85 mg/L × min at 5°C  
547 and pH 6 and about 1 mg/mL × min at 5°C and pH 8. In their study on HEV, Girones  
548 et al. (2014) do not specify the pH and temperature conditions under which they

549 performed their experiments so the comparison with HAV is not easy but if we  
550 suppose that the conditions were close to those of Sobsey et al., then we can  
551 conclude that HEV is more sensitive to chlorination than HAV.

552 HEV thus seems to be quite sensitive to chlorination, as it is more susceptible  
553 than HAdV2 and HAV. However, this needs to be confirmed by further studies carried  
554 out with more harmonized conditions (temperature, pH) and different types of HEV in  
555 different types of environment (e.g., “clean” water, sewage-containing water, etc.).

556

557 All in all, it seems that HEV does not exhibit a particularly high level of  
558 resistance to the main inactivating factors for viruses in water (i.e. heat, UV and  
559 chlorine) compared to classical models of enteric viruses, namely MS2 phage or F-  
560 specific RNA phages, or to other pathogenic enteric viruses such as HAV. The  
561 classical multi-barrier treatments to prevent enteric virus transmission to humans  
562 should thus be effective for HEV. The question is still under discussion for animals. In  
563 order to expand our knowledge of HEV resistance to treatments, a method to assess  
564 rapidly and with high sensitivity and specificity the quantity of infectious viruses would  
565 be needed and, as much as possible, standardized studies need to be conducted.  
566 Harmonizing temperature or chlorine concentration is difficult to achieve but some  
567 efforts can be made on the duration of study or on the UV wavelength and fluence.

568 **6. Physical removal in the environment and water treatment**

569 The behaviour of virus particles in the environment depends mainly on their  
570 size and on their surface properties, including their electrostatic charge and  
571 hydrophobicity.

572 The size of HEV is similar to that of most other pathogenic enteric viruses  
573 such as HAV (27-33 nm) or NoV (27 nm) and phages used as models such as F-  
574 specific RNA (20-30 nm) (Hartard et al., 2016; Kapikian et al., 1972; Kuzmanovic et  
575 al., 2003; Rumnieks and Tars, 2017; Yokosuka, 2000).

576 It is known that the virus charge depends on its isoelectric point  
577 (abbreviations: pl or IEP) and on the pH of the medium. A negative charge is  
578 observed if the pH is above the pl and, conversely, a positive charge is observed  
579 when the pH is below the pl (Gerba, 1984). To date, nothing is known about the pl of  
580 HEV, even less so about its charge under environmental conditions. Defining total  
581 charge is far from an easy task because viruses are soft particles for which the  
582 genome may have an impact on the surface charge (Dika et al., 2011; Langlet et al.,  
583 2008a). The variability of HEV may also impact its pl and charge. Indeed, as there  
584 are different genotypes and subgenotypes, a quasispecies distribution, quasi-  
585 enveloped and non-enveloped forms, the virus can be found under different forms  
586 within environment waters. This can have an impact on its hydrophobicity and  
587 charge, and thus on its behaviour. Charge greatly influences virus behaviour  
588 (Bhattacharjee et al., 2002); thus, high negative charge or low pl leads to low  
589 adhesion/aggregation capacity. In a review written in 2010, a summary of the  
590 isoelectric points of various viruses was constructed. From this summary it was  
591 concluded that the pl is essential but not sufficient to predict adhesion (Michen and  
592 Graule, 2010). In fact, hydrophobicity has also proved to be highly important in the  
593 adhesion/aggregation process and may be of great importance in water treatment  
594 processes for example (Boudaud et al., 2012). Hydrophobic interactions are short  
595 distance interactions which may take place when the electrostatic charge of viral  
596 particles is low because of the virus type, the medium pH close to the pl or a high

597 ionic strength leading to ions neutralizing the particle charge (Dika et al., 2015;  
598 Langlet et al., 2008b). Hydrophobicity of the different amino acids can also have an  
599 impact on surface adhesion, as shown for NoV (Samandoulgou et al., 2015).  
600 Changes in physico-chemical conditions, e.g. ionic strength or temperature, can  
601 affect the secondary or tertiary structure and change which amino acids are exposed  
602 on the surface, thus modifying hydrophobicity and adhesion to hydrophobic surfaces.  
603 As discussed before, HEV can exist under non-enveloped or quasi-enveloped forms;  
604 the presence of the quasi-envelope means that there are lipids, which should make  
605 the virus more hydrophobic, thus modifying its physico-chemical characteristics, and  
606 therefore its behaviour. Again, very little is known about HEV hydrophobicity. To  
607 begin addressing the question of the impact of hydrophobicity, we compared ORF2  
608 sequences between the different HEV genotypes using the AnTheProt software. As  
609 already stated in the second paragraph and as shown in Figure 2, subgenotypes 3  
610 showed a large number of differences in their hydrophobicity profiles between  
611 residues 68 and 100. The hydrophobicity profiles obtained for G3f and G3h were  
612 compared, which showed major differences. For subgenotypes 4, differences in their  
613 hydrophobicity profiles were observed between residues 67 and 74, and residues 95  
614 and 100 (data not shown).

615 To conclude on this part, HEV is about the same size as many pathogenic  
616 enteric viruses and bacteriophages, from which we could infer some behavioural  
617 data. However, its charge, hydrophobicity and adhesion are still unknown and, given  
618 its high variability (intergenotype, intragenotype, quasispecies and different forms),  
619 these criteria could vary, which could impact the virus behaviour and transmission.

620 **7. How may we include the variability of HEV in future studies concerning  
621 water?**

622 In the future, the investigation into HEV variability concerning water will be  
623 facilitated by a number of increasingly sensitive molecular methods, designed to  
624 analyse viral genomic plasticity. These methods could prove useful for the study of  
625 the sequences of different HEV isolates, coming from different types of samples, in  
626 order to better understand the virus circulation. Concentration and extraction  
627 methods are difficult to implement: charge, size and hydrophobicity of the virus are  
628 variable.

629 So far, it has been possible to use direct sequencing (Sanger sequencing) to  
630 sequence a variable part of the genome and then to study phylogenetic links based on  
631 the results, and to determine the genotype and sometimes the subgenotype (Xia et  
632 al., 2015). That method only shows the main HEV population, but allows for the direct  
633 comparison between the sequences of different samples.

634 When it comes to quasispecies study, cloning-sequencing or Ultra-Deep  
635 sequencing (UDS) can be used. Cloning-sequencing is considered to be a reference  
636 method in the exploration of viral quasispecies, but this method has drawbacks: it is  
637 time consuming, expensive and the number of clones that are studied limits its  
638 sensitivity (Ramírez et al., 2013). In the best-case scenario, about 100 clones can be  
639 sequenced, which poorly represents a population of millions (or even more) of viral  
640 particles. UDS method allows the production of millions of sequences within one run,  
641 and is less expensive than previous methods. Many different UDS techniques have  
642 been developed such as Illumina (sequencing using synthesis), Ion Torrent or  
643 pyrosequencing. Each one of them has its own advantages and drawbacks and the  
644 choice of technique used will depend on the local equipment, personal experience  
645 and the type of microorganism being studied (Quail et al., 2012).

646 The outputs from the Sanger sequencing and UDS should be analysed by

647 informatics tools. Analyses can be done on the resulting data, keeping in mind the  
648 pros and cons of each sequencing technique. These different techniques can be  
649 used together and compared. According to the data, comparisons can be made  
650 between the sequences of different samples and between the different  
651 subpopulations within one sample. The diversity and complexity can be calculated  
652 between different subpopulations (Lhomme et al., 2012).

653 Various protein properties such as the antigenicity profile can be predicted  
654 through use of the AnTheProt software as discussed above (Parker et al., 1986).  
655 This approach has also been used in some studies and helps the determination of  
656 whether or not an amino acid substitution has consequences on the function of the  
657 protein (Schvoerer et al., 2013). It can help the determination of viral behaviour and  
658 give information on the transmission (which can be completed by analysis of different  
659 sample types such as human or animal samples).

660 However, there is a need for a method that would allow the concentration of  
661 HEV. As many of the virus characteristics vary, it would be complicated to use a  
662 method based on viral size, charge or hydrophobicity. As long as many of HEV  
663 characteristics remain unknown or poorly known, methods to study the virus will be  
664 difficult to implement.

665

666 **Conclusion**

667 The transmission and persistence of HEV in the environment are still poorly  
668 understood. As explained in the present review, HEV seems to be quite resistant to  
669 high temperatures in its non-enveloped form and as resistant as HCV in its quasi-  
670 enveloped form. We point out that the few studies performed on UV treatment  
671 suggest that HEV has the same susceptibility as HAV, which is a UV-sensitive virus  
672 compared to MS2 phage, and that HEV is sensitive to chlorination. The classical  
673 treatments and surveillance methods to fight against enteric viruses should be  
674 effective against HEV.

675 We are dealing with a highly variable virus: there are different genotypes and  
676 subgenotypes, a quasispecies distribution and quasi-enveloped and non-enveloped  
677 forms. Different particles can thus be found in environmental waters depending on  
678 the geographic location or the origin and type of pollution. This can have an impact  
679 on the virus quantity, hydrophobicity, charge and infectivity, and therefore on its  
680 behaviour and resistance to treatments. Virus culture from water samples would be  
681 informative but currently seems rather ambitious. More information is also needed  
682 concerning HEV surface properties (isoelectric point, hydrophobicity) to better predict  
683 its behaviour in the environment and develop optimized concentration techniques in  
684 order to study it. This knowledge will help sanitary authorities to monitor the  
685 circulation of HEV and to develop measures to prevent human contamination.

686

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690

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1073 **Figure 1:** A: phylogenetic tree of amino acids of the ORF2 M domain of G1 reference  
1074 sequences (Smith et al., 2016a). B: phylogenetic tree of amino acids of the ORF2 P  
1075 domain of G1 reference sequences (Smith et al., 2016a). C: phylogenetic tree of  
1076 amino acids of the ORF2 M domain of G3 reference sequences (Smith et al., 2016a).  
1077 D: phylogenetic tree of amino acids of the ORF2 P domain of G3 reference  
1078 sequences (Smith et al., 2016a). Overall mean distance: 0.015 (G1) versus 0.004  
1079 (G3) for M domain and 0.026 (G1) versus 0.010 (G3) for P domain.

1080 **Figure 2:** AnTheProt hydrophobicity profiles for HEV genotypes 3f and 3h on HEV  
1081 ORF2. We worked on 25 amino acid sequences of ORF2 of the different HEV  
1082 genotypes. The results are given for subgenotypes 3f and 3h (ref 3f: KT591534; 3h:  
1083 JQ013794) (Smith et al., 2016a). Black circles highlight the variations in the upper  
1084 part of the image. aa: amino acids. At the bottom: superposed profiles; subgenotype  
1085 3f is in black and subgenotype 3h is in grey. The black arrows show the differences.  
1086 Most differences are seen at the N-terminal end and, to a lesser extent, C-terminal  
1087 end of the protein.

1088 **Figure 3:** HEV replication and its possible paths after cell egress. **1:** Initial fixation to  
1089 hepatocytes using heparan sulfate as a non-specific receptor of HEV. Specific  
1090 receptor(s) is (are) still unknown. **2:** Specific fixation to clathrin and dynamin-2. **2':**  
1091 For quasi-enveloped HEV, cell entry could require two GTPases (Rab5 and Rab7),  
1092 an endosomal acidification and the lipid degradation activity in the lysosome. **3:**  
1093 Endocytosis. **4:** HEV replication. **5:** HEV leaves the cell in a quasi-enveloped form. **6:**  
1094 The virus quasi-envelope is destroyed in the bile after exposure to intestinal  
1095 deoxycholic acid and proteases (Yin et al., 2016a) and non-enveloped HEV can be  
1096 found in the faeces. **7:** The quasi-enveloped HEV may be a key form in cell-to-cell  
1097 infection.

1098 **Figure 4:** HEV circulation in the environment and between its hosts. **1:** Pollution of  
 1099 human origin that arrives in wastewater treatment plants is mainly composed of non-  
 1100 enveloped faecal HEV particles. **2:** When infected pigs are slaughtered, pollution  
 1101 both composed of non- and quasi-enveloped HEV is introduced in wastewater  
 1102 treatment plants. **3:** Discharge of treated wastewater, the quality of which can even  
 1103 be altered in case of malfunction (e.g., bypass of untreated water due to heavy rain).  
 1104 **4:** Infected pig stool can contaminate environmental waters through manure runoff,  
 1105 HEV is then in a non-enveloped form. **5:** Shellfish that grow in environmental waters  
 1106 can then be contaminated by HEV. **5bis:** Humans could possibly get infected by  
 1107 consumption of contaminated shellfish. **6:** Humans and animals could get infected  
 1108 through consumption of contaminated water. **7:** Crops can be contaminated by  
 1109 irrigation. **7bis:** Humans and animals that consume the crops could possibly get  
 1110 infected. **8:** When eating meat of infected animals, humans can be theoretically  
 1111 contaminated by quasi-enveloped HEV. **9:** Contamination occurs between animals.  
 1112 **10:** Contamination can also take place between humans through blood transfusion  
 1113 by quasi-enveloped HEV.

1114

1115 **Table 1:** Various studies conducted around the world concerning HEV prevalence in  
 1116 untreated sewage water. NA: not applicable, ND: not done, ng: not given, gc:  
 1117 genome copies, y: year, m: month.

1118 **Table 2:** Various studies conducted around the world concerning HEV prevalence in  
 1119 treated sewage water. NA: not applicable, ND: not done y: year, m: month.

1120 **Table 3:** Various studies conducted around the world concerning HEV prevalence in  
1121 bivalve molluscan shellfish. NA: not applicable, ND: not done; PDU: PCR detectable  
1122 units, ng: not given, gc: genome copies, y: year, m: month.

**Table 1:** Various studies conducted around the world concerning HEV prevalence in untreated sewage water. NA: not applicable, ND: not done, ng: not given, gc: genome copies, y: year, m: month.

Location	Volume analysed	Sampling period	Occurrence	Positive/Total	Genotype (n)	Viral load (gc/L)	Reference
<b>Europe</b>							
France		1 m	25%	1/4	3 (1)	ND	Clemente-Casares et al., 2003
France	1 L	3 m	22.2%	4/18	3f (4)	$1 \times 10^3$ - $6.3 \times 10^5$	Miura et al., 2016
Greece		2 m	0%	0/5	NA	ND	Clemente-Casares et al., 2003
Greece	100 mL	1 y 9 m	0%	0/48	NA	NA	Kokkinos et al., 2011
Italy	10 mL	1 y	16%	19/118	1 (18), 3 (1)	ND	La Rosa et al., 2010
Italy	500 mL	1 y	4.76%	1/21	3 (1)	ND	Iaconelli et al., 2015
Norway	40 mL	1 y	8%	4/50	Probable 3 (2)	ND	Myrmel et al., 2015
Portugal	60 mL	2 m	6.7%	2/30	3 (2)	ND	Matos et al., 2016
Spain	40 mL	7 y 2 m	43.5%	20/46	3 (6)	ND	Clemente-Casares et al., 2003
Spain	42 mL	7 y 2 m	31.8%	29/91	1 (1), 3 (14)	$3.2 \times 10^4$	Rodriguez-Manzano et al., 2010
Spain	50 mL	1 y 6 m	13.5%	5/37	ND	ND	Rusiñol et al., 2015
Sweden		2 m	0%	0/4	NA	ND	Clemente-Casares et al., 2003

<b>Sweden</b>	5 L	1 m	33.3%	1/3	ND	$4.5 \times 10^4$	Wang et al., 2018
<b>Switzerland</b>	500 mL	1 y	32.3%	40/124	1 (1)	$7.8 \times 10^4$	Mascaux et al., 2013
<b>Switzerland</b>	90 mL	1 y	24.1%	15/62			Mascaux et al., 2013
<b>UK</b>	20 mL	9 m	93%	14/15	3 (14)	ND	Smith et al., 2016b
<b>North America</b>							
<b>USA</b>	40 mL	1 m	20%	1/5	3 (1)	ND	Clemente-Casares et al., 2003
<b>South America</b>							
<b>Argentina</b>	1,500 mL	4 y	6.3%	3/48	3 (3)	ND	Martínez Wassaf et al., 2014
<b>Columbia</b>	2 L	6 m	30%	3/10	3 (3)	ND	Baez et al., 2017
	50- 1,500 mL	3 m	0%	0/10		NA	Baez et al., 2017
		17 m	20%	2/10		NA	Baez et al., 2017
<b>Africa – Middle East</b>							
<b>Egypt</b>	4 L	1 y 2 m	0%	0/76	NA	NA	Kamel et al., 2011
<b>Egypt</b>	5 L	5 m	23.1%	3/13	ND	$6.9 \times 10^2$	El-Senousy and Abou-Elela, 2017
<b>Israel</b>	1 L	2 y	8.3%	14/169	3 (5)	ND	Ram et al., 2016
<b>Tunisia</b>	80 mL	ng	0%	0/24	NA	NA	Hmaied et al., 2015
<b>Asia</b>							
<b>India</b>	500 mL	3 m	100%	9/9	ND	ND	Jothikumar et al., 1993
<b>China</b>	10 mL	3 m	1.3%	2/152	4 (2)	ND	Li et al., 2017

<b>India</b>	200 µL- 10 mL	1 y	11%	9/82	1a (10)	ND	Vaidya et al., 2002
<b>India</b>	32 mL	2 y	41%	79/192	1 (6)	ND	Ippagunta et al., 2007
<b>Japan</b>	50 mL	1 y 6 m	1.6%	1/62	3 (1)	ND	Ishida et al., 2012
<b>Japan</b>	50 mL	11 m	11%	3/28	ND	$1.7 \times 10^5$ - $1.8 \times 10^6$	Kobayashi et al., 2017

**Table 2:** Various studies conducted around the world concerning HEV prevalence in treated sewage water. NA: not applicable, ND: not done y: year, m: month.

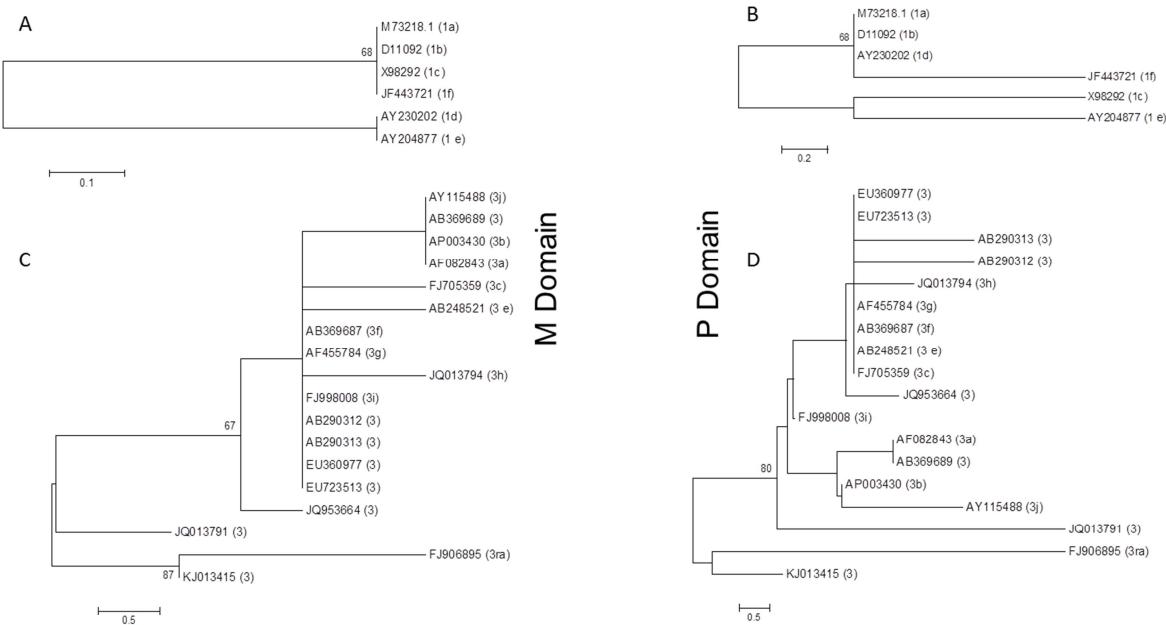
Location	Volume analysed	Sample period	Occurrence	Positive/Total	Genotype (n)	Viral load	Reference
<b>Europe</b>							
<b>Italy</b>	500 mL	1 y	0%	0/21	NA	NA	Iaconelli et al., 2015
<b>Italy</b>	500 mL	1 y	0%	0/21	NA	NA	Iaconelli et al., 2017
<b>Norway</b>	40 mL	1 y	8%	4/50	1 (1) 3 (1)	ND	Myrmel et al., 2015
<b>Portugal</b>	Not specified	4 m	0%	0/60	NA	NA	Matos et al., 2016
<b>Spain</b>	50 mL (pre-treated)	1 y 6 m	12.5%	4/32	ND	ND	Rusiñol et al., 2015
<b>Spain</b>	10 L	1 y 6 m	0%	0/22	NA	NA	Rusiñol et al., 2015
<b>Switzerland</b>	500 mL	1 y	0%	0/25	NA	NA	Mascaux et al., 2013
<b>Africa</b>							
<b>Egypt</b>	2 L	1 y 2 m	0%	0/38	NA	NA	Kamel et al., 2011
<b>Asia</b>							
<b>India</b>	10 mL-200 µL	1 y	10.88%	5/46	1a	ND	Vaidya et al., 2002
<b>Japan</b>	200-500 mL	1 y 6 m	0%	0/53	NA	NA	Ishida et al., 2012

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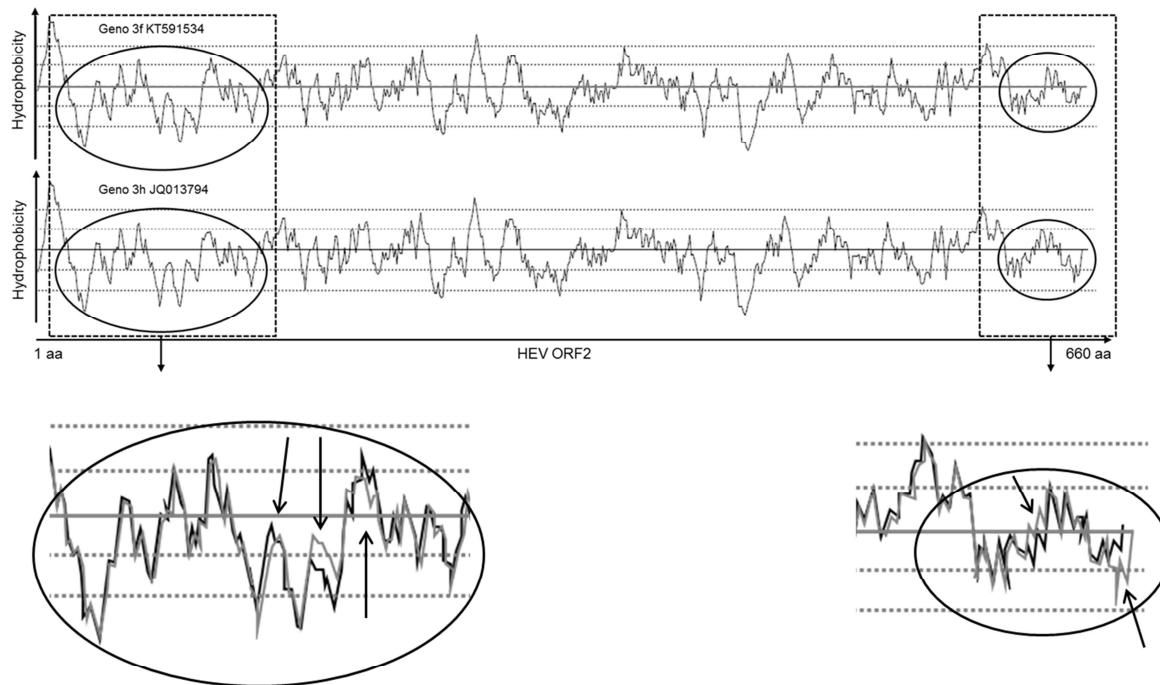
**Table 3:** Various studies conducted around the world concerning HEV prevalence in bivalve molluscan shellfish. NA: not applicable, ND: not done; PDU: PCR detectable units, ng: not given, gc: genome copies, y: year, m: month.

Location	Sample period	Animal	Occurrence	Positive/Total	Genotype (n)	Viral load	Reference
<b>Europe</b>							
<b>Finland (imported from Denmark)</b>	8 m	Mussels	6%	3/51	ND	$1.3 \times 10^2$ - $3.5 \times 10^2$ PDU/g	Diez-Valcarce et al., 2012
<b>France</b>	2 y	Cockles	0%	0/49	NA	NA	Grodzki et al., 2014
<b>France</b>	2 y	Oysters	0%	0/66	NA	NA	Grodzki et al., 2014
<b>France</b>	2 y	Clams	0%	0/65	NA	NA	Grodzki et al., 2014
<b>France</b>	2 y 6 m	Mussels	0%	0/94	NA	NA	Grodzki et al., 2014
<b>Italy</b>	Punctual	Mussels	8.1%	3/37	1	ND	Donia et al., 2012
<b>Italy</b>	ng	Mussels	2.7%	8/298	3 (2)	$3 \times 10^1$ - $9 \times 10^1$ gc/g of digestive tissue	La Rosa et al., 2018
<b>Italy</b>	ng	Razor clams	4.9%	2/41			La Rosa et al., 2018
<b>Italy</b>	ng	Mussels	0%	0/9	NA	NA	La Rosa et al., 2012
<b>Italy</b>	ng	Clams	0%	0/2	NA	NA	La Rosa et al., 2012
<b>Italy</b>	2 y 7 m	Mussels	0%	0/50	NA	NA	Iaconelli et al., 2015
<b>Italy</b>	2 y 7 m	Clams	0%	0/6	NA	NA	Iaconelli et al., 2015
<b>Italy</b>	2 y	Mussels	0%	0/108	NA	NA	Fusco et al., 2017

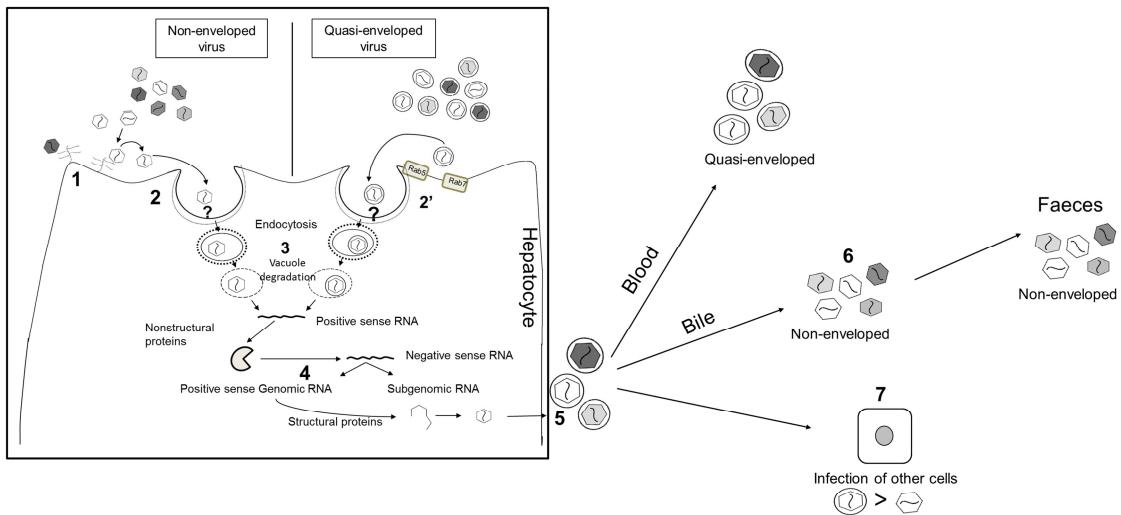
<b>Scotland</b>	ng	Mussels	85.4%	41/48	3 (11)	$5.4 \times 10^3$ - $1.6 \times 10^5$ IU/mL	Crossan et al., 2012
<b>Scotland</b>	ng	Mussels	3.0%	8/270	3 (3)	62.4 IU/mL (1 sample)	O'Hara et al. 2018
<b>Scotland</b>	ng	Oysters	2.5%	1/40	ND		O'Hara et al., 2018
<b>Spain</b>	8 m	Mussels	0%	0/51	NA	NA	Diez-Valcarce et al., 2012
<b>Spain</b>	18 m	Mussels	14.8% batches	12 batches/81	3e (6)	$6.7 \times 10^1$ to $8.6 \times 10^4$ gc/g of digestive tissue	Mesquita et al., 2016
<b>Asia</b>							
<b>China</b>	1 y 10 m	<i>A. granosa</i>	14.3%/kg	Ng		ND	Gao et al., 2015
<b>China</b>	1 y 10 m	<i>S. subcrenata</i>	28.2%/kg	Ng		ND	Gao et al., 2015
<b>China</b>	1 y 10 m	<i>R. philippinarum</i>	11.5%/kg	Ng	4 (ng)	ND	Gao et al., 2015
<b>Japan</b>	4 m	Yamato-Shijimi	6.25% packages	2 packages/32	3 (ng)	ND	Li et al., 2007
<b>Korea</b>	ng	Oysters	8.7%	14/161	3a (14)	ND	Song et al., 2010b
<b>Thailand</b>	2 y	Cockles	0%	0/69	NA	NA	Namsai et al., 2011
<b>Thailand</b>	2 y	Oysters	0%	0/52	NA	NA	Namsai et al., 2011
<b>Thailand</b>	2 y	Mussels	0%	0/92	NA	NA	Namsai et al., 2011



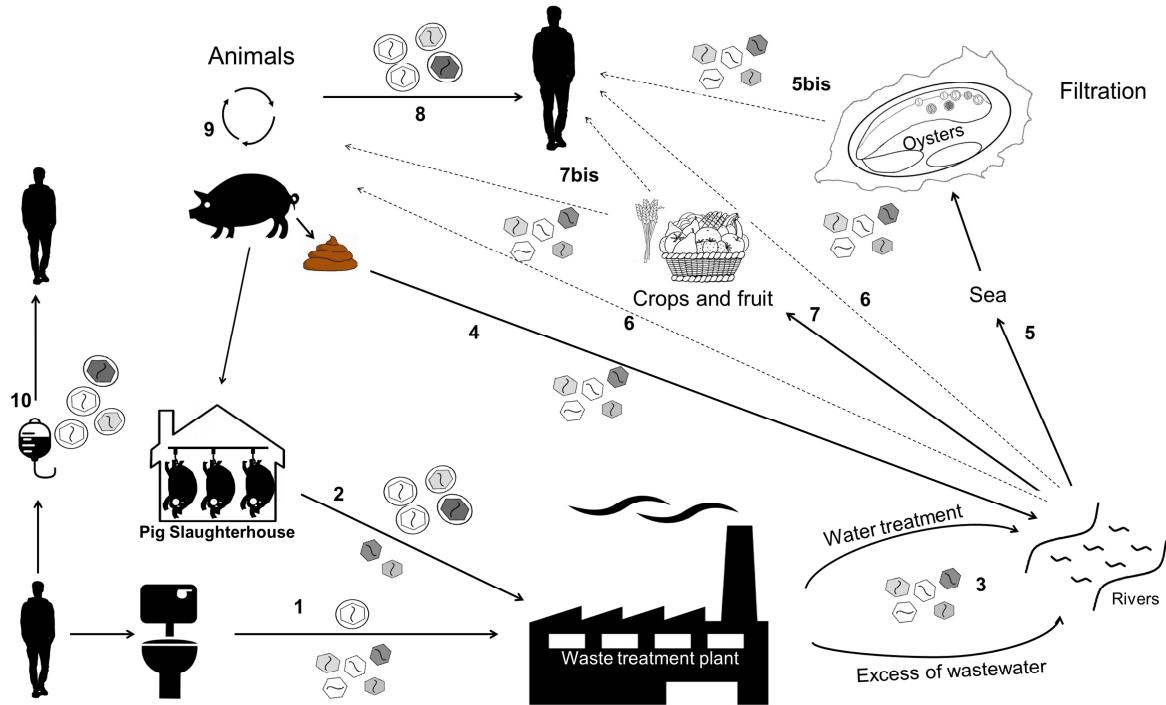
**Figure 1:** A: phylogenetic tree of amino acids of the ORF2 M domain of G1 reference sequences (Smith et al., 2016a). B: phylogenetic tree of amino acids of the ORF2 P domain of G1 reference sequences (Smith et al., 2016a). C: phylogenetic tree of amino acids of the ORF2 M domain of G3 reference sequences (Smith et al., 2016a). D: phylogenetic tree of amino acids of the ORF2 P domain of G3 reference sequences (Smith et al., 2016a). Overall mean distance: 0.015 (G1) versus 0.004 (G3) for M domain and 0.026 (G1) versus 0.010 (G3) for P domain.



**Figure 2:** AnTheProt hydrophobicity profiles for HEV genotypes 3f and 3h on HEV ORF2. We worked on 25 amino acid sequences of ORF2 of the different HEV genotypes. The results are given for subgenotypes 3f and 3h (ref 3f: KT591534; 3h: JQ013794) (Smith et al., 2016a). Black circles highlight the variations in the upper part of the image. aa: amino acids. At the bottom: superposed profiles; subgenotype 3f is in black and subgenotype 3h is in grey. The black arrows show the differences. Most differences are seen at the N-terminal end and, to a lesser extent, C-terminal end of the protein.



**Figure 3:** HEV replication and its possible paths after cell egress. **1:** Initial fixation to hepatocytes using heparan sulfate as a non-specific receptor of HEV. Specific receptor(s) is (are) still unknown. **2:** Specific fixation to clathrin and dynamin-2. **2':** For quasi-enveloped HEV, cell entry could require two GTPases (Rab5 and Rab7), an endosomal acidification and the lipid degradation activity in the lysosome. **3:** Endocytosis. **4:** HEV replication. **5:** HEV leaves the cell in a quasi-enveloped form. **6:** The virus quasi-envelope is destroyed in the bile after exposure to intestinal deoxycholic acid and proteases (Yin et al., 2016a) and non-enveloped HEV can be found in the faeces. **7:** The quasi-enveloped HEV may be a key form in cell-to-cell infection.



**Figure 4:** HEV circulation in the environment and between its hosts. 1: Pollution of human origin that arrives in wastewater treatment plants is mainly composed of non-enveloped faecal HEV particles. 2: When infected pigs are slaughtered, pollution both composed of non- and quasi-enveloped HEV is introduced in wastewater treatment plants. 3: Discharge of treated wastewater, the quality of which can even be altered in case of malfunction (e.g., bypass of untreated water due to heavy rain). 4: Infected pig stool can contaminate environmental waters through manure runoff, HEV is then in a non-enveloped form. 5: Shellfish that grow in environmental waters can then be contaminated by HEV. 5bis: Humans could possibly get infected by consumption of contaminated shellfish. 6: Humans and animals could get infected through consumption of contaminated water. 7: Crops can be contaminated by irrigation. 7bis: Humans and animals that consume the crops could possibly get infected. 8: When eating meat of infected animals, humans can be theoretically contaminated by quasi-enveloped HEV. 9: Contamination occurs between animals. 10: Contamination can also take place between humans through blood transfusion by quasi-enveloped HEV.

**Title**

Transmission of hepatitis E virus by water: an issue still pending in industrialized countries

**Highlights:**

- Hepatitis E virus (HEV) is a highly variable virus, waterborne in some cases.
- HEV seems sensitive to water treatments compared to other enteric viruses.
- Cell culture methods should be improved especially for HEV detection in water.
- Role of water for animal transmission of HEV should be investigated.
- Presence of enveloped HEV particles in the environment may be explored.