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1 **Relationships between host-species specificities and glycan specificities of**  
2 **pathogenic and non-pathogenic lagoviruses**

3

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36 #Adress correspondance to Jacques Le Pendu, jacques.le-pendu@inserm.fr

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## 39 **Abstract**

40 The rabbit hemorrhagic disease virus (RHDV) and the European brown hare syndrome  
41 virus (EBHSV) are two lagoviruses from the family *Caliciviridae* that cause fatal  
42 diseases in two leporid genera, *Oryctolagus* and *Lepus*, respectively. In the last few  
43 years, several examples of host jumps of lagoviruses among leporids were recorded. In  
44 addition, a new pathogenic genotype of RHDV emerged and many non-pathogenic  
45 strains of lagoviruses have been described. The molecular mechanisms behind host  
46 shifts and the emergence of virulence are unknown. Since RHDV uses glycans of the  
47 histo-blood group antigen type as attachment factors to initiate infection, we studied if  
48 glycan specificities of the new pathogenic RHDV genotype, non-pathogenic lagoviruses  
49 and EBHSV potentially play a role in determining host range and virulence of  
50 lagoviruses. We observed binding to A, B or H antigens of the histo-blood group family  
51 for all strains known to primarily infect European rabbits (*Oryctolagus cuniculus*), that  
52 have recently been classified as GI strains. Yet, it failed to explain the emergence of  
53 virulence since similar glycan specificities were found between several pathogenic and  
54 non-pathogenic strains. EBHSV, recently classified as GII.1, instead, bound to terminal  
55  $\beta$ -linked N-acetylglucosamine residues of O-glycans. Expression of these attachment  
56 factors in the upper respiratory and digestive tracts in three lagomorph species  
57 (*Oryctolagus cuniculus*, *Lepus europaeus* and *Sylvilagus floridanus*) showed species-  
58 specific patterns in relation with the susceptibility to these viruses, indicating that  
59 species-specific glycan expression is likely a major contributor of lagoviruses host  
60 specificity and range.

61

## 62 **Importance**

63 Lagoviruses constitute a genus of the *Caliciviridae* family, comprising highly  
64 pathogenic viruses, RHDV and EBHSV, which infect rabbits and hares, respectively.

65 Recently, non-pathogenic strains were discovered and new pathogenic strains have  
66 emerged. In addition, host jumps between lagomorphs are observed. The mechanisms  
67 responsible for the emergence of pathogenicity and host-species range are unknown.  
68 Previous studies showed that RHDV strains attach to glycans expressed in the upper  
69 respiratory and digestive tracts of rabbits, the likely doors of virus entry. Here we  
70 studied the glycan-binding properties of novel pathogenic and non-pathogenic strains  
71 looking for a link between glycan-binding and virulence or between glycan specificity  
72 and host range. We found that glycan binding did not match with virulence. However,  
73 expression of glycan motifs in the upper respiratory and digestive tracts of lagomorphs  
74 revealed species-specific patterns associated with the host range of the virus strains,  
75 suggesting that glycan diversity contributes to lagoviruses' host range.

## 76 **Introduction**

77 High mutation rates, vast population sizes and short generation times make RNA viruses  
78 capable of rapidly adapting to a large number of hosts and thus prone to cross species  
79 boundaries (1, 2). Viruses are most likely to jump between closely related species (3) and this  
80 may result in unique spill-over infections or severe epidemics, depending on how successfully  
81 the virus adapts to the new host population. Host switching constitutes an important  
82 mechanism of virus evolution (4) and is important in several families of viruses, including  
83 *Caliciviridae*. These non-enveloped viruses have a single-stranded, positive-sense RNA  
84 genome. Caliciviruses comprise five recognized genera: *Norovirus*, *Sapovirus*, *Vesivirus*,  
85 *Nebovirus* and *Lagovirus* (5). The genus *Lagovirus* encompasses two presently recognized  
86 species: rabbit hemorrhagic disease virus (RHDV), highly fatal to the European rabbit  
87 (*Oryctolagus cuniculus*), and European brown hare syndrome virus (EBHSV), that affects  
88 European brown and mountain hares (*Lepus europaeus* and *L. timidus*, respectively) (6).

89 Both RHDV and EBHSV emerged in the 1980s (7, 8) and are similar in terms of morphology,  
90 genome organization and epidemiological course of the respective diseases, rabbit  
91 hemorrhagic disease (RHD) and European brown hare syndrome (EBHS) (9, 10). For  
92 EBHSV, only a single serotype is known (11) and cases of non-pathogenic forms of the virus  
93 circulating in hare populations have just been described (12). In contrast, several serological  
94 subgroups are recognized for RHDV (13) including the antigenic variant RHDVa (14), and  
95 several non-pathogenic and moderately pathogenic strains also circulating in European rabbit  
96 populations from different parts of the world (15-19). In 2010, new pathogenic RHDV strains  
97 emerged and rapidly spread throughout Europe and Australia (20-28). Due to the large  
98 number of new lagovirus strains described, confusion in the extant nomenclature and close  
99 relationship between RHDV and EBHSV, a new classification of lagoviruses has recently  
100 been proposed distinguishing a single species with two genogroups and several genotypes

101 within these genogroups (29). In this new nomenclature, all classical RHDV strains are  
102 classified as GI.1, the new pathogenic strains (called RHDV2 or RHDVb) are classified as  
103 GI.2, whilst the related non-pathogenic strains (RCV-E and RCV-A) are classified as GI.3  
104 and GI.4. All pathogenic EBHSV strains fall into the GII.1 genotype.

105 Virus attachment to the cells of any new host is an initial first step for virus entry and  
106 subsequent replication, and thus constitutes a crucial step for a species jump. Attachment  
107 factors can include proteins, carbohydrates and lipids. Regarding caliciviruses, the most  
108 common ligands are carbohydrates: murine norovirus and feline caliciviruses use sialic acid  
109 (30, 31), human noroviruses recognize heparan sulfate (32) and histo-blood group antigens  
110 (HBGAs) are used by noroviruses and RHDV (33-35).

111 In Europe, three genera of lagomorphs exist: *Oryctolagus* (rabbits), *Lepus* (hares) and  
112 *Sylvilagus* (cottontails), which diverged about 12 million years ago (36). *Sylvilagus* is a genus  
113 native to the Americas, of which one species, eastern cottontail (*S. floridanus*) has been  
114 introduced in Europe. In Italy, eastern cottontails were successfully introduced in the Po  
115 valley in the 1960s and may have caused a decline in hare populations due to competition with  
116 this native lagomorph (37). Massive introductions took place in France in the 1970s and  
117 1980s but the species failed to establish (38). The European rabbit (*Oryctolagus cuniculus*) is  
118 widely distributed across Europe and may occur in sympatry with hares and locally in Italy  
119 with cottontails.

120 Experimental cross-infections with RHDV and EBHSV in hares and rabbits have been  
121 attempted, but results were quite disparate with some studies failing to induce disease (39-41),  
122 while others reported successful cross-infection (42, 43). Eastern cottontail challenges with  
123 EBHSV resulted in infection and death of one animal (44). Recently, several cases of cross-  
124 species infection occurring in natural conditions were reported: Iberian hare (*L. granatensis*)  
125 with RHDV infection (45), eastern cottontail (*S. floridanus*) susceptible to EBHSV (44) and

126 *L. capensis*, *L. corsicanus* and *L. europaeus* were found to be fatally susceptible to the new  
127 RHDV genotype (GI.2) (46-49).

128 Several studies have detected the presence of low levels of RHDV RNA in micromammals  
129 such as mice, voles and shrews sharing a habitat with RHDV affected rabbits (50, 51). While  
130 these findings, a likely result of ingestion of RHDV contaminated materials, indicate the  
131 possibility of micromammals to act as a mechanical vector for RHDV, no conclusive  
132 evidence has been presented so far suggesting that lagoviruses can productively infect species  
133 outside the lagomorph family.

134 Two non-mutually exclusive hypotheses for the emergence of pathogenicity in lagoviruses are  
135 currently proposed. The first suggests the emergence of virulence from non-pathogenic  
136 circulating viruses through acquisition of key mutations that, for reasons not directly related  
137 to the host, resulted in high virulence. This hypothesis is supported by the detection of  
138 antibodies against RHDV and EBHSV in samples collected before pathogenic virus  
139 emergence (52, 53) and by the characterization of widespread non-pathogenic forms (12, 15-  
140 18, 54). The other hypothesis involves a species jump, most likely from *S. floridanus* (25, 55),  
141 in which viruses would likely circulate as benign forms. This is consistent with the dates of  
142 introduction of *S. floridanus* in Europe and subsequent emergence of RHD and EBHS (7, 8,  
143 56, 57), although lagoviruses have not yet been reported in cottontails in their native range.

144 We showed earlier that RHDV strains recognized fucosylated glycans of the HBGA type (58).  
145 Later on, binding was observed to blood group B, A and H type 2 epitopes in a strain-  
146 dependent manner with slight differences in specificity for A, B or H epitopes so that not all  
147 animals were equally recognized by a single strain. Synthesis of these carbohydrate antigens  
148 proceeds by stepwise addition of monosaccharides to precursor disaccharides such as the  
149 Gal $\beta$ 4GlcNAc so-called type 2 precursor that appeared to be the main precursor in rabbits  
150 (59). Addition of a fucose in  $\alpha$ 1,2 linkage to its galactose residue generates the H type 2

151 epitope, which itself serves as a precursor for the A and B epitopes characterized by an  
152 additional N-acetylgalactosamine or galactose, respectively, linked in  $\alpha$ 1,3 to the galactose of  
153 the precursor (60). Following devastating outbreaks, selection of resistant animals based on  
154 their weak expression of these attachment factors could be documented, showing the role of  
155 these HBGAs as functional virus ligands and of their intra-species polymorphism in  
156 contributing to susceptibility or resistance (35, 59, 61, 62). Considering the recent reports of  
157 lagovirus species jumps, the close phylogenetic relationship of leporids (36), together with  
158 their overlapping geographic range (63) and an overall conservation of glycans among  
159 vertebrates, we sought to investigate the potential role of host glycan recognition in lagovirus  
160 cross-species jumps. With this aim, the ability of the new RHDV genotype (GI.2), EBHSV  
161 (GII.1) and non-pathogenic lagoviruses from Europe and Australia (GI.3 and GI.4) to  
162 recognize glycans was examined in addition to that of classical RHDV strains (GI.1).  
163 Furthermore, we investigated the expression of the corresponding glycans epitopes in tissues  
164 from European rabbits (*O. cuniculus*), European brown hares (*L. europaeus*) and eastern  
165 cottontails (*S. floridanus*) in order to relate the expression of these glycans to the documented  
166 susceptible host species.

167

168



## 169 **Results**

### 170 **Lagovirus GI strains attach to HBGA-type glycans**

171 We previously showed that classical strains of RHDV (GI.1) recognized glycans of the  
172 HBGA type and that their ability to recognize individual rabbits depended on the animals A,B  
173 phenotypes. Rabbits can be classified as A+B+, A+B-, A-B+ and A-B- depending on their  
174 expression of the A and B histo-blood group antigens in the gut. In order to determine if the  
175 other lagovirus strains were also influenced by the A,B type of rabbits, we analyzed the  
176 binding of VLPs prepared from the new RHDV genotype (GI.2), EBHSV (GII.1) and non-  
177 pathogenic rabbit strains (GI.3 and GI.4) to duodenum scrapings of A+B+, A+B- and A-B-  
178 rabbits. Due to its very low frequency, the A-B+ subgroup of rabbits was not used. A classical  
179 strain (GI.1d or RHDV G5) was used as a control. Positions of the used strains in the  
180 lagovirus phylogenetic tree are shown in Fig. 1. As depicted in Fig. 2, binding of the classical  
181 strain (GI.1d) to tissue extracts of A+B+ animals was significantly stronger than binding to  
182 tissue extracts of A-B- animals. Binding to A+B- animals was also stronger than to A-B-  
183 animals, consistent with the previously reported preference of that strain for the B antigen  
184 over the A antigen and its weak ability to recognize the H antigen, which constitutes the  
185 precursor of both the A and B antigens. All three non-pathogenic strains tested (GI.4a, GI.4d  
186 and GI.3) presented a similar pattern of binding, showing a preferred recognition of A+B+  
187 animals over A+B- and a poor recognition of A-B- animals. By contrast, both the new  
188 pathogenic variant RHDV2 (GI.2) and EBHSV (GII.1) did not present this pattern of  
189 recognition. For these viruses, binding to rabbit tissues occurred independently of the histo-  
190 blood group A,B type, suggesting either an equal recognition of the A, B and H motifs or  
191 binding to an unrelated ligand.

192 To gain further insight into the glycans potentially recognized by these lagoviruses, we  
193 assayed their binding to a printed glycan array that displays a large number of glycan motifs  
194 (Tables S1 A-F). Since binding of the VLPs was detected using polyclonal anti-sera, with the  
195 exception of the non-pathogenic RCV-A1 strain (GI.4a) strain that was detected using a  
196 monoclonal antibody, natural anti-carbohydrate antibodies present in the sera gave a relatively  
197 high and uneven background. We therefore applied a stringent selection criteria for specificity  
198 by considering only the glycan motifs for which a fluorescence intensity ratio  $>10$  between  
199 the assay performed in the presence or in the absence of VLPs was obtained. In these  
200 conditions, although we might have missed some weakly bound glycan motifs, the major  
201 specific ligands could be detected (Fig. 3). For all strains, the highest signal was obtained with  
202 heparin, indicating strong recognition of this sulfated polysaccharide. Some strains also bound  
203 to sulfated oligosaccharides (Supplementary material). The classical RHDV strain (GI.1a), as  
204 well as the non-pathogenic strains (GI.4a, GI.4d and GI.3) and the new pathogenic strain  
205 (GI.2 or RHDV2) additionally bound to HBGA motifs, mainly to the B type 2 epitope for the  
206 classical RHDV strain, as well as for the non-pathogenic strains GI.4a and GI.4d. For the  
207 latter strains, a much weaker binding to the A type 2 motif was observed, consistent with their  
208 weaker ability to bind to duodenal tissue extracts from animals lacking the B epitope. The  
209 new pathogenic strain GI.2 and the non-pathogenic strain GI.3 showed equally strong signals  
210 on the B type 2, A type 2 and H type 2 motifs. Importantly, in this experiment, for the GI.3  
211 VLPs, the signal was saturating, making it impossible to determine if a differential  
212 recognition of the A, B and H type 2 epitopes could occur. We therefore tested the binding of  
213 the three strains GI.2, GI.3 and GI.4d to the same oligosaccharides coupled to polyacrylamide  
214 by ELISA (Fig. 4). In these conditions the new RHDV genotype GI.2 showed a strong  
215 binding to A, B and H type 2. This was confirmed by assaying another strain of the same  
216 genotype on a set of HBGA-related oligosaccharides immobilized on ELISA plates

217 (structures given in Table S2). Both RHDV2 (GI.2) strains showed strong binding to A, B and  
218 H type 2. A weak binding to the Lewis Y difucosylated motif was additionally observed  
219 (Fig.5). The much stronger binding to B type 2 over A type 2 and the very weak binding to H  
220 type 2 of the non-pathogenic GI.4d strain was confirmed (Fig. 4), whilst the non pathogenic  
221 GI.3 VLPs showed a strong binding to B type 2 only, indicating a similar strong preference  
222 for this motif that could not be seen on the data from the printed glycan microarray due to the  
223 signal saturation.

224 Overall these data are consistent with a preferential recognition of the B epitope presented on  
225 rabbit gut tissue by the classical RHDV strains and the non-pathogenic strains. The new  
226 pathogenic strain GI.2 also recognizes HBGA motifs. Yet, its preference for the B epitope  
227 over the A and H epitopes is much less pronounced, consistent with its ability to attach to  
228 duodenum extracts regardless of the A,B phenotypes.

#### 229 **EBHSV (GII.1) attaches to a distinct glycan motif**

230 When testing the binding of GII.1 VLPs by ELISA to the set of HBGA-related glycans  
231 presented in Table S2, we failed to detect any signal (data not shown). However, on the  
232 printed glycan array, beside that of heparin, a strong signal was observed for several  
233 structures composed of N-acetylglucosamines in  $\beta$ -anomeric linkage (Fig. 3). The printed  
234 glycan microarray also confirmed the lack of binding to HBGA-related motifs. Thus, the  
235 GII.1 strain presented a clearly distinct glycan specificity compared to those of the GI strains.  
236 We have previously shown that the HBGA motifs recognized by classical strains of RHDV  
237 were mainly expressed on O-glycans in the rabbit duodenum (59). To determine whether the  
238 motifs recognized by the GII.1 strain were preferentially expressed on N-linked or O-linked  
239 glycan chains, a European brown hare duodenal extract was treated with either PGNase F or  
240 O-sialoglycoprotein endopeptidase (OSGE) in order to selectively remove N-linked and O-

241 linked glycans, respectively. Following treatment, VLPs were incubated and their binding  
242 detected. As shown in Fig. 6a, PNGase treatment resulted in a substantial decrease (50%) of  
243 ConA lectin binding, used as a control for efficacy of enzyme treatment. Yet, it had no effect  
244 on GII.1 VLPs attachment. By contrast, OSGE treatment resulted in a near complete loss of  
245 anti-A antibody binding used as a control for enzyme efficacy, as well as in a strongly  
246 decreased attachment of VLPs, indicating that GII.1 VLPs attach to O-glycans on duodenal  
247 extracts from hares, similar to the previously described attachment to O-glycans of rabbit  
248 tissue by GI strains (59).

249 Since the data from printed glycan arrays indicated a specificity of the GII.1 strain for  $\beta$ -  
250 linked N-acetylglucosamine residues such as  $\text{GlcNAc}\beta\text{4GlcNAc}\beta\text{-R}$  that are not present on  
251 O-glycans, we hypothesized that the ligands present on hare tissues could comprise an  
252 accessible  $\beta$ -linked N-acetylglucosamine residue. We thus examined the expression of  
253 terminal N-acetylglucosamine residues on tissues from European hares (*L. europaeus*) as well  
254 as from the European rabbit (*O. cuniculus*) and the eastern cottontail (*S. floridanus*) using the  
255 succinylated lectin WGA (sWGA). As shown in Fig. 6b, binding sites for this terminal N-  
256 acetylglucosamine-specific lectin were observed on surface epithelial cells of the duodenum  
257 from the three lagomorph species. Staining was also observed on the tracheal surface  
258 epithelial cells, albeit of *Lepus europaeus* only. It was undetectable on the same tissues from  
259 the other two species. Staining by sWGA of the duodenal epithelium appeared weaker on the  
260 *Oryctolagus cuniculus* tissue sections. That was confirmed by ELISA using duodenal tissue  
261 extracts from the three lagomorph species (Fig. 6c).

262 Having observed that terminal N-acetylglucosamine residues are present in a species-specific  
263 manner on the trachea and to some extent on the duodenum of leporids, we next sought to  
264 determine whether GII.1 VLPs did attach to these epitopes on hare tissues. To this aim, VLPs

265 were incubated on tissue sections from hares and the same binding pattern was observed (not  
266 shown). Terminal N-acetylglucosamine residues were removed by pre-treatment of the tissue  
267 sections using a  $\beta$ -hexosaminidase prior to incubation with the VLPs. The enzyme treatment  
268 partly removed the sWGA binding sites, ascertaining efficacy (Fig. 7). Compared with mock  
269 treated sections (Fig. 7b), staining by GII.1 VLPs of the treated sections was also clearly  
270 diminished (Fig. 7c). Next, trachea tissue sections were pre-incubated in the presence of  
271 unlabeled sWGA or a fucose-specific lectin (AAL) prior to incubation with the VLPs. In this  
272 condition, VLP binding was clearly decreased on the sWGA pre-treated sections as compared  
273 to control section or to sections pre-treated with AAL, indicating blocking of their binding  
274 sites by sWGA (Fig. 7d, e, f). These results indicate that the GII.1 VLPs (EBHSV) recognize  
275 terminal  $\beta$ -linked N-acetylglucosamine residues present on O-glycans that are preferentially  
276 expressed on the trachea and in the small intestine of hares as compared to the same tissues in  
277 rabbits.

### 278 **Variations in histo-blood group antigens expression across lagomorph species**

279 The differences in expression of the N-acetylglucosamine residues recognized by the GII.1  
280 VLPs between hares and rabbits were associated with the host-species specificity of EBHSV  
281 (GII.1) that readily infects European brown hares but is not known to infect European rabbits.  
282 This prompted us to examine the expression of the A, B and H motifs in the same lagomorph  
283 tissues in search of potential host preferences based on the ability of the diverse virus strains  
284 to recognize these glycan motifs. The results are summarized on Fig. 8 and Table 1.

285 Major differences in A, B, or H antigens expression were observed across the three species.  
286 Indeed, A antigen expression was clearly confirmed in the trachea and the small intestine of  
287 *O. cuniculus* and *L. europaeus*, although not all individual *O. cuniculus* expressed the antigen  
288 due to the genetic polymorphism of the A antigen expression. Accordingly, the animals which

289 expressed the A antigen did so both in the trachea and the small intestine. By contrast, none of  
290 the six *S. floridanus* tested expressed the A antigen in the trachea, despite a clear expression  
291 in the gut by four of them. The B antigen was not detected in the trachea of *O. cuniculus*  
292 although it was strongly expressed in the small intestine of 16 out of 21 animals tested. The  
293 remaining 5 animals were classified as B- as previously described (59). The same antigen was  
294 completely absent from all six *L. europaeus* tested, but it was detected both in the trachea and  
295 the small intestine of all *S. floridanus* individuals. Interestingly, as previously described (59),  
296 expression of the B antigen in *O. cuniculus* small intestine was patchy and heterogeneous,  
297 with areas of strong expression among negative areas. At variance, in *S. floridanus* staining  
298 by the anti-B was always strong and homogeneous. Finally, the H type 2 antigen, detected by  
299 the UEA-I lectin was found strongly expressed in the trachea and the small intestine of both  
300 *O. cuniculus* and *S. floridanus*, but not in the corresponding tissues of *L. europaeus* where it  
301 was either completely undetectable or present at very low levels but not at the apical surface  
302 of cells. These data indicate that beside the intra-specific genetic polymorphism of the A  
303 and/or B antigen expression, there are overarching species-specific features. Strikingly, hares  
304 lack both the B and H antigens in the trachea and the small intestine. *O. cuniculus* and *S.*  
305 *floridanus* can express both the A and B antigen in the intestine, but in the trachea, only the A  
306 antigen is present in *O. cuniculus* and inversely, only the B antigen is present in *S. floridanus*.  
307 Trachea and small intestine were chosen as they are easy to sample on wild animals captured  
308 *in naturo*. Expression of the same glycans in the nose of two hares and two European rabbits  
309 was also assessed to confirm that their expression was similar to that of the trachea. The  
310 results indicated that trachea glycan expression corresponds to that in the nasal epithelium, a  
311 likely door of entry of lagoviruses in a natural setting (Fig. 9).

312

313

## 314 **Discussion**

315 RNA viruses have commonly crossed species barriers, probably because of their high  
316 mutation rates, short generation times and large population sizes that enable them to quickly  
317 adapt to new hosts (1). Phylogenetically related species are more likely to experience species  
318 jumps as they have more similarity in cell receptors and other components critical to the viral  
319 replication (64). Caliciviruses appear to be good models to study the role of molecular factors  
320 involved in species jumps as they are fast evolving single-stranded RNA viruses and as  
321 several instances of likely host-species jumps have been reported within this virus family, in  
322 particular the vesiviruses and noroviruses (65-67). Within the *Lagovirus* genus, cross-species  
323 infections involving closely related host species of the Leporidae family (order Lagomorpha)  
324 have recently been reported, including the classical RHDV (GI.1) in *L. granatensis*, the new  
325 RHDV genotype (GI.2) infecting several hare species and EBHSV (GII.1) infecting *S.*  
326 *floridanus* (44-49). Previous studies conducted on RHDV showed involvement of glycans of  
327 the HBGA type in attachment of the virus to epithelia of the upper respiratory tract, or of the  
328 gut, that constitute the most likely common doors of entry of the virus (35, 58, 59, 61, 62).  
329 HBGA structures are highly conserved among vertebrates and this conservation might  
330 facilitate cross-species infections. However, species differences also exist in terms of HBGA  
331 expression. These include the absence of motifs based on type 1 precursor in many species,  
332 loss of the alphaGal motif in apes, or differences in cellular distribution, such as the lack of  
333 ABH antigens on erythrocytes of most mammals (68-70). Nevertheless, comparative analysis  
334 of the expression of these glycan epitopes in lagomorph species had never been performed.  
335 The relationship between the host range of lagoviruses and their glycan ligands was thus  
336 particularly interesting to analyse. Here we analyzed the expression of these glycans in the  
337 trachea, small intestine and nose.

338 Overall, our observations are consistent with a role of the glycan attachment factors in  
339 determining lagoviruses host specificity (or lack thereof) in three species of leporids.

340 Indeed, we observed that all GI strains (pathogenic or not) could attach to *O. cuniculus*  
341 epithelial cells through attachment to either the A or the H antigens in the trachea and to the B  
342 antigen in the small intestine. We had previously observed that European rabbits' survival to  
343 outbreaks of RHD was associated with the absence or low expression of these antigens,  
344 clearly establishing their role in the infection process *in vivo* (35, 59, 61, 62).

345 The lack of infection of hares by many GI strains might reflect the lack of expression of the B  
346 antigen and the lack of accessibility of the H antigen since in hares, among HBGAs, only the  
347 A antigen appears available at cell or tissue surfaces. Early strains of RHDV (GI.1c) that  
348 emerged during the second half of the 1980s did not recognize the A antigen at all (59), which  
349 might explain why these strains did not infect hares even in experimental conditions (39-41).  
350 However, the virus evolved to progressively acquire recognition of the A antigen, diminishing  
351 in parallel its ability to recognize the H antigen (59). This led to the circulation of strains such  
352 as those that we used in the present study that can bind to A+B- animals, but hardly recognize  
353 A-B- animals. This newly evolved ability of RHDV (GI.1) strains to bind to the A antigen  
354 might explain the recent report of natural infection of hares in the Iberian Peninsula (45).

355 The broad HBGA specificity of the new RHDV genotype (GI.2) that recognizes A, B, and H  
356 type 2 epitopes almost equally well is associated with its ability to infect both European  
357 rabbits and hares, the latter species being recognized through the presence of the A antigen on  
358 the trachea and the small intestine. Interestingly, evidence for the development of genetic  
359 resistance to classical RHDV (GI.1) has been obtained in Australia (71) and selection of  
360 genetically resistant rabbits involving the B- phenotype was observed in Australia and France  
361 (35, 59), suggesting that the broad HBGA specificity of the GI.2 strains might allow infection



362 of animals resistant to GI.1 strains. This could help to explain why the GI.2 virus has been  
363 spreading so successfully and supplanting GI.1 strains. By contrast, the exclusive expression  
364 of terminal N-acetylglucosamine residues on the trachea of hares and their lower expression  
365 in the gut of European rabbits correlate with the host species-specificity of EBHSV (GII.1)  
366 that does not infect European rabbits. In terms of glycan specificity, EBHSV thus appears  
367 quite distinct from the GI lagoviruses. Nonetheless, both types of strains attach to O-glycans  
368 expressed at the surface of the upper respiratory tract and of the small intestine, indicating a  
369 common mechanism of infection. Unlike RHDV, EBHSV did not show any agglutination of  
370 human erythrocytes regardless of their ABO phenotype (data not shown) or binding to the  
371 polymorphic HBGAs. Instead, it attached to terminal  $\beta$ -linked N-acetylglucosamine residues  
372 that are present in glycans of all species in all cell types. These motifs are generally masked  
373 by addition of other monosaccharides. The patterns of binding to tissues observed using the  
374 lectin sWGA and VLPs from EBHSV indicate restriction in their availability as ligands. In a  
375 previous study, the occurrence of O-glycans from rabbits' small intestine presenting terminal  
376  $\beta$ -GlcNAc residues was observed by mass-spectrometry (59). Yet, these structures were of  
377 relatively low abundance. It would be interesting to perform the same type of analysis on hare  
378 tissues in order to determine the precise O-glycan oligosaccharides that harbor these terminal  
379  $\beta$ -GlcNAc residues and their abundance. It would also be interesting to determine if an intra-  
380 specific polymorphism of their expression exists that might contribute to generate different  
381 individual susceptibilities to the virus, similar to what was observed between HBGAs and  
382 susceptibility to RHDV.

383 Glycan specificity of lagoviruses nevertheless fails to explain the lack of infection of *S.*  
384 *floridanus* by classical RHDV (GI.1) both experimentally and *in naturo* in northern Italy  
385 where European rabbits and free living cottontail rabbits live in sympatry (44), despite strong  
386 binding to B antigen expressed in the trachea and the small intestine of that potential host

387 species. It is also unclear why infection of *S. floridanus* by EBHSV occurs (44) despite the  
388 absence of expression of terminal  $\beta$ -GlcNAc residues in the trachea of these animals. In that  
389 case the role of glycan recognition cannot be excluded since the virus strain that we used was  
390 older than the strain for which records of cross-infection have been obtained and differential  
391 glycan specificity has evolved since then (35). Alternatively, an intra-specific genetic  
392 polymorphism of  $\beta$ -GlcNAc expression might also exist, but too few animals were studied to  
393 investigate this possibility, which warrants further studies. Regardless, it is most likely that  
394 absence of cross-infection involves factors unrelated to the initial attachment step, but rather  
395 subsequent steps within the infection cycle such as entry receptor incompatibility, replication  
396 mechanism incompatibilities or the presence of species-specific anti-viral factors.

397 Printed glycan microarrays, revealed that a common feature of all virus strains that we  
398 examined was a strong binding to heparin. Binding to heparin cannot be compared to those on  
399 the other glycans that were printed on the microarray since it is a polysaccharide constituted  
400 of a large number of repeating units. It is structurally similar to heparan sulfate that is  
401 expressed by all animal tissues. Heparan sulfate is a complex polysaccharide composed of  
402 repeating variably sulfated disaccharide units that can display a remarkable structural  
403 diversity (72). Interactions of heparan sulfate with proteins are established mainly through  
404 electrostatic interactions of its negatively charged sulfates with basic amino acids (73).  
405 Heparan sulfate is a primary or co-receptor for viruses from various families, including  
406 *Parvoviridae*, *Retroviridae*, *Herpetoviridae* as well as *Filoviridae* (74, 75). Within the  
407 *Caliciviridae* family, binding of GII noroviruses to heparan sulfate has been reported (32).  
408 However, its exact role in the infection process remains unknown. Our observation of the  
409 binding of diverse lagoviruses to heparin suggests a shared property between lagoviruses and  
410 other noroviruses, but its functional importance remains to be examined.

411 Highly virulent lagoviruses emerged independently on at least three separate occasions, first  
412 in the early 1980s with EBHSV (GII.1) and RHDV (GI.1) and then in 2010 with the new  
413 RHDV genotype (GI.2). The causes for the emergence of these highly pathogenic strains in a  
414 short interval are not known at present. Clearly, the shared ability to recognize HBGA glycan  
415 motifs by pathogenic and non-pathogenic strains indicates that it does not constitute a  
416 virulence factor and therefore cannot explain the acquisition of virulence by some strains. It  
417 has been proposed that the emergence of virulence might have involved a species jump, *S.*  
418 *floridanus* being a possible candidate species of origin because of the concomitance between  
419 its repeated introductions in Europe from the 1970s and the emergence of highly pathogenic  
420 lagoviruses (55). Here we observed that the B histo-blood group antigen, which is the  
421 preferred ligand of all GI strains, is strongly and homogeneously expressed in the trachea and  
422 small intestine of *S. floridanus*, thus compatible with this hypothesis. Regardless of the  
423 answer to this difficult question, the data presented here strongly indicate that species-specific  
424 glycan expression represents an important element of the host species-specificity and range of  
425 lagoviruses.

426

427

428

## 429 **Materials and Methods**

### 430 **Virus-like particles (VLPs) and virus preparations**

431 VLPs from the first non-pathogenic lagovirus strain described in Australia were prepared and  
432 described earlier (79). The strain was originally called RCV-A1 (accession number  
433 EU871528) and is now called GI.4a in the new proposed nomenclature. VLPs of seven other  
434 strains of lagoviruses were generated using a previously described method (80). Recombinant  
435 baculoviruses were generated containing the VP1 sequence from the following viruses: two  
436 classical RHDV strains from France, a GI.1d and a GI.1a strain in the new proposed  
437 nomenclature (previously G5 and G6 or RHDVa), accession numbers AM085133 and  
438 AJ969628, respectively; two strains of the new pathogenic genotype from France (GI.2 in the  
439 new proposed nomenclature, previously RHDV2 or RHDVb), strains 10.28 (accession  
440 number HE800531) and 10.32 (accession number HE800532); two non-pathogenic strains  
441 from France including a GI.3 strain previously called RCV-E1, strain 06-11 (accession  
442 number AM268419) and a GI.4d strain, previously called RCV-E2, strain B09/08-117  
443 (accession number LT708121); one strain from EBHSV, GII.1 in the new proposed  
444 nomenclature, strain B/EBHS/6 from France (accession number KY801206). Briefly,  
445 recombinant baculoviruses expressing the VLPs were used to infect Sf9 cells. Cellular debris  
446 and baculovirus were removed by centrifugation (10,000 rpm, 30 minutes) and freeze-  
447 thawing cycles released VLPs from the cells. The supernatant was once again centrifuged at  
448 27,000 rpm for 3h and pellets were resuspended in 200  $\mu$ L PBS. A caesium chloride solution  
449 (0.4g/mL) was added to the preparation and ultracentrifuged for 18h at 36,000 rpm. VLP  
450 fractions were collected by puncture and dialyzed against PBS. Caesium chloride was  
451 eliminated through serial washes on Vivaspin columns 30000 MWCO PES. The integrity and

452 quality of the VLPs was checked by Coomassie blue staining of SDS-PAGE gels and Western  
453 blot. Protein amounts were quantified using a nanodrop 2000 (ThermoFisher scientific).  
454 In some experiments, virus samples were used. They were obtained from liver homogenates  
455 (10% (w/v) in PBS) of dead animals prepared as previously described (59).

456

#### 457 **Antibodies to VLPs**

458 A previously prepared high-titered rabbit sera Lp4 was used for RHDV G5 and RHDV G6  
459 (GI.1d and GI.1a, respectively, in the new proposed nomenclature) detection (59). A  
460 hyperimmune serum that recognizes the new RHDV genotype (GI.2 in the new proposed  
461 nomenclature), and the non-pathogenic lagoviruses RCV-E1 and RCV-E2 (GI.3 and GI.4d in  
462 the new proposed nomenclature) was produced. For this, two rats were serially inoculated  
463 with the VLP from the 10-28 strain (GI.2). The anti-EBHSV polyclonal antibody was  
464 generated by serial inoculation of two rats with VLPs from EBHSV (GII.1 in the new  
465 proposed nomenclature). Recognition of target VLPs using the antibodies generated was  
466 confirmed using ELISA. Rat inoculations were performed at the animal experimentation core  
467 facility of the University of Nantes (IRT-UN facility agreement number 4478) and were  
468 approved by the national ethic review board from the French Ministry of Enseignement  
469 Supérieur et de la Recherche (project license number CEEA.2012.83). The animal care and  
470 use protocol adhered to the European Directive number 2010/063 and to the national French  
471 regulation (Décret n°2013-118 du 1<sup>er</sup> février 2013 relatif à la protection des animaux utilisés à  
472 des fins scientifiques). A monoclonal antibody that specifically recognizes the Australian  
473 rabbit calicivirus RCV-A1 (GI.4a in the new proposed nomenclature) was also used (MAb  
474 11F12) for detection of RCV-A1 VLPs (79). Isolation of the first non-pathogenic Australian  
475 lagovirus strain (RCV-A1) that allowed preparation of VLPs and their antibodies was  
476 approved by the CSIRO Sustainable Ecosystems Animal Ethics Committee (SEAEC # 06-31)

477 and performed using the guidelines of the Australian code of practice for the care and use of  
478 animals for scientific purposes.

479

#### 480 **Tissue sampling**

481 Trachea and duodenum tissue samples from European rabbits, hares and cottontails were used  
482 for histochemistry following fixation in formalin for 48-96h and paraffin embedding.  
483 Duodenum mucosa extracts were prepared as follows: the first 5 cm posterior to the  
484 gastroduodenal junction were removed after clearing the section from intestinal contents, the  
485 sample was vigorously rinsed in PBS and stored in RNAlater (Ambion, Life Technologies,  
486 Paisley, UK) at -20°C. One cm sections of the duodenum were then rinsed in PBS, opened  
487 and scraped into RLT lysis buffer (Qiagen, Hilden, Germany) containing  $\beta$ -mercaptoethanol.  
488 The tissue scrapings were homogenized and boiled for 10 minutes. After clearing, protein  
489 contents were determined using a nanodrop 2000 apparatus and kept at -20°C prior to being  
490 used for ELISA.

491 For analysis of the classical RHDV G5 strain (GI.1d in the new proposed nomenclature), 103  
492 wild rabbits (*O. cuniculus*) hunting-harvested in Southern France were used. For the other  
493 analyses, rabbit samples were collected from 12 domestic animals and 9 wild animals from  
494 western France that had been freshly killed by hunters. Four samples of European brown  
495 hares (*L. europaeus*) from Spain reared in captivity were collected. Samples from two  
496 additional *L. europaeus* hunted in western France were also used and six captivity reared  
497 eastern cottontails (*S. floridanus*) were bought from a French farm.

498 The use of domestic European rabbits was carried out in a group V animal facility (agreement  
499 N° 44267), and approved under specific agreement N° 006933 by the National Committee of  
500 Ethics on Animal Experiments of the French Ministry of Higher Education and Research.

501 Breeders from whom animals were obtained approved the study. Animal care and handling  
502 were performed in strict accordance with the recommendations of the French National Guide  
503 for the Ethics of Animal Experiments and euthanasia was performed under xylazine and  
504 ketamine anesthesia. Tissues from wild European rabbits and hares were taken from animals  
505 killed by hunters during rabbit and hare hunting seasons in the Aveyron area, France. No wild  
506 animal has been killed specifically for the purpose of this study and hunters approved the  
507 study. Therefore no animal ethics permit was required.

508

### 509 **Phenotyping ELISA**

510 Duodenum scrapings were phenotyped using ELISA. Briefly, the duodenum scrapings were  
511 diluted in duplicates in eleven two-fold dilutions with final dilutions ranging from 1/100 to  
512 1/102,400 in 0.1 M sodium carbonate buffer pH 9.5 on a Nunc MaxiSorp plate (ThermoFisher  
513 scientific, Waltham, MA). Antibody dependent assays were blocked with 5% non-fat dry milk  
514 (Régilait, Saint-Martin-Belle-Roche, France) diluted in PBS while lectin assays were blocked  
515 with synblock (AbD serotec, Oxford UK). The A antigen was detected using mouse  
516 monoclonal anti-A antibody 2A21, and the B antigen was detected using a specific mouse  
517 monoclonal antibody B49 (81). H type 2 expression was determined using the HRP  
518 conjugated lectin *Ulex europaeus*-I (UEA-I) at 2 µg/mL (Sigma-Aldrich, St. Louis, MO).  
519 Expression of terminal β-linked N-acetylglucosamine residues was determined using  
520 succinylated wheat germ agglutinin (sWGA) at 10 µg/mL (Vector Laboratories, Burlingame,  
521 CA). Secondary horseradish peroxidase (HRP) conjugated anti-mouse (Uptima/Interchim,  
522 Montlucon FR) was used for detection of anti-A and anti-B primary antibodies. HRP-avidin D  
523 (Vector laboratories) was used for detection of UEA-I and sWGA. TMB (BD Bioscience, San  
524 Jose CA) was used as a substrate for all assays and O.D. values were measured at 450nm.

525

526 **VLPs and virus binding assays**

527 VLP binding to animal duodenum scrapings or synthetic sugars was analyzed as previously  
528 described (59, 82). Briefly, duodenum scrapings, normalized for protein concentrations, were  
529 coated diluted in a range of dilutions in 0.1M sodium carbonate buffer or 1 µg of synthetic  
530 sugars was coated in the same buffer. Plates were blocked with 5% non-fat dry milk diluted in  
531 PBS or distilled water. VLPs at 8 µg/mL were then added to the plate. Binding of VLPs was  
532 detected using primary antibodies against the respective strains described above. Secondary  
533 anti-rabbit, anti-rat or anti-mouse antibodies conjugated with HRP were then used according  
534 to the primary antibodies. TMB (BD Bioscience, San Jose CA) was used as a substrate for all  
535 assays and O.D. values were measured at 450 nm.

536 In the case of EBHSV (GII.1 in the new proposed nomenclature), additional assays were  
537 performed in order to test the effect of enzyme treatments on binding as follows. Hare  
538 duodenum tissue extracts were coated in Nunc Maxisorp plates at 1/400 dilution at 4 °C  
539 overnight. Prior to the blocking step, plates were incubated six hours at 37 °C with 1000U of  
540 PNGase F (New England Biolabs, Evry, France). After incubation with the enzyme, ELISA  
541 steps were performed as described above for binding to tissue scrapings. For treatment with  
542 O-sialoglycoprotein endopeptidase (OSGE; Cedarlane, Burlington, Canada) a slightly  
543 modified protocol was adopted. Hare duodenum samples were incubated overnight at 37 °C  
544 with 15 µL (36 µg) of the enzyme and then coated in Nunc Maxisorp plates at 4 °C overnight.  
545 After blocking the plate, binding was carried out as described above.

546

547 **Printed glycan microarray assay**

548 Printed glycan array slides were manufactured and profiled as described in (83). Briefly, six  
549 replicates of 353 mono- and oligosaccharides (Table S1) 50 µM as ω-aminopropyl glycosides



550 of 95-98% purity were diluted in 300 mM PBS/0.001 % Tween 20 (pH 8.5) and printed by  
551 robotic pin deposition on *N*-hydroxysuccinimide activated glass slides (Schott Nexteron™);  
552 the array also contained 150 bacterial polysaccharides (not shown in Table S1). Free *N*-  
553 hydroxysuccinimide activated groups were blocked with 25 mM ethanolamine in 100 mM  
554 boric acid with 0.2 % Tween 20 at a final pH of 8.5. Slides were then rinsed with MilliQ-  
555 grade water, dried and stored at 4°C in a dessicator.

556 Each VLP diluted in PBS-Tween BSA (Sigma, St Louis, MO) (0.1 % (v/v) Tween 20 and 1 %  
557 (w/v) BSA) was incubated on slides in humid chamber overnight at 4°C with gentle shaking.  
558 Monoclonal antibodies or hyperimmune sera also diluted in PBS-Tween BSA were incubated  
559 at 37°C for 60 minutes with gentle shaking. A final incubation with Cy5-labeled secondary  
560 antibodies diluted in PBS-Tween (0.01 % (v/v) Tween 20) was performed at room  
561 temperature for 60 minutes at 37°C. In between incubations, slides were washed with a series  
562 of 0.1% and 0.01% Tween 20 in PBS. Fluorescence signals were measured with an Agilent  
563 scanner G2565CA and analyzed using the ImaGene analysis software version 7.5  
564 (BioDiscovery, El Segundo, USA).

565

## 566 **Immunohistochemistry**

567 Tissue sections were used either individually, or tissue blocks were used to prepare a tissue  
568 microarray that contained duplicate tissue samples from trachea and duodenum from 10  
569 rabbits, 8 hares and 6 cottontail rabbits. Sections were de-paraffinated through baths of LMR-  
570 SOL (1-Bromopropane, 2-Methylpropane-2-ol and Acetonitrile) followed by re-hydration  
571 with successive baths of 100, 90, 70 and 50% ethanol. Endogenous peroxidase activity was  
572 blocked with 0.3% hydrogen peroxide in PBS. Non-specific binding was blocked with 3%  
573 BSA in PBS. HRP conjugated UEA-I (Sigma-Aldrich, St. Louis, MO) at 0.8 µg/mL, HRP  
574 conjugated succinylated Wheat Germ Agglutinin (sWGA; EY-Laboratories, Burlingame, CA)

575 at 2  $\mu\text{g}/\text{mL}$ , anti-A monoclonal antibody 2A21 and anti B monoclonal antibody B49 were  
576 used for binding to H antigen, A antigen and B antigen respectively. Lectins and antibodies  
577 were incubated with the tissue sections in 1% BSA in PBS at 4°C (UEA-I), at room  
578 temperature (sWGA) or at 37°C (antibodies) overnight. After three washes in PBS, a  
579 biotinylated anti-mouse antibody (Vector laboratories, Burlingame, CA) diluted in 1% BSA  
580 in PBS was added to the assays with primary mouse antibodies. Sections were washed three  
581 times in PBS prior to addition of HRP-conjugated avidin D (Vector laboratories, Burlingame,  
582 CA) also diluted in 1% BSA in PBS. Substrate was added to the slides (AEC kit, Vector  
583 laboratories, Burlingame, CA) followed by Mayer's haematoxylin solution (Merck,  
584 Whitehouse Station, NJ) for contrast staining.

585 In order to confirm the role of terminal  $\beta$ -linked N-acetylglucosamine residues in EBHSV  
586 attachment, deparaffinated and hydrogen peroxide blocked tissue sections were treated with  
587 25U  $\beta$ -N-Hexosaminidase<sub>f</sub> (New England Biolabs, Ipswich, MA) for 2 hours at 37°C. Fresh  
588 enzyme was then added and sections were further incubated overnight at 37°C. Control  
589 sections were made in parallel with the corresponding enzyme buffers (Sodium Citrate pH  
590 4.5). After overnight incubation, sections were washed twice in PBS and blocked with PBS-  
591 5% BSA for 1h at room temperature. A competition assay was also performed by pre-  
592 incubating sections with unlabeled sWGA (Vector Labs) or the fucose-specific lectin AAL  
593 (Vector Labs) as a control at 10  $\mu\text{g}/\text{mL}$  in PBS-1% BSA for 1h at room temperature prior to  
594 incubation with EBHSV. B/EBHS/6 infected liver homogenate diluted 1/5 in PBS-1% BSA  
595 was then added and incubated at 4°C overnight. After 3 washes with PBS, monoclonal anti-  
596 EBHSV antibody (5F5, a kind gift from Dr. L. Capucci, IZSLER, Brescia, Italy) was added at  
597 a 1/100 dilution for 2h at 37°C, followed by 3 washes with PBS and incubation with  
598 biotinylated anti-mouse antibody (Vector laboratories) at a 1/1000 dilution for 2h at 37°C.  
599 The continuation of the protocol was as described above.

600

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621 **References**

- 622 1. Woolhouse MEJ, Haydon, DT, Antia, R. 2005. Emerging pathogens: the  
623 epidemiology and evolution of species jump. *Trends ecol evol* 20:238-244.
- 624 2. Geoghegan JL, Duchene, S, Holmes, EC. 2017. Comparative analysis estimates the  
625 relative frequencies of co-divergence and cross-species transmission within viral  
626 families. *PLoS Pathog* 13:e1006215.
- 627 3. Longdon B, Brockhurst, MA, Russell, CA, Welch, JJ, Jiggins, FM. 2014. The  
628 evolution and genetics of virus host shifts. *PLoS Pathog* 10:e1004395.
- 629 4. Longdon B, Hadfield, JD, Webster, CL, Obbard, DJ, Jiggins, FM. 2011. Host  
630 phylogeny determines viral persistence and replication in novel hosts. *PLoS*  
631 *Pathog* 7:e1002260.
- 632 5. Clarke IN, Estes, MK, Green, KY, Hansman, GS, Knowles, NJ, Koopmans, MK,  
633 Matson, DO, Meyers, G, Neill, JD, Radford, A, Smith, AW, Studdert, MJ, Thiel, H-J,  
634 Vinjé, J. 2012. Family Caliciviridae. In King AMQ, Adams MJ, Carstens EB,  
635 Lefkowitz EJ (ed), *Virus taxonomy Classification and nomenclature of viruses*  
636 *Ninth report of the international committee on taxonomy of viruses*. Elsevier  
637 Academic Press.
- 638 6. Abrantes J, van der Loo, W, Le Pendu, J, Esteves, PJ. 2012. Rabbit haemorrhagic  
639 disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. *Vet Res*  
640 43:1-19.
- 641 7. Liu SJ, Xue, HP, Pu, BQ, Quian, NH. 1984. A new viral disease in rabbit. *Anim Husb*  
642 *Vet Med* 16:235-255.
- 643 8. Gavier-Widén D, Mörner, T. 1991. Epidemiology and diagnosis of the European  
644 brown hare syndrome in Scandinavian countries: a review. *Revue Scientifique et*  
645 *Technique - Office International des Epizooties* 10:453-458.
- 646 9. Capucci L, Scicluna, M, T, Lavazza, A. 1991. Diagnosis of viral haemorrhagic  
647 disease of rabbits and the European brown hare syndrome. *Rev Sci Tech Off Int*  
648 *Epiz* 10:347-370.
- 649 10. Laurent S, Vautherot, JF, Le Gall, G, Madelaine, MF, Rasschaert, D. 1997.  
650 Structural, antigenic and immunogenic relationships between European brown  
651 hare syndrome virus and rabbit haemorrhagic disease virus. *J Gen Virol* 78 ( Pt  
652 11):2803-11.
- 653 11. Lopes AM, Capucci, L, Gavier-Widén, D, Le Gall-Reculé, G, Brocchi, E, Barbieri, I,  
654 Quemener, A, Le Pendu, J, Geoghegan, J, Holmes, EC, Esteves, PJ, Abrantes, J.  
655 2014. Molecular evolution and antigenic variation of European brown hare  
656 syndrome virus (EBHSV). *Virology* 468-470:104-112.
- 657 12. Cavadini P, Molinari, S, Pezzoni, G, Chiari, M, Brocchi, E, Lavazza, A, Capucci, L.  
658 Identification of a New Non-Pathogenic Lagovirus in Brown Hares (*Lepus*  
659 *europaeus*), p. In Kelly P, Phillips S, Smith A, Browning C (ed), *California State*  
660 *University Stanislaus*,
- 661 13. Le Gall-Reculé G, Zwingelstein, F, Laurent, S, De Boisseron, C, Portejoie, Y,  
662 Rasschaert, D. 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus  
663 in France between 1993 and 2000 and the characterization of RHDV antigenic  
664 variants. *Arch Virol* 148:65-81.
- 665 14. Capucci L, Fallacara, F, Grazioli, S, Lavazza, A, Pacciarini, ML, Brocchi, E. 1998. A  
666 further step in the evolution of rabbit hemorrhagic disease virus: the appearance  
667 of the first consistent antigenic variant. *Virus Research* 58:115-126.

- 668 15. Bergin IL, Wise, AG, Bolin, SR, Mullaney, TP, Kiupel, M, Maes, RK. 2009. Novel  
669 calicivirus identified in rabbits, Michigan, USA. *Emerg Infect Dis* 15:1955-1962.
- 670 16. Capucci L, Fusi, P, Lavazza, A, Pacciarini, M, L, Rossi, C. 1996. Detection and  
671 preliminary characterization of a new rabbit calicivirus related to Rabbit  
672 Haemorrhagic Disease Virus but non pathogenic. *J Virol* 70:8614-8623.
- 673 17. Le Gall-Reculé G, Zwingelstein, F, Fages, MP, Bertagnoli, S, Gelfi, J, Aubineau, J,  
674 Roobrouck, A, Botti, G, Lavazza, A, Marchandeanu, S. 2011. Characterisation of a  
675 non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV.  
676 *Virology* 410:395-402.
- 677 18. Strive T, Wright, JD, Robinson, AJ. 2009. Identification and partial  
678 characterisation of a new Lagovirus in Australian wild rabbits. *Virology* 384:97-  
679 105.
- 680 19. Nicholson LJ, Mahar, JE, Strive, T, Zheng, T, Holmes, EC, Ward, VK, Duckworth, JA.  
681 2017. Discovery and characterization of a benign rabbit calicivirus in New  
682 Zealand. *Appl Environ Microbiol* in press.
- 683 20. Le Gall-Reculé G, Zwingelstein, F, Boucher, S, Le Normand, B, Plassiart, G,  
684 Portejoie, Y, Decors, A, Bertagnoli, S, Guérin, JL, Marchandeanu, S. 2011. Detection  
685 of a new variant of rabbit haemorrhagic disease virus in France. *Vet Rec* 168:137-  
686 138.
- 687 21. Le Gall-Reculé G, Lavazza, A, Marchandeanu, S, Bertagnoli, S, Zwingelstein, F,  
688 Cavadini, P, Martinelli, N, Lombardi, G, Guérin, JL, Lemaitre, E, Decors, A, Boucher,  
689 S, Le Normand, B, Capucci, L. 2013. Emergence of a new lagovirus related to  
690 rabbit hemorrhagic disease virus. *Vet Res* 44:81.
- 691 22. Dalton KP, Nicieza, I, Balseiro, A, Muguerza, MA, Rosell, JM, Casais, R, Alvarez, AL,  
692 Parra, F. 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain.  
693 *Emerg Infect Dis* 18:2009-2012.
- 694 23. Abrantes J, Lopes, AM, Dalton, KP, Melo, P, Correia, JJ, Ramada, M, Alves, PC, Parra,  
695 F, Esteves, PJ. 2013. New variant of rabbit hemorrhagic disease virus, Portugal,  
696 2012-2013. *Emerg Infect Dis* 19:1900-2.
- 697 24. Westcott DG, Frossard, JP, Everest, D, Dastjerdi, A, Duff, JP, Steinbach, F,  
698 Choudhury, B. 2014. Incursion of RHDV2-like variant in Great Britain. *Vet Rec*  
699 174:333.
- 700 25. Almeida T, Lopes, AM, Magalhaes, MJ, Neves, F, Pinheiro, A, Goncalves, D, Leitao,  
701 M, Esteves, PJ, Abrantes, J. 2015. Tracking the evolution of the G1/RHDVb  
702 recombinant strains introduced from the Iberian Peninsula to the Azores islands,  
703 Portugal. *Infect Genet Evol* 34:307-13.
- 704 26. Duarte M, Henriques, M, Barros, SC, Fagulha, T, Ramos, F, Luis, T, Fevereiro, M,  
705 Benevides, S, Flor, L, Barros, SV, Bernardo, S. 2015. Detection of RHDV variant 2  
706 in the Azores. *Vet Rec* 176:130.
- 707 27. Neimanis AS, Ahola, H, Zohari, S, Larsson Pettersson, U, Brojer, C, Capucci, L,  
708 Gavier-Widen, D. 2017. Arrival of rabbit haemorrhagic disease virus 2 to  
709 northern Europe: Emergence and outbreaks in wild and domestic rabbits  
710 (*Oryctolagus cuniculus*) in Sweden. *Transbound Emerg Dis*.
- 711 28. Hall RN, Mahar, JE, Haboury, S, Stevens, V, Holmes, EC, Strive, T. 2015. Emerging  
712 Rabbit Hemorrhagic Disease Virus 2 (RHDVb), Australia. *Emerg Infect Dis*  
713 21:2276-2278.
- 714 29. Le Pendu J, Abrantes, J, Bertagnoli, S, Guiton, JS, Le Gall-Reculé, G, Lopes, AM,  
715 Marchandeanu, S, Alda, F, Almeida, T, Alves, PC, Barcena, J, Burmakina, G, Blanco, E,  
716 Calvete, C, Cavadini, P, Cooke, B, Dalton, KP, Delibes, MM, Deptula, W, Eden, JS,

- 717 Fang, W, Ferreira, CC, Fereira, P, Foronda, P, Gonçalves, D, Gavier-Widén, D, Hall,  
718 R, Hukowska-Szematowicz, B, Kerr, P, Kovaliski, J, Lavazza, A, Mahar, JE,  
719 Malogolovkin, A, Marques, R, Marques, S, Martin-Alonso, A, Monterroso, P,  
720 Moreno, S, Mutze, G, Neimanis, AS, Niedzwiedzka-Rystwej, P, Peacock, DE, Parra,  
721 F, Rocchi, M, Rouco, C, Ruvöen-Clouet, N, Silva, E, Silvério, D, Strive, T, Thompson,  
722 G, et al. 2017. Proposal for a unified classification system and nomenclature of  
723 lagoviruses. *J Gen Virol* 98:1658-1666.
- 724 30. Stuart AD, Brown, TD. 2007. Alpha2,6-linked sialic acid acts as a receptor for  
725 Feline calicivirus. *J Gen Virol* 88:177-86.
- 726 31. Taube S, Perry, JW, Yetming, K, Patel, SP, Auble, H, Shu, L, Nawar, HF, Lee, CH,  
727 Connell, TD, Shayman, JA, Wobus, CE. 2009. Ganglioside-linked terminal sialic  
728 acid moieties on murine macrophages function as attachment receptors for  
729 murine noroviruses. *J Virol* 83:4092-101.
- 730 32. Tamura M, Natori, K, Kobayashi, M, Miyamura, T, Takeda, N. 2004. Genogroup II  
731 noroviruses efficiently bind to heparan sulfate proteoglycan associated with the  
732 cellular membrane. *J Virol* 78:3817-3826.
- 733 33. Ruvöen-Clouet N, Belliot, G, Le Pendu, J. 2013. Noroviruses and histo-blood  
734 groups: the impact of common host genetic polymorphisms on virus  
735 transmission and evolution. *Rev Med Virol* 23:355-366.
- 736 34. Tan M, Jiang, X. 2014. Histo-blood group antigens: a common niche for norovirus  
737 and rotavirus. *Expert Rev Mol Med* 16:e5.
- 738 35. Le Pendu J, Nystrom, K, Ruvöen-Clouet, N. 2014. Host-pathogen co-evolution and  
739 glycan interactions. *Curr Opin Virol* 7:88-94.
- 740 36. Matthee CA, van Vuuren, BJ, Bell, D, Robinson, TJ. 2004. A molecular supermatrix  
741 of the rabbits and hares (Leporidae) allows for the identification of five  
742 intercontinental exchanges during the Miocene. *Syst Biol* 53:443-447.
- 743 37. Rosin A, Gilio, N, Merrigi, A. 2008. Introduced Lagomorphs as a threat to "native"  
744 Lagomorphs: the case of the Eastern Cottontail (*Sylvilagus floridanus*) in  
745 Northern Italy., p 153-164. In Alves P, Ferrand N, Hackländer K (ed), *Lagomorph*  
746 *Biology*. Springer-Verlag, Berlin, Heidelberg.
- 747 38. Sasse C. 1983. Ecological consequences of the introduction of the American  
748 cottontail, *Sylvilagus floridanus*, into Europe, p. 33, Report Council of Europe SN-  
749 US, 83, vol. 83. Research Institute for Nature Management, Leersum, The  
750 Netherlands.
- 751 39. Lavazza A, Scicluna, M, T, Capucci, L. 1996. Susceptibility of hares and rabbits to  
752 the European Brown hare syndrome virus (EBHSV) and rabbit haemorrhagic  
753 disease virus (RHDV) under experimental conditions. *Zentralbl Veterinarmed B*  
754 43:401-410.
- 755 40. Chasey D, Lucas, M, Westcott, D, Williams, M. 1992. European brown hare  
756 syndrome in the U.K.; a calicivirus related to but distinct from that of viral  
757 haemorrhagic disease in rabbits. *Arch Virol* 124:363-70.
- 758 41. Nauwynck H, Callebaut, P, Peeters, J, Ducatelle, R, Uyttebroek, E. 1993.  
759 Susceptibility of hares and rabbits to a Belgian isolate of European brown hare  
760 syndrome virus. *J Wildl Dis* 29:203-8.
- 761 42. di Modugno G, Nasti, R. 1990. La Malattia emorragica virale in Puglia. Contributo  
762 sperimentale. *Riv Conigliol* 27:25-32.
- 763 43. Morisse J-P, Picault, J-P, Boilletot, E, Morin, M. 1990. Relations étiologiques entre  
764 le syndrome du lièvre brun européen (EBHS) et la maladie hémorragique virale  
765 du lapin (VHD). *Rev Med Vet* 141:463-467.

- 766 44. Lavazza A, Cavadini, P, Barbieri, I, Tizzani, P, Pinheiro, A, Abrantes, J, Esteves, PJ,  
767 Grilli, G, Gioia, E, Zanoni, M, Meneguz, P, Guitton, JS, Marchandeanu, S, Chiari, M,  
768 Capucci, L. 2015. Field and experimental data indicate that the eastern cottontail  
769 (*Sylvilagus floridanus*) is susceptible to infection with European brown hare  
770 syndrome (EBHS) virus and not with rabbit hemorrhagic disease (RHD) virus.  
771 *Vet Res* 46:13.
- 772 45. Lopes AM, Marques, S, Silva, E, Magalhaes, MJ, Pinheiro, A, Alves, PC, Le Pendu, J,  
773 Esteves, PJ, Thompson, G, Abrantes, J. 2014. Detection of RHDV strains in the  
774 Iberian hare (*Lepus granatensis*): earliest evidence of rabbit lagovirus cross-  
775 species infection. *Vet Res* 45:94.
- 776 46. Puggioni G, Cavadini, P, Maestrone, C, Scivoli, R, Botti, G, Ligios, C, Le Gall-Reculé,  
777 G, Lavazza, A, Capucci, L. 2013. The new French 2010 rabbit hemorrhagic disease  
778 virus causes an RHD-like disease in the Sardinian Cape hare (*Lepus capensis*  
779 *mediterraneus*). *Vet Res* 44:96.
- 780 47. Camarda A, Pugliese, N, Cavadini, P, Circella, E, Capucci, L, Caroli, A, Legretto, M,  
781 Mallia, E, Lavazza, A. 2014. Detection of the new emerging rabbit haemorrhagic  
782 disease type 2 virus (RHDV2) in Sicily from rabbit (*Oryctolagus cuniculus*) and  
783 Italian hare (*Lepus corsicanus*). *Res Vet Sci* 97:642-645.
- 784 48. Hall RN, Peacock, DE, Kovaliski, J, Mahar, JE, Mourant, R, Piper, M, Strive, T. 2016.  
785 Detection of RHDV2 in European brown hares (*Lepus europaeus*) in Australia. .  
786 *Veterinary Record VetRec-2016-104304*.
- 787 49. Velarde R, Cavadini, P, Neimanis, AS, Cabezon, O, Chiari, M, Gaffuri, A, Lavin, S,  
788 Grilli, G, Gavier-Widén, D, Lavazza, A, Capucci, L. 2016. Spillover events of  
789 infection of Brown hares (*Lepus europaeus*) with Rabbit haemorrhagic disease  
790 type 2 virus (RHDV2) caused sporadic cases of an European brown hare  
791 syndrome-like disease in Italy and Spain. . *Transbound Emerg Dis*.
- 792 50. Merchan T, Rocha, G, Alda, F, Silva, E, Thompson, G, de Trucios, SH, Pages, A.  
793 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific  
794 vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*).  
795 *Infect Genet Evol* 11:1469-1474.
- 796 51. Rocha G, Alda, F, Pagés, A, Merchán, T. 2016. Experimental transmission of rabbit  
797 haemorrhagic disease virus (RHDVa) from rabbit to wild mice (*Mus spretus* and  
798 *Apodemus sylvaticus*) under laboratory conditions. *Infect Genet Evol* 47:94-98.
- 799 52. Rodak L, Smid, B, Valicek, L, Vesely, T, Stepanek, J, Hampl, J, Jurak, E. 1990.  
800 Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic  
801 disease virus and determination of its major structural proteins. *J Gen Virol*  
802 71:1075-1080.
- 803 53. Robinson AJ, Kirkland, PD, Forrester, RI, Capucci, L, Cooke, BD, Philbey, AW.  
804 2002. Serological evidence for the presence of a calicivirus in Australian wild  
805 rabbits, *Oryctolagus cuniculus*, before the introduction of rabbit haemorrhagic  
806 disease virus (RHDV): its potential influence on the specificity of a competitive  
807 ELISA for RHDV. . *Wildlife Res* 29:655-662.
- 808 54. Forrester NL, Trout, RC, Gould, EA. 2007. Benign circulation of rabbit  
809 haemorrhagic disease virus on Lambay Island, Eire. *Virology* 358:18-22.
- 810 55. Esteves PJ, Abrantes, J, Bertagnoli, S, Cavadini, P, Gavier-Widen, D, Guitton, JS,  
811 Lavazza, A, Lemaitre, E, Letty, J, Lopes, AM, Neimanis, AS, Ruvoen-Clouet, N, Le  
812 Pendu, J, Marchandeanu, S, Le Gall-Recule, G. 2015. Emergence of Pathogenicity in  
813 Lagoviruses: Evolution from Pre-existing Nonpathogenic Strains or through a  
814 Species Jump? *PLoS Pathog* 11:e1005087.



- 815 56. Arthur CP, Chapuis, JL. 1983. L'introduction de *Sylvilagus floridanus* en France:  
816 historique, dangers et expérimentation en cours. . CR Soc Biogeograph 59:333-  
817 356.
- 818 57. Mussa PP. 1996. Il Silvilago in provincia di Torino. Habitat:5-11.
- 819 58. Ruvoën-Clouet N, Ganière, JP, André-Fontaine, G, Blanchard, D, Le Pendu, J. 2000.  
820 Binding of Rabbit Hemorrhagic Disease Virus to antigens of the ABH histo-blood  
821 group family. J Virol 74:11950-11954.
- 822 59. Nyström K, Le Gall-Reculé, G, Grassi, P, Abrantes, J, Ruvoën-Clouet, N, Le Moullac-  
823 Vaidye, B, Lopes, AM, Esteves, PJ, Strive, T, Marchandeu, S, Dell, A, Haslam, SM,  
824 Le Pendu , J. 2011. Histo-Blood Group Antigens act as attachment factors of  
825 Rabbit Hemorrhagic Disease Virus infection in a virus strain-dependent manner.  
826 PLoS Pathogens 7:e1002188.
- 827 60. Marionneau S, Cailleau-Thomas, A, Rocher, J, Le Moullac-Vaidye, B, Ruvoën-  
828 clouet, N, Clément, M, Le Pendu, J. 2001. ABH and Lewis histo-blood group  
829 antigens, a model for the meaning of oligosaccharide diversity in the face of a  
830 changing world. Biochimie 83:565-573.
- 831 61. Guillon P, Ruvöen-Clouet, N, Le Moullac-Vaidye, B, Marchandeu, S, Le Pendu , J.  
832 2009. Association between expression of the H histo-blood group antigen,  $\alpha$ 1,2  
833 fucosyltransferases polymorphism of wild rabbits, and sensitivity to rabbit  
834 hemorrhagic disease virus. Glycobiology 19:21-28.
- 835 62. Nystrom K, Abrantes, J, Lopes, AM, Le Moullac-Vaidye, B, Marchandeu, S, Rocher,  
836 J, Ruvoen-Clouet, N, Esteves, PJ, Le Pendu, J. 2015. Neofunctionalization of the  
837 Sec1  $\alpha$ 1,2fucosyltransferase paralogue in leporids contributes to glycan  
838 polymorphism and resistance to rabbit hemorrhagic disease virus. PLoS Pathog  
839 11:e1004759.
- 840 63. Alves PC, Hackländer, K. 2008. Lagomorph species: Geographical distribution  
841 and conservation status, p 395-405. In Alves PC, Ferrand N, Hackländer K (ed),  
842 Lagomorph Biology. Springer-Verlag, Berlin, Heidelberg.
- 843 64. Holmes EC. 2013. What can we predict about viral evolution and emergence?  
844 Curr Opin Virol 3:180-4.
- 845 65. Smith AW, Skilling, DE, Cherry, N, Mead, JH, Matson, DO. 1998. Caliciviruses  
846 emergence from ocean reservir: zoonotic and interspecies movements. Emerg  
847 Infect Dis 4:13-20.
- 848 66. Di Martino B, Di Rocco, C, Ceci, C, Marsilio, F. 2009. Characterization of a strain of  
849 feline calicivirus isolated from a dog faecal sample. Vet Microbiol 139:52-7.
- 850 67. Caddy SL, de Rougemont, A, Emmott, E, El-Attar, L, Mitchell, JA, Hollinshead, M,  
851 Belliot, G, Brownlie, J, Le Pendu, J, Goodfellow, I. 2015. Evidence for human  
852 norovirus infection of dogs in the United kingdom. J Clin Microbiol 53:1873-83.
- 853 68. Oriol R, Candelier, JJ, Taniguchi, S, Balanzino, L, Peters, L, Niekrasz, M, Hammer, C,  
854 Cooper, DK. 1999. Major carbohydrate epitopes in tissues of domestic and  
855 African wild animals of potential interest for xenotransplantation research.  
856 Xenotransplantation 6:79-89.
- 857 69. Oriol R, Mollicone, R, Couillin, P, Dalix, AM, Candelier, JJ. 1992. Genetic regulation  
858 of the expression of ABH and Lewis antigens in tissues. APMIS 100, Supp 27:28-  
859 38.
- 860 70. Macher BA, Galili, U. 2008. The Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-R ( $\alpha$ -Gal) epitope: A  
861 carbohydrate of unique evolution and clinical relevance. Biochem Biophys Acta  
862 1780:75-88.

- 863 71. Elsworth PG, Kovaliski, J, Cooke, BD. 2012. Rabbit haemorrhagic disease: are  
864 Australian rabbits (*Oryctolagus cuniculus*) evolving resistance to infection with  
865 Czech CAPM 351 RHDV? *Epidemiol Infect* 140:1972-81.
- 866 72. Esko JD, Lindahl, U. 2001. Molecular diversity of hepan sulfate. *J Clin Invest*  
867 108:169-173.
- 868 73. Meneghetti MC, Hughes, AJ, Rudd, TR, Nader, HB, Powell, AK, Yates, EA, Lima, MA.  
869 2015. Heparan sulfate and heparin interactions with proteins. *J R Soc Interface*  
870 12:0589.
- 871 74. Olofsson S, Bergström, T. 2005. Glycoconjugate glycans as viral receptors. *Annals*  
872 *of Medicine* 37:154-172.
- 873 75. Salvador B, Sexton, NR, Carrion, R, Jr., Nunneley, J, Patterson, JL, Steffen, I, Lu, K,  
874 Muench, MO, Lembo, D, Simmons, G. 2013. Filoviruses utilize  
875 glycosaminoglycans for their attachment to target cells. *J Virol* 87:3295-304.
- 876 76. Mahar JE, Nicholson, L, Eden, JS, Duchêne, S, Kerr, PJ, Duckworth, J, Ward, VK,  
877 Holmes, EC, Strive, T. 2016. Benign rabbit caliciviruses exhibit evolutionary  
878 dynamics similar to those of their virulent relatives. *J Virol* 90:9317-9329.
- 879 77. Lopes AM, Dalton, KP, Magalhaes, MJ, Parra, F, Esteves, PJ, Holmes, EC, Abrantes,  
880 J. 2015. Full genomic analysis of a new variant rabbit hemorrhagic disease virus  
881 revealed multiple recombination events. *J Gen Virol* 96:1309-1319.
- 882 78. Lopes AM, Silvério, D, M.J, M, Areal, H, Alves, PC, Esteves, PJ, Abrantes, J. 2017.  
883 Characterization of RHDV strains by complete genome sequencing identifies a  
884 novel genetic group. *Scientific Reports* in press.
- 885 79. Liu J, Kerr, PJ, Wright, JD, Strive, T. 2012. Serological assays to discriminate  
886 rabbit haemorrhagic disease virus from Australian non-pathogenic rabbit  
887 calicivirus. *Vet Microbiol* 157:345-54.
- 888 80. Jiang X, Wang, M, Graham, DY, Estes, MK. 1992. Expression, self-assembly, and  
889 antigenicity of the Norwalk virus capsid protein. *J Virol* 66:6527-6532.
- 890 81. Le Pendu J, Le Cabellec, M, Bara, J. 1997. Immunohistological analysis of  
891 antibodies against ABH and other glycoconjugates in normal human pyloric and  
892 duodenal mucosae. *Transfus Clin Biol* 1:41-46.
- 893 82. Galanina OE, Chinarev, AA, Sholova, NV, Sablina, MA, Bovin, NV. 2012.  
894 Immobilization of polyacrylamide-based glycoconjugates on solid phase in  
895 immunosorbent assays. *Methods Mol Biol* 808:167-182.
- 896 83. Huflejt ME, Vuskovic, M, Vasiliu, D, Xu, H, Obukhova, P, Shilova, N, Tuzikov, A,  
897 Galanina, O, Arun, B, Lu, K, Bovin, N. 2009. Anti-carbohydrate antibodies of  
898 normal sera: findings, surprises and challenges. *Mol Immunol* 46:3037-49.
- 899  
900

901

902 **Table 1: Expression of ABH antigens in tissues of lagomorphs**

	Trachea <sup>a</sup>			Small intestine <sup>b</sup>		
	<i>Oryctolagus</i>	<i>Lepus</i>	<i>Sylvilagus</i>	<i>Oryctolagus</i>	<i>Lepus</i>	<i>Sylvilagus</i>
anti-A	5/21 <sup>c,d</sup>	6/6	0/6	5/21 <sup>d</sup>	6/6	4/6
anti-B	0/21	0/6	6/6	16/21 <sup>d</sup>	0/6	6/6
UEA-I	20/21	0/6 <sup>e</sup>	6/6	19/21	0/6 <sup>f</sup>	6/6

903 <sup>a</sup>Labeling of the epithelium

904 <sup>b</sup>Labeling of villi and crypts epithelial cells

905 <sup>c</sup> Number of animals with positive staining over total number tested

906 <sup>d</sup>A and B antigens expression is polymorphic with variable frequencies between  
 907 populations (59)

908 <sup>e</sup>Weak labelling of glandular cells of the sub-mucosa only

909 <sup>f</sup> Weak irregular labelling only

910

911 **Figure legends**

912 **Figure 1: Phylogenetic relationships between the lagoviruses studied for their glycan**  
913 **attachment properties.** The maximum-likelihood (ML) phylogenetic tree was built as  
914 previously described (29). Red stars indicate strains that had been previously studied (59).  
915 Blue stars indicate new strains studied in the present manuscript.

916 **Figure 2: Histo-blood group A,B phenotype dependence of the binding of lagovirus**  
917 **strains to duodenum extracts of rabbits (*Oryctolagus cuniculus*).** VLPs from each strain  
918 were incubated on ~3 µg/mL proteins coated tissue extracts from animals of the A-B-, A+B-  
919 and A+B+ phenotypes and their binding quantified by ELISA. To account for variations in  
920 extract material concentrations, data points correspond to normalized mean OD values for  
921 each animal. For GI.1d (RHDV G5) values were normalized to ConA binding values as  
922 previously described (59). For the other strains, OD values were normalized to protein  
923 concentrations of the extracts. Horizontal bars represent mean values and SD. Statistically  
924 significant differences between groups are shown: \*\* p<0.01, \*\*\* p<0.0001 (Two-tailed  
925 Mann-Whitney).

926

927 **Figure 3: Results from the printed glycan microarrays.** Direct fluorescence measurements  
928 obtained from the printed glycan microarrays incubated with VLPs of each virus strain. Only  
929 glycans to which specific binding by at least one of the strains was observed are shown. They  
930 are either HBGA-related structures (black bars), or GlcNAc-terminated structures (grey bars).  
931 Except that of heparin (white bars) to which all strains bound most strongly, their structures  
932 are shown on the right hand side (blue square = N-acetylglucosamine, yellow circle =  
933 galactose, red triangle = fucose, yellow square = N-acetylgalactosamine). The full list of

934 arrayed oligosaccharides (n=360) is presented on Table S1. Results are shown as mean values  
935 of six replicates; error bars represent S.D.

936

937 **Figure 4: Comparison of the binding of VLPs from three lagovirus strains to A, B and H**  
938 **type 2.** Following incubation of VLPs to polyacrylamide-conjugated glycans coated on  
939 ELISA plates, binding was detected using rat polyclonal antibodies. Data are shown as mean  
940 values of triplicates. The negative control (C-) corresponds to OD values obtained in the  
941 absence of VLPs.

942

943 **Figure 5: Comparison of the HBGA specificity of two strains of the new RHDV variant**  
944 **(RHDV2 or GI.2).** Neoglycoconjugates either as polyacrylamide (R1) or human serum  
945 albumin conjugates (R2) were immobilized on ELISA plates. After incubation with VLPs  
946 from strain 10.28 or 10.32, binding was detected using a specific rat antiserum. Other  
947 structures represent the mean OD values of 48 other HBGA-related neoglycoconjugates to  
948 which no binding was observed (structures given on Table S2).

949

950 **Figure 6: EBHSV binding to hare duodenum tissue extracts following enzyme**  
951 **treatments and expression of terminal GlcNAc residues in lagomorph tissues.** ELISA  
952 plates were coated with duodenum mucosa extracts and treated with either PNGase F or O-  
953 sialoglycoprotein endopeptidase (OSGE). Untreated control wells were incubated in the  
954 presence of the enzyme buffers only. Following treatments, tissue extracts were incubated  
955 with biotinylated ConA, an anti-A blood group mAb or EBHSV. Data are shown as means of  
956 duplicate percentages of binding in treated wells (white bars) versus untreated wells (black  
957 bars). Ratio of untreated wells OD values to those of negative controls in absence of either  
958 ConA, the anti-A or EBHSV were 9.8, 11.7 and 6.4, respectively (**a**). Staining of *Oryctolagus*

959 *cuniculus*, *Lepus europaeus* and *Sylvilagus floridanus* duodenum and trachea was performed  
960 using biotinylated succinylated wheat germ agglutinin (sWGA). Tissues from 6 animals of  
961 each species were analyzed. Representative images from each species corresponding to a  
962 x200 magnification are shown. Specificity of sWGA attachment was performed by co-  
963 incubation with chitobiose-polyacrylamide conjugate that completely inhibited the staining  
964 unlike a disaccharide conjugate without N-acetylglucosamine (not shown) (b). sWGA  
965 binding was quantified by ELISA on duodenum extracts coated at ~3 µg/mL proteins. Data  
966 represent mean OD values normalized for protein content variations for each individual  
967 animal. Statistically significant differences between groups are shown: \*\* p<0.01, (Two-  
968 tailed Mann-Whitney) (c).

969

970 **Figure 7: Blocking terminal GlcNAc residues decreases EBHSV binding.** Upper panel:  
971 *Lepus europaeus* trachea tissue sections were either left untreated (b) or treated with β-  
972 hexosaminidase (c) prior to incubation with sWGA, showing a decrease of accessible terminal  
973 N-acetylglucosamine residues following treatment. Middle panel: tissue sections were either  
974 left untreated (d) or treated with β-hexosaminidase (e). Detection of virus binding was then  
975 performed using a monoclonal anti-EBHSV antibody (5F5). Lower panel: *Lepus europaeus*  
976 tissue sections were incubated with the virus alone (f), the virus in the presence of the  
977 unlabeled fucose specific lectin AAL (g) or the virus in the presence of unlabeled sWGA (h).  
978 For negative control sections, all incubation steps were followed, but the virus was omitted  
979 (a).

980

981 **Figure 8: Expression of A, B and H antigens in lagomorph tissues.** Staining of section of  
982 trachea (upper panel) and duodenum (lower panel) from 3 species of lagomorphs was  
983 performed using specific monoclonal antibodies for A and B antigens (anti-A, anti-B) and the

984 lectin I from *Ulex europaeus* (Ulex) for H antigen. Tissues from 21 *Oryctolagus cuniculus*  
985 individuals, 6 *Lepus europaeus* and 6 *Sylvilagus floridanus* were analyzed. Representative  
986 images corresponding to a x400 magnification (upper panel) or a x200 magnification (lower  
987 panel) from each species are shown. No detectable staining was visible on negative controls  
988 in performed in the presence of irrelevant primary antibodies (not shown).

989

990 **Figure 9: Glycan expression in the trachea matches that in the nose.** Staining of the nose  
991 epithelium of European rabbit (*Oryctolagus cuniculus*) and European brown hare (*Lepus*  
992 *europaeus*) by the UEA-I and sWGA lectins illustrating binding patterns as compared to the  
993 trachea. Stars indicate glandular ducts, strongly stained by UEA-I in *Oryctolagus cuniculus*  
994 only and inversely by sWGA in *Lepus europaeus* only. Arrows show the dying and dead cells  
995 of the stratum granulosa and stratum corneum layers of the nose stratified epithelium that are  
996 labelled by the UEA-I lectin in both species.

997

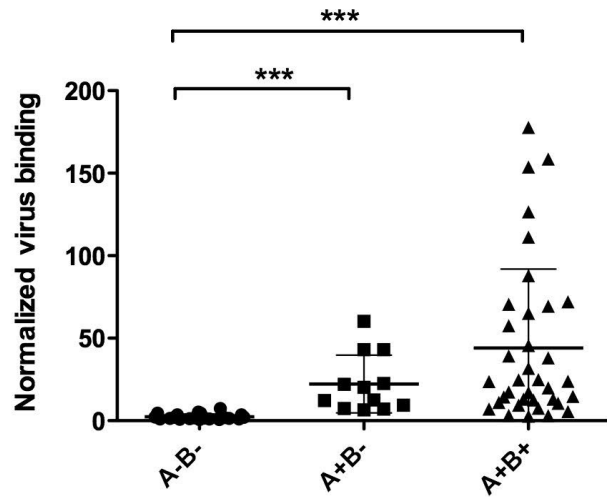
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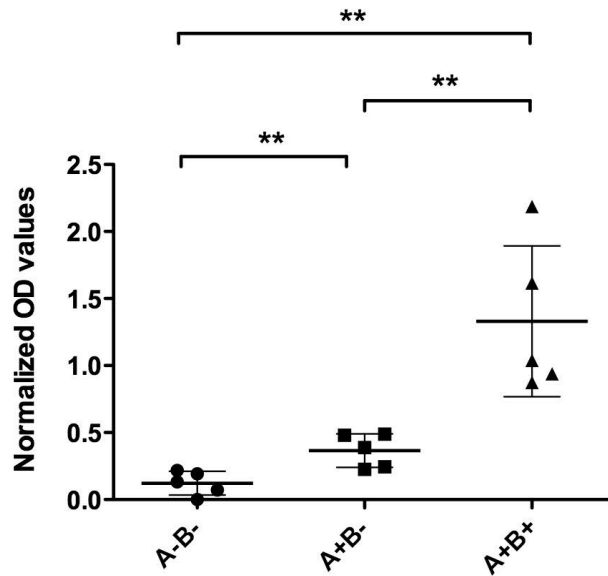
0.09

(GII.2)

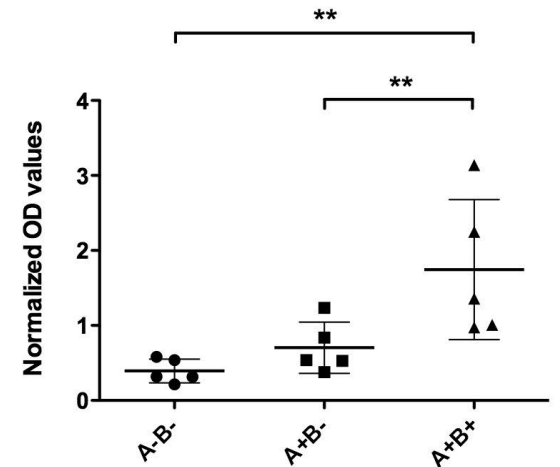




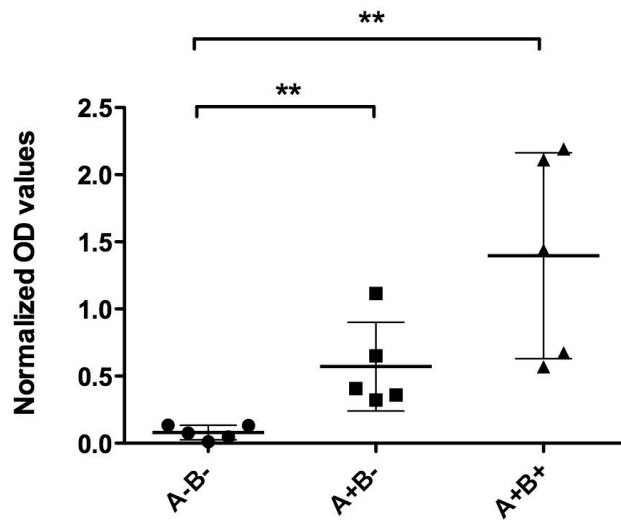
GI.1d (RHDV G5)



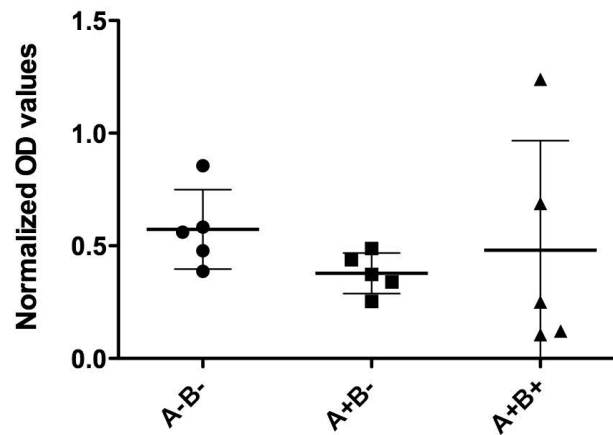
GI.4a (RCV-A1)



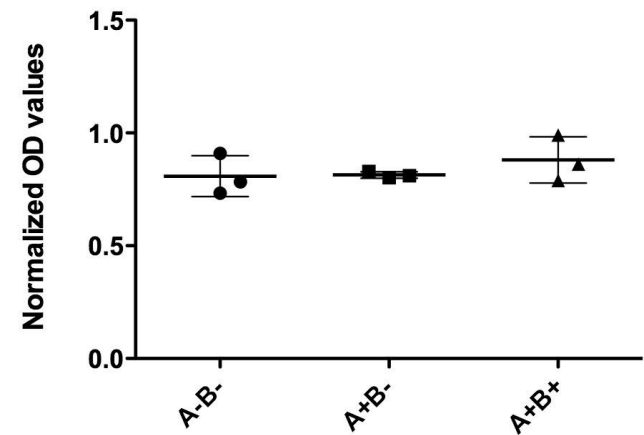
GI.4d (RCV-E2)



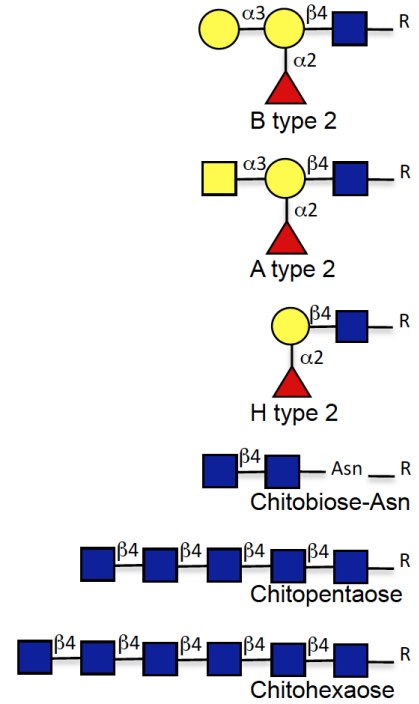
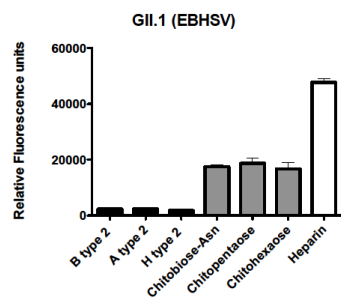
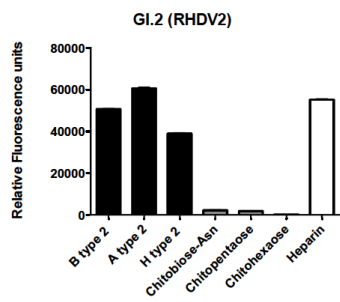
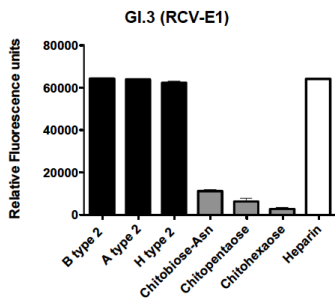
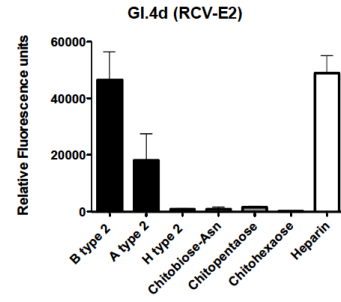
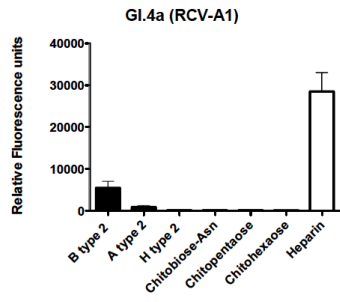
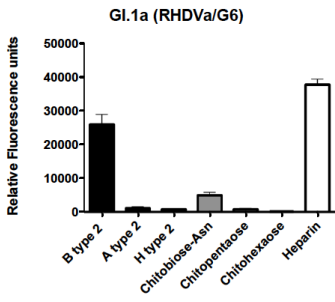
GI.3 (RCV-E1)



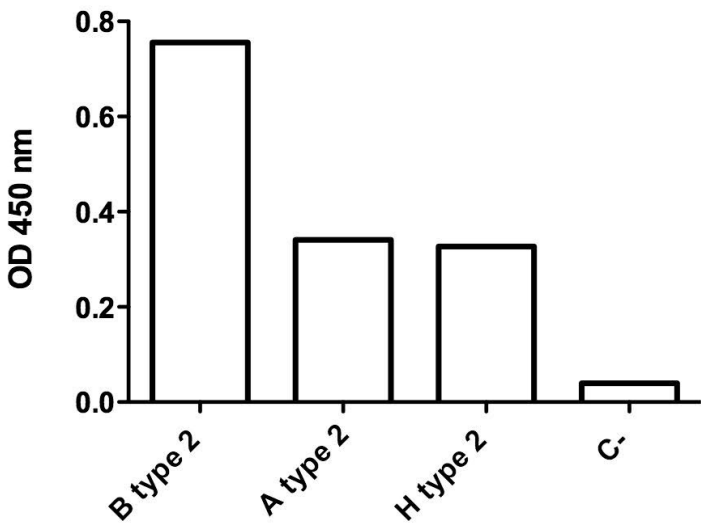
GI.2 (RHDV2)



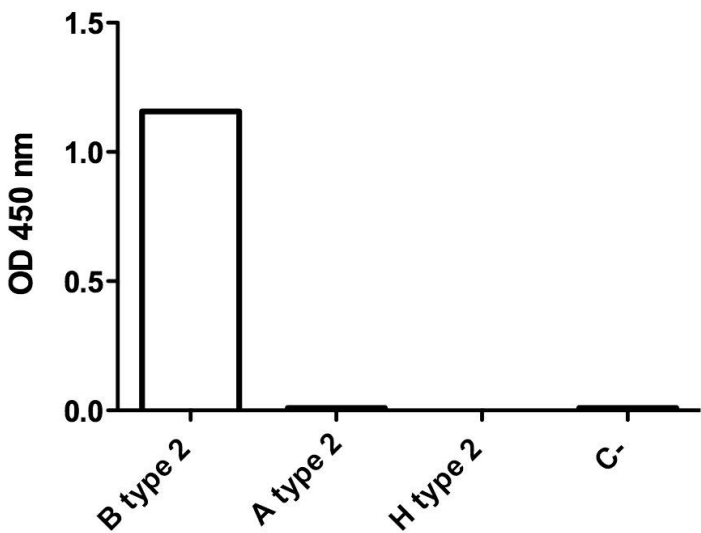
GII.1 (EBHSV)



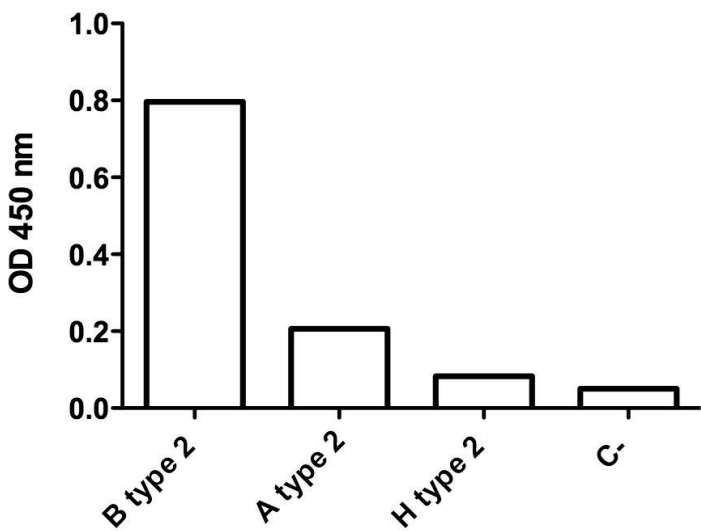
### GI.2 (RHDV2)

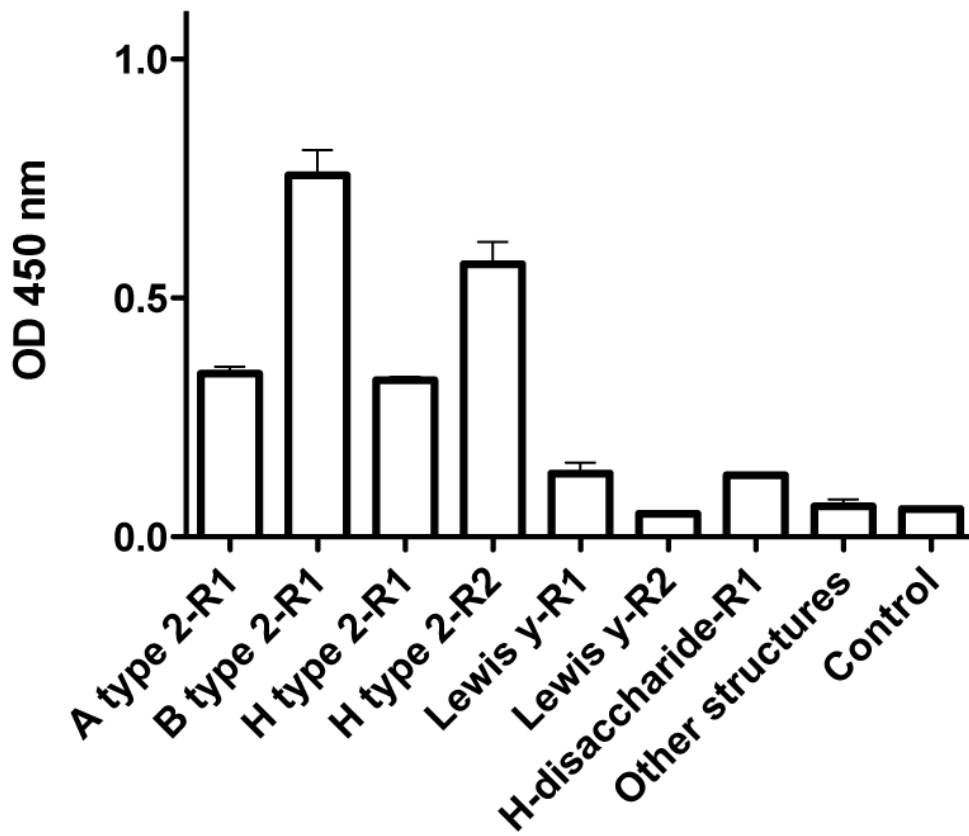


### GI.3 (RCV-E1)

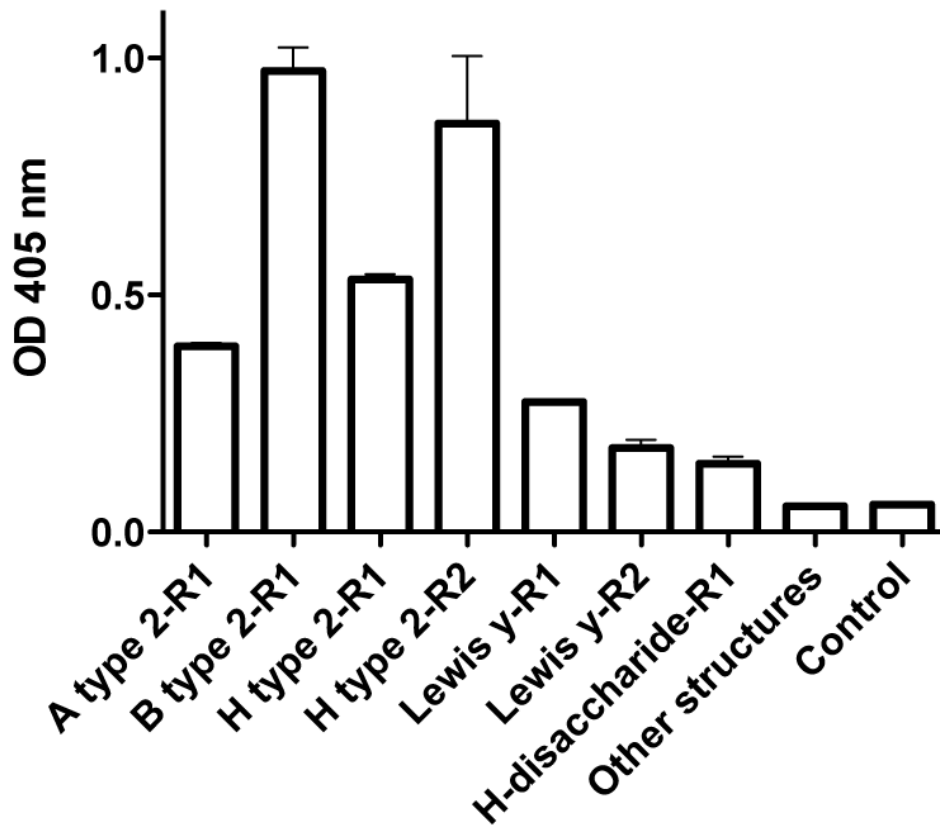


### GI.4d (RCV-E2)

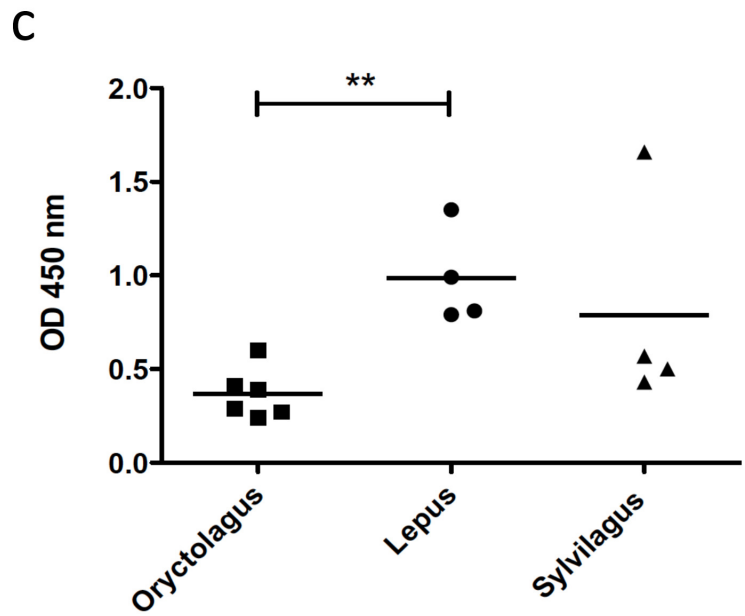
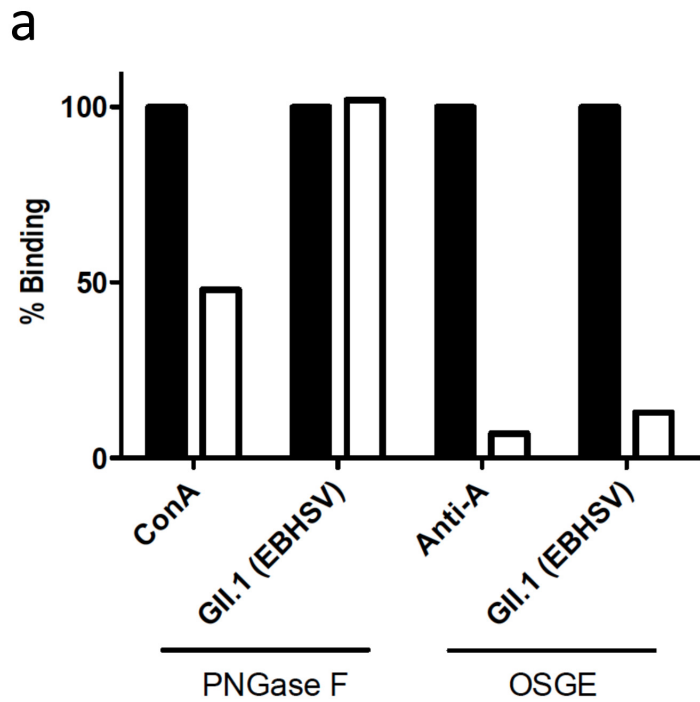




**Strain 10.28**

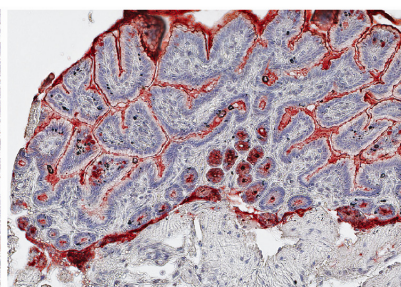
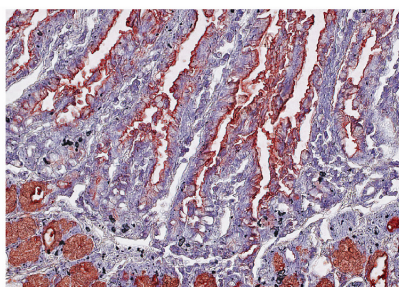
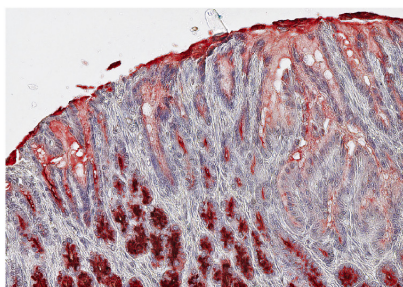


**Strain 10.32**

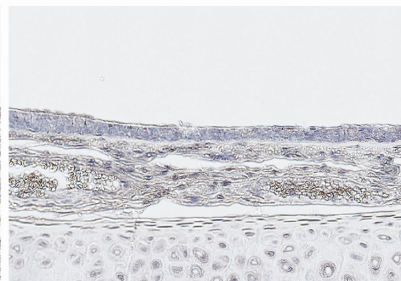
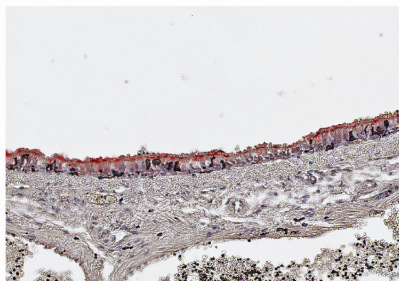
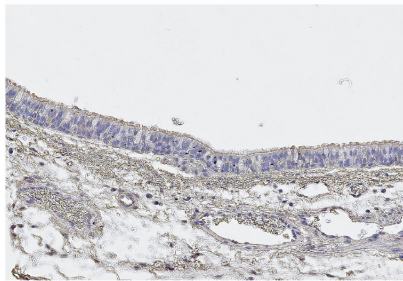


**b** *Oryctolagus* *Lepus* *Sylvilagus*

Duodenum

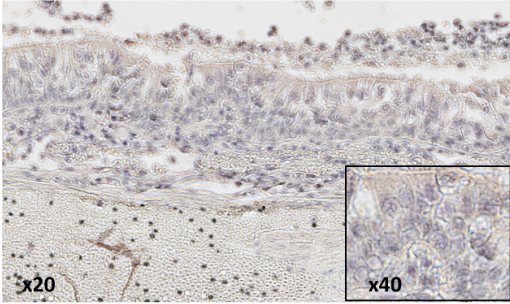


Trachea

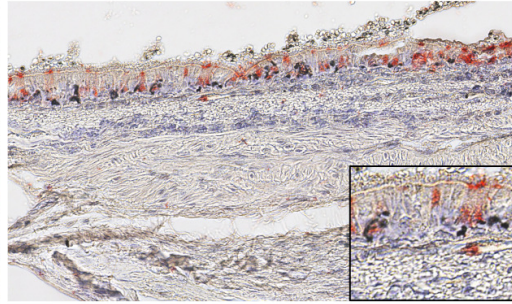




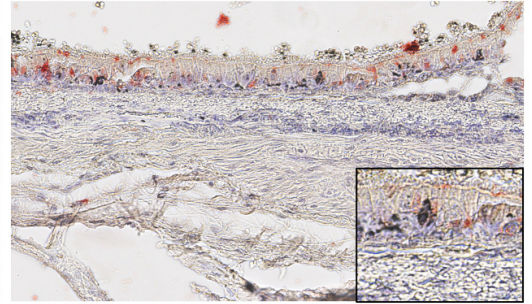
a



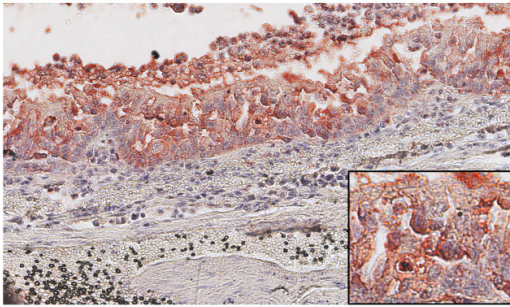
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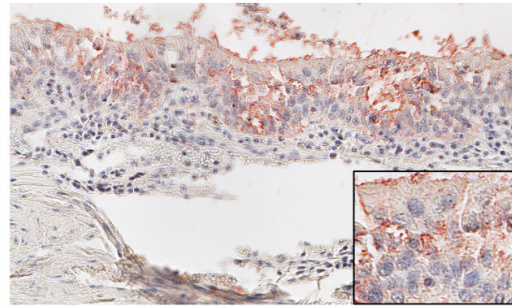
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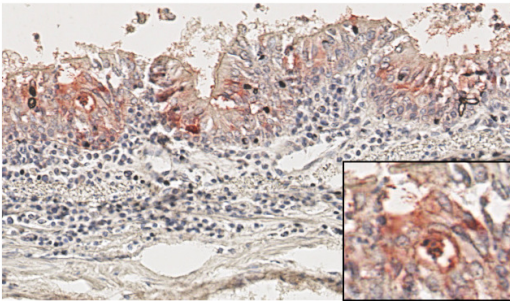
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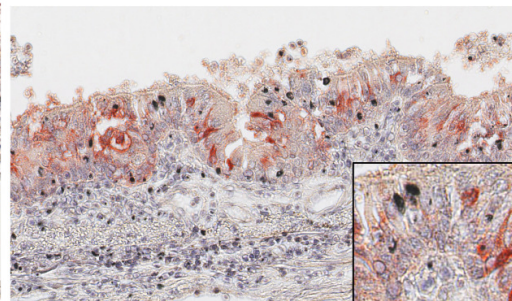
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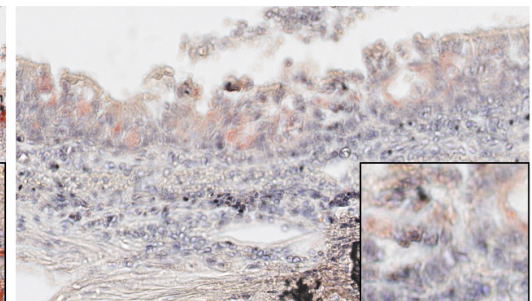
f



g



h



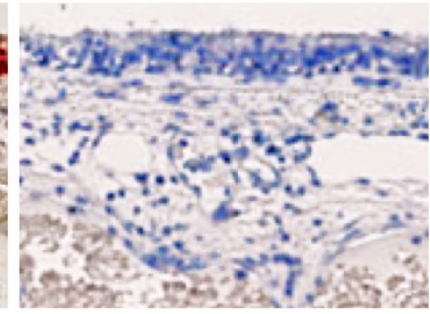
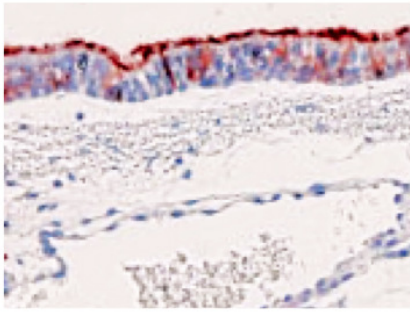


*Oryctolagus*

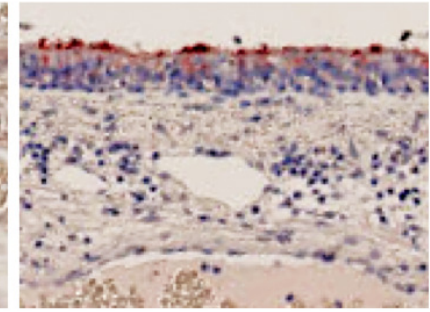
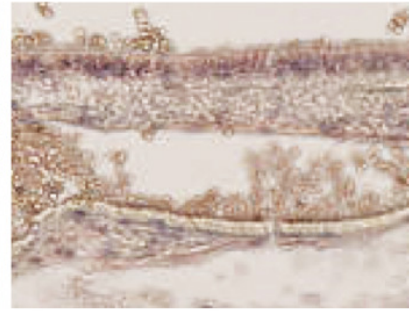
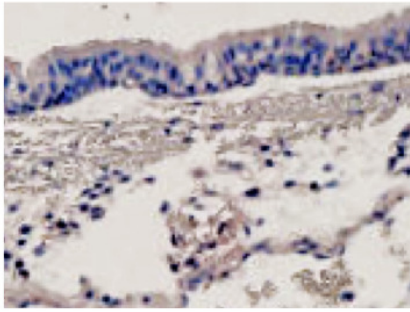
*Lepus*

*Sylvilagus*

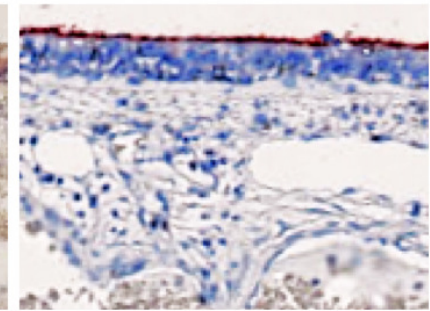
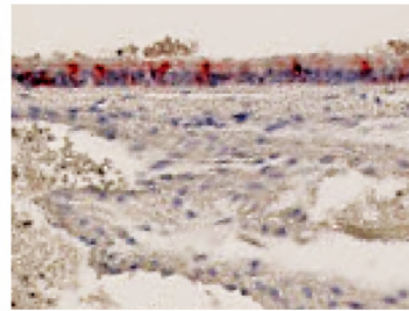
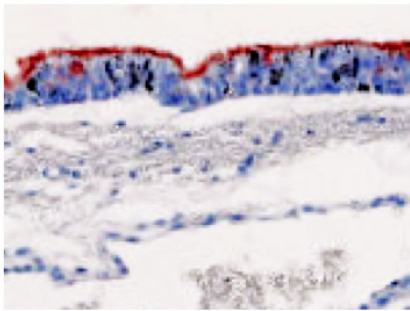
Anti-A



Anti-B



Ulex

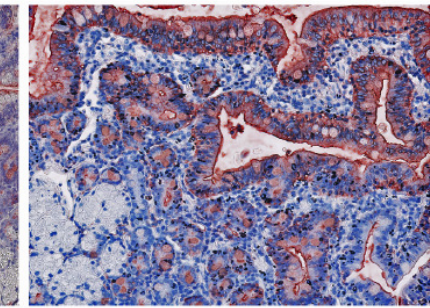
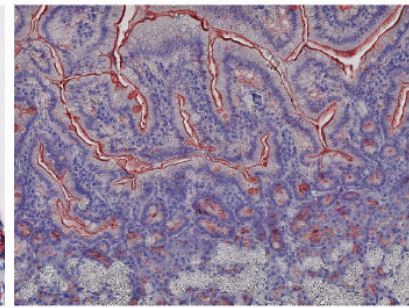
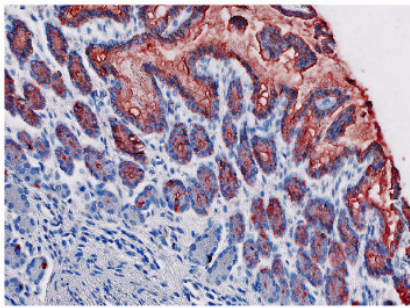


*Oryctolagus*

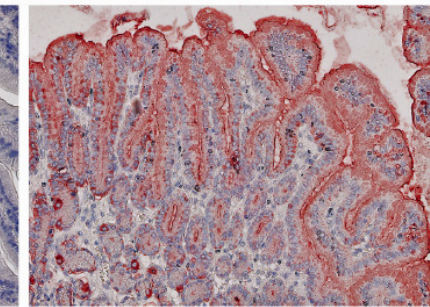
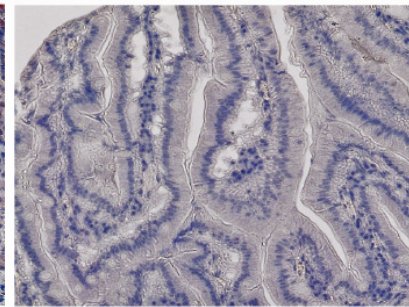
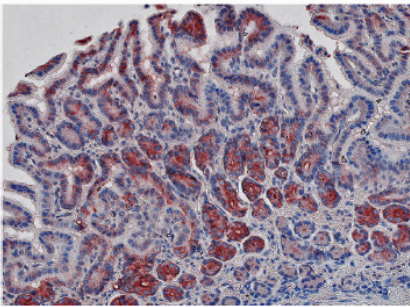
*Lepus*

*Sylvilagus*

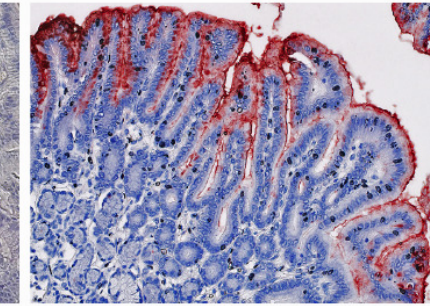
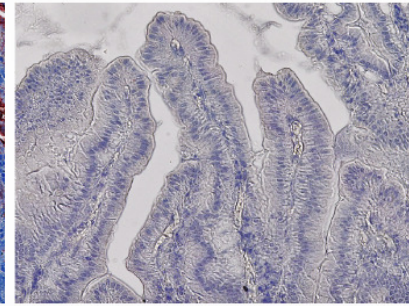
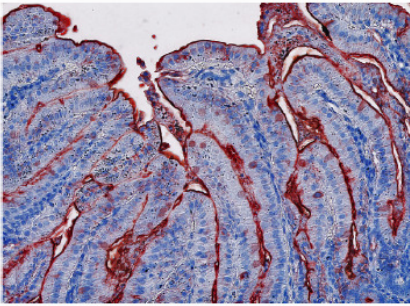
Anti-A



Anti-B



Ulex

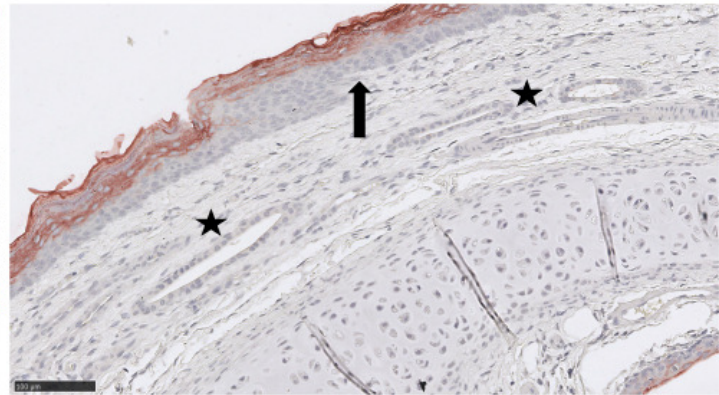
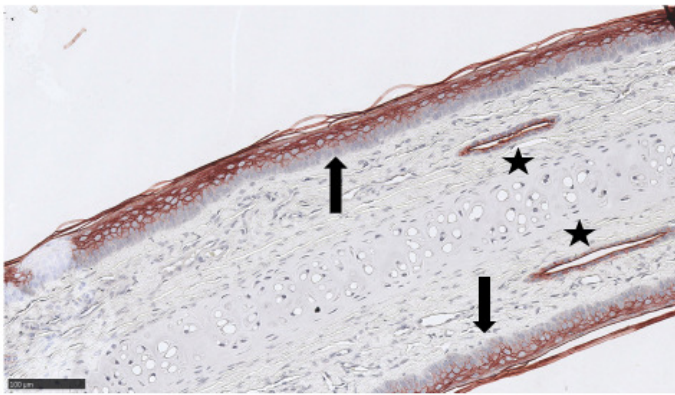




*Oryctolagus*

*Lepus*

UEA-I



sWGA

