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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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38

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 β -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 β 4GlcNAc so-called type 2 precursor that appeared to be the main precursor in rabbits
150 (59). Addition of a fucose in α 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 α 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 β -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 β -
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 β -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 β -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 β -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 β -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 β -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 β -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 β -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 β -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 μ 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^{er} 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 β -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 β -linked N-acetylglucosamine residues in EBHSV
586 attachment, deparaffinated and hydrogen peroxide blocked tissue sections were treated with
587 25U β -N-Hexosaminidase_f (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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620

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- 899
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901

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

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

903 ^aLabeling of the epithelium

904 ^bLabeling of villi and crypts epithelial cells

905 ^c Number of animals with positive staining over total number tested

906 ^dA and B antigens expression is polymorphic with variable frequencies between
 907 populations (59)

908 ^eWeak labelling of glandular cells of the sub-mucosa only

909 ^f 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.

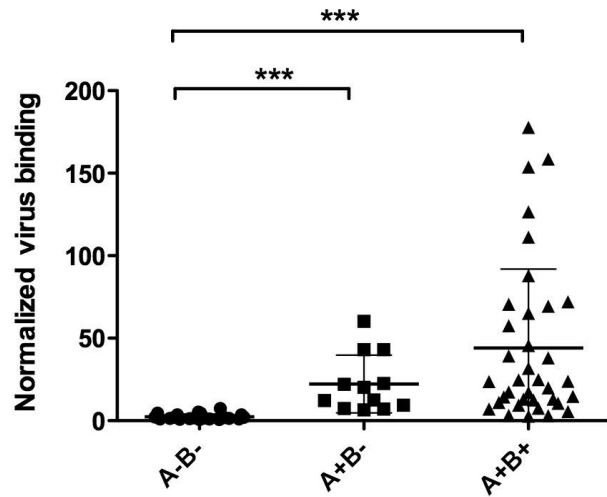
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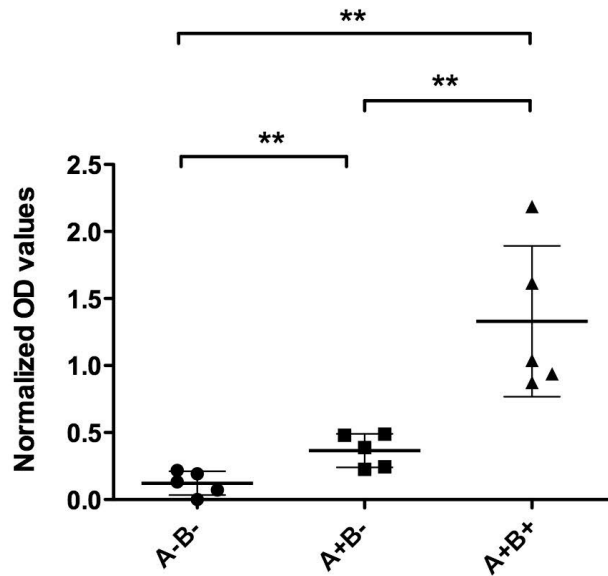


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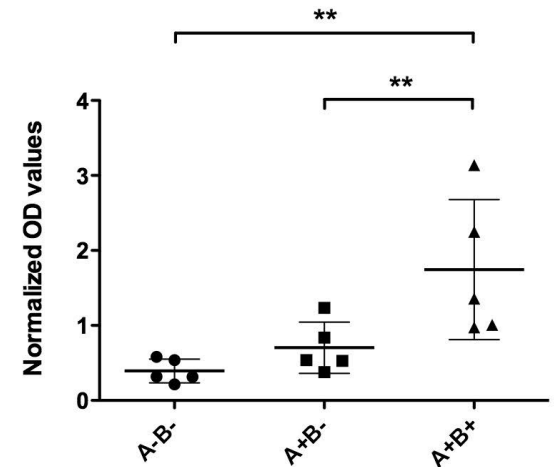
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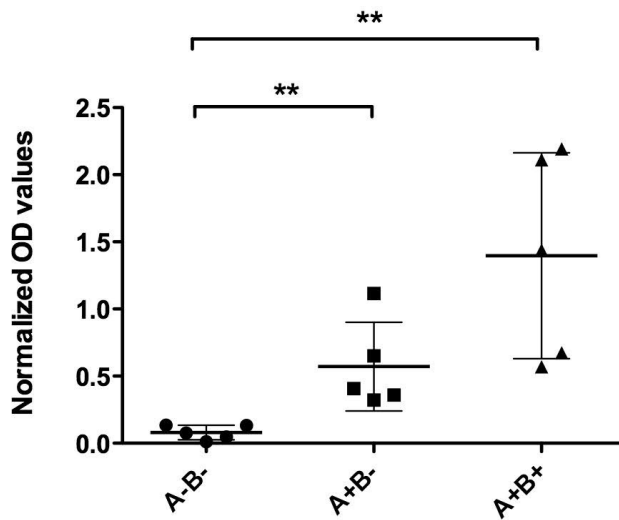
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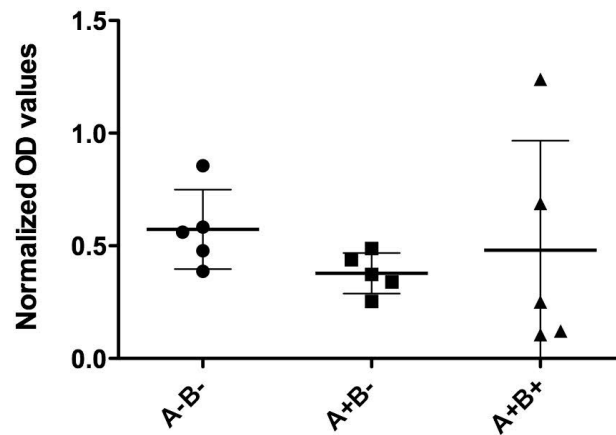
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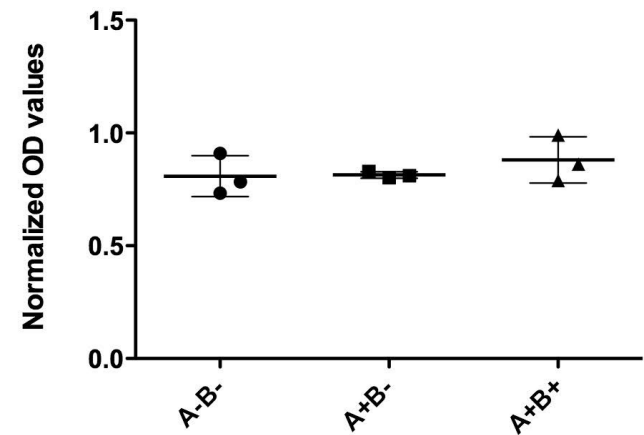
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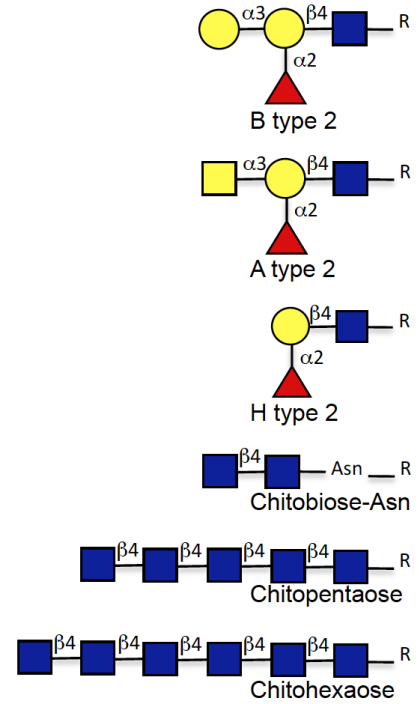
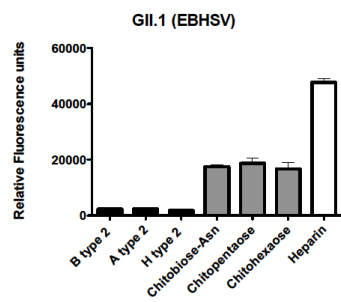
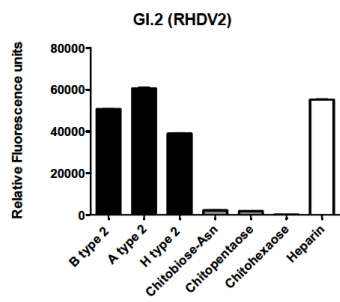
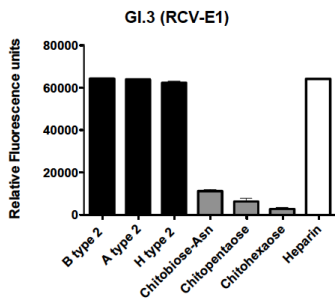
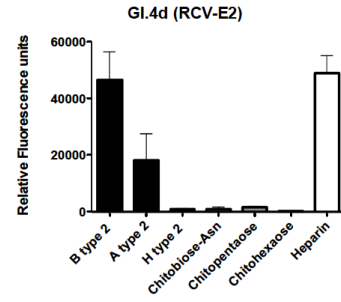
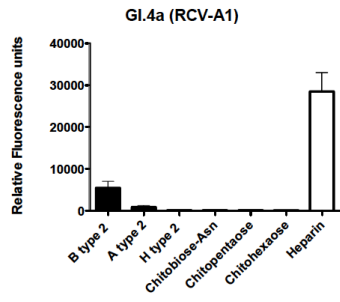
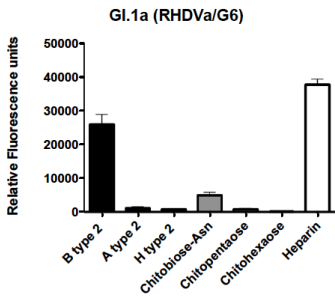
GI.3 (RCV-E1)



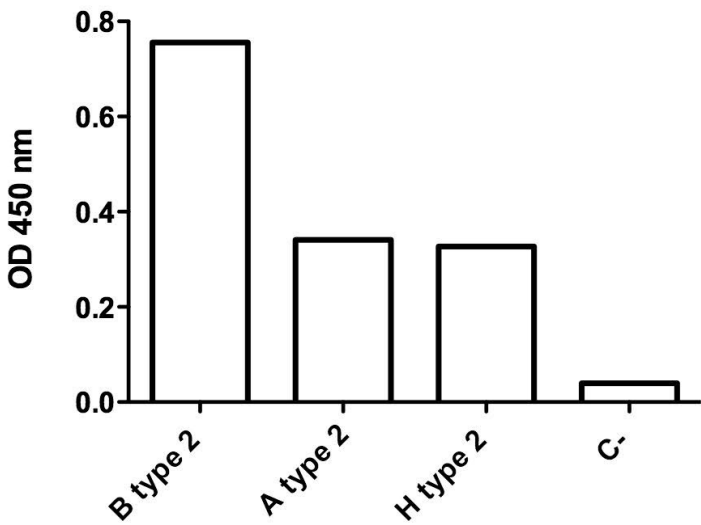
GI.2 (RHDV2)



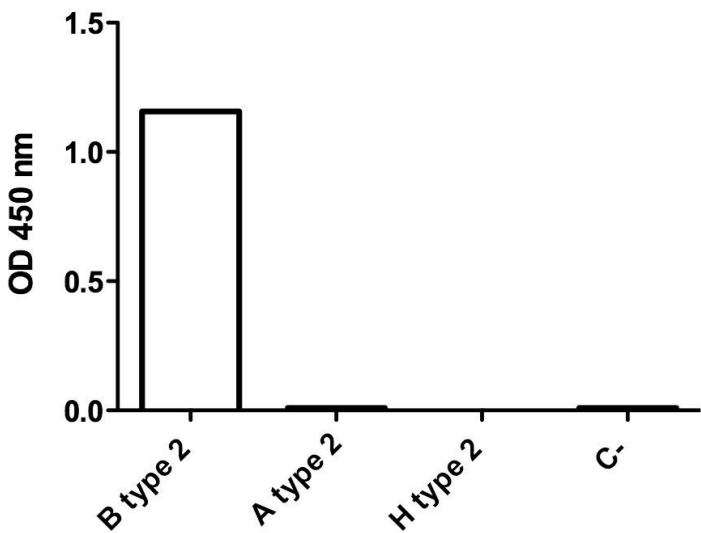
GII.1 (EBHSV)



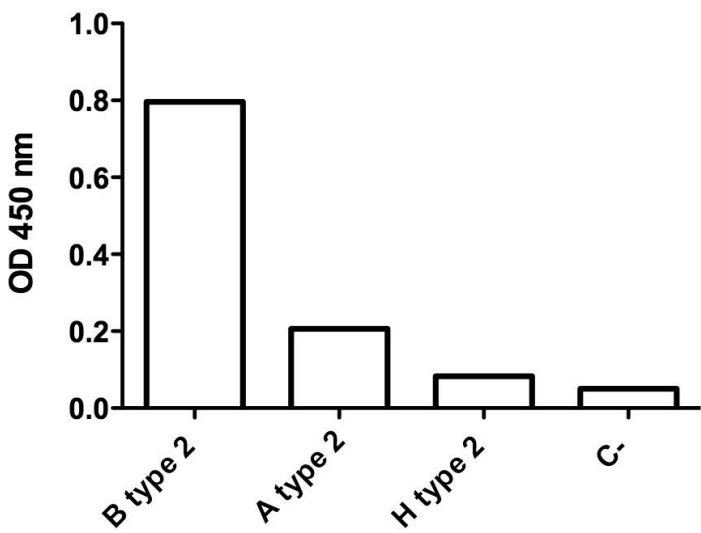
GI.2 (RHDV2)

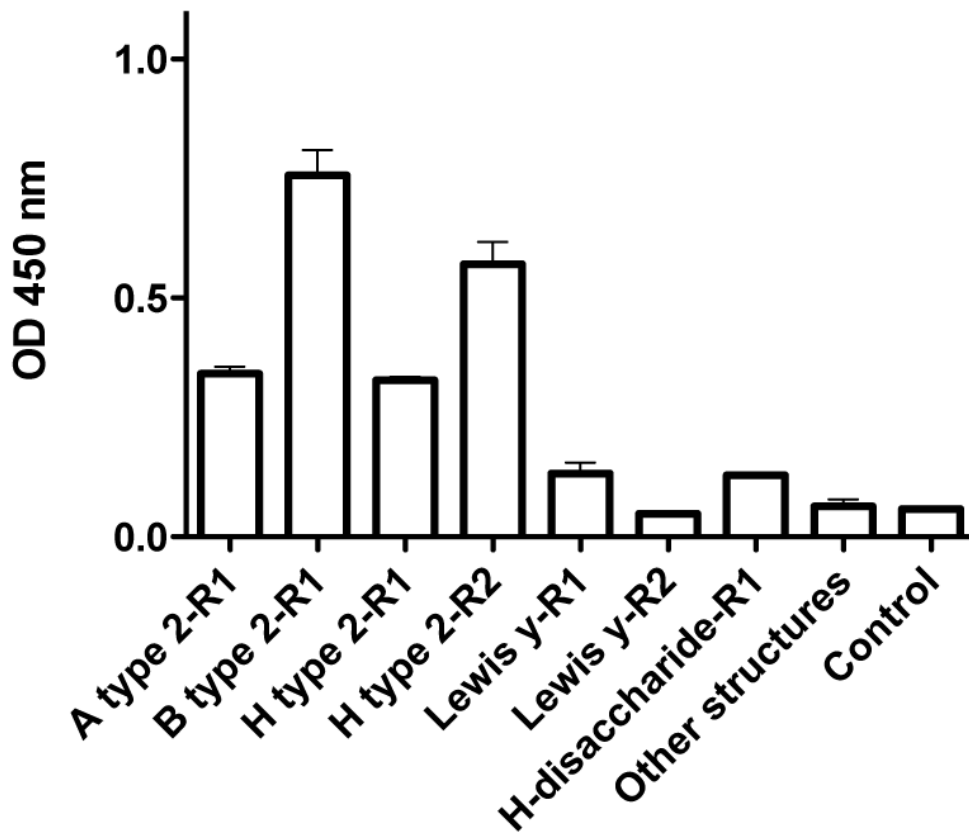


GI.3 (RCV-E1)

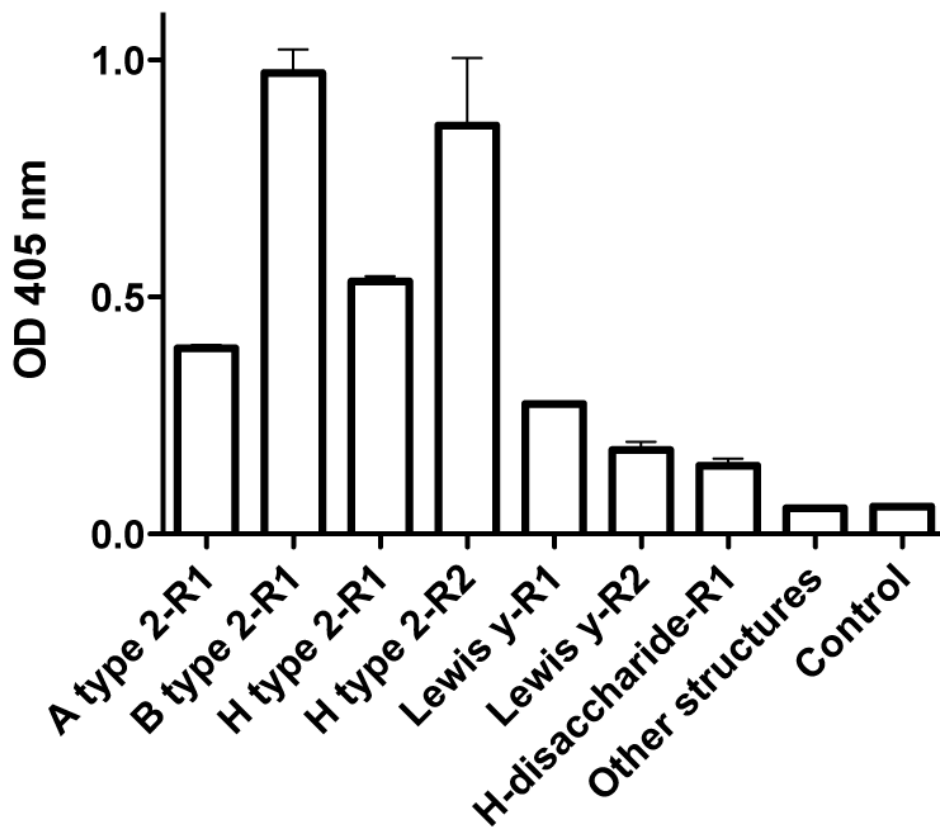


GI.4d (RCV-E2)

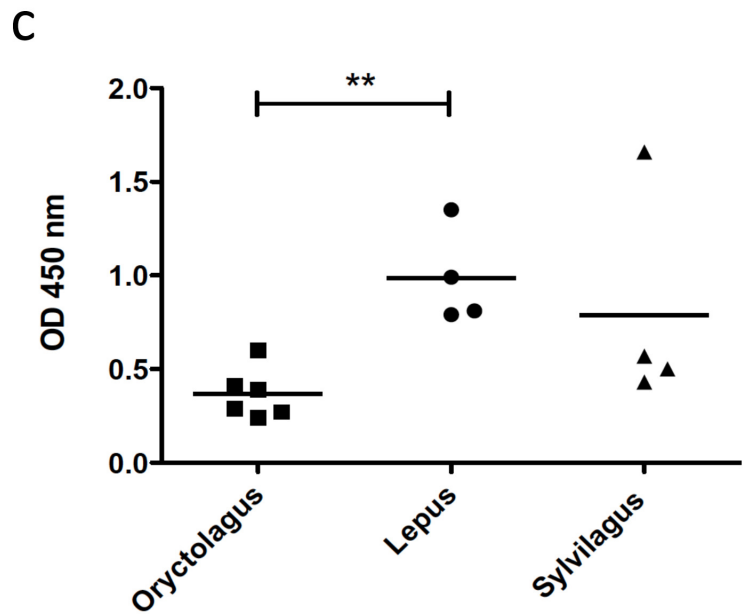
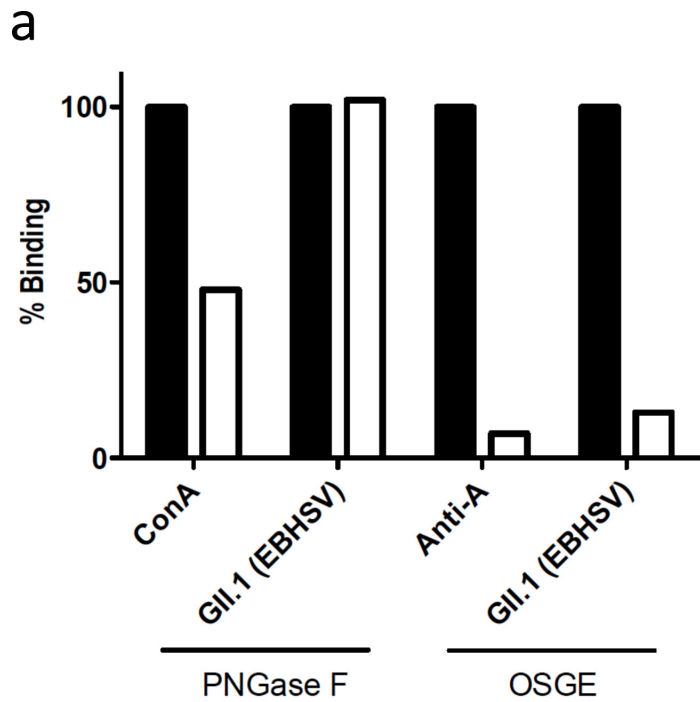




Strain 10.28

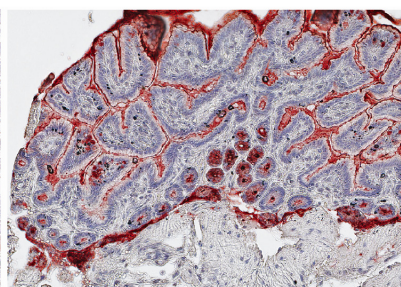
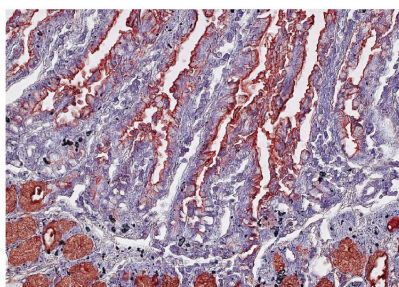
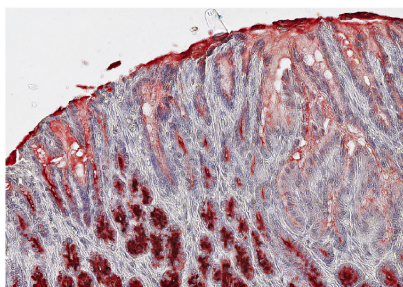


Strain 10.32

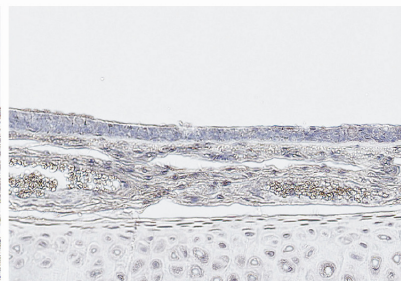
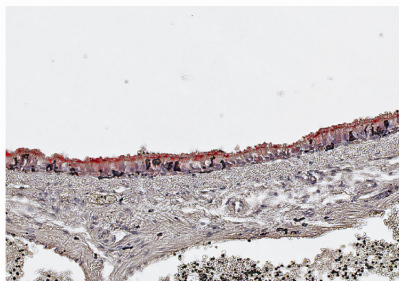
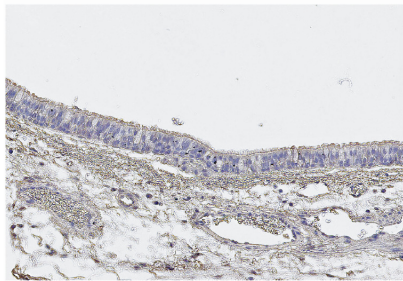


b *Oryctolagus* *Lepus* *Sylvilagus*

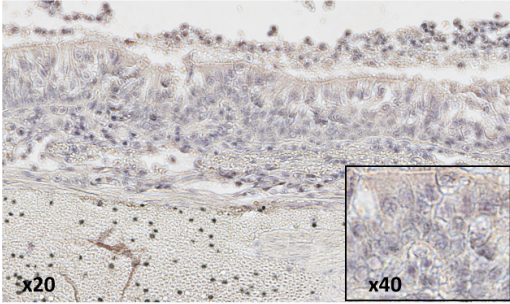
Duodenum



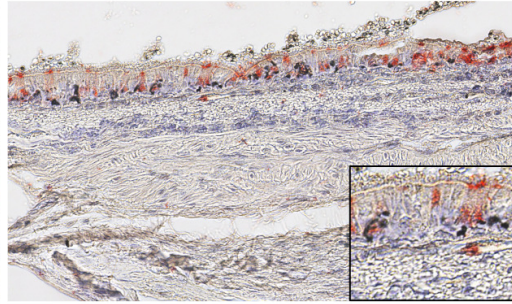
Trachea



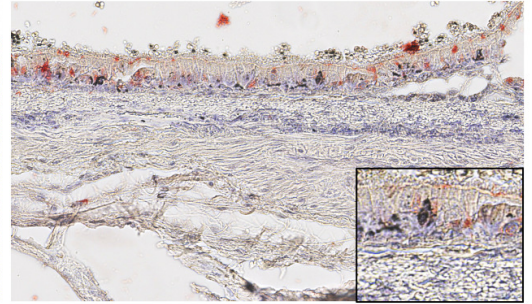
a



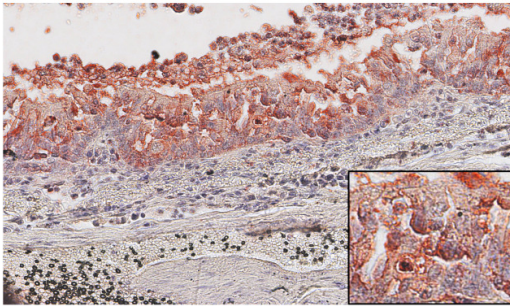
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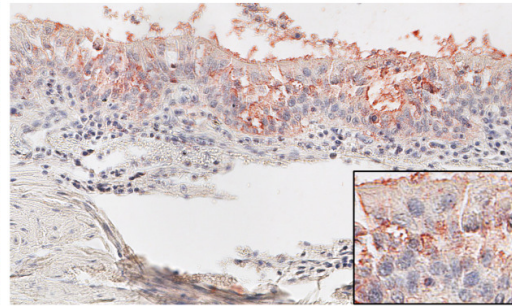
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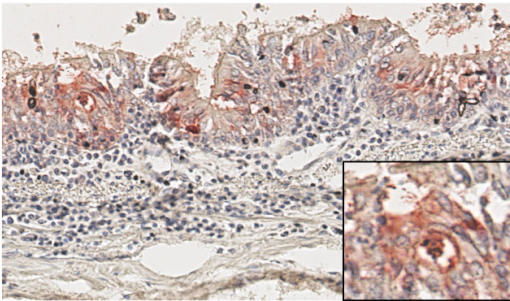
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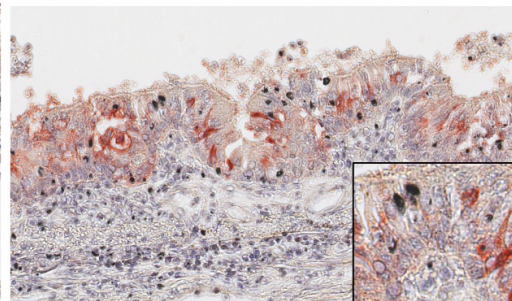
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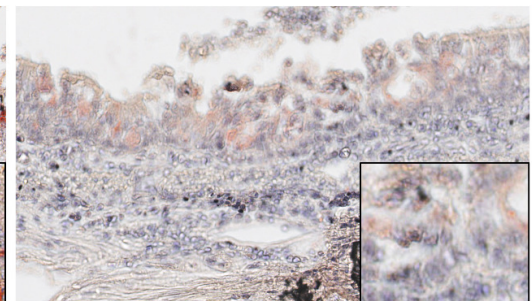
f



g



h

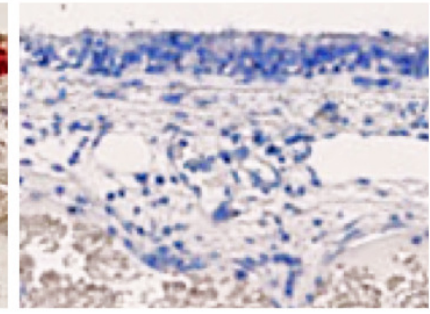
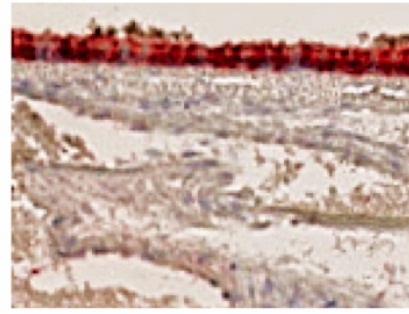
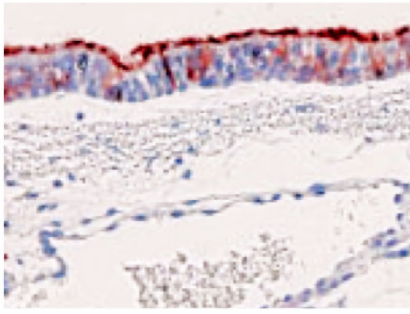


Oryctolagus

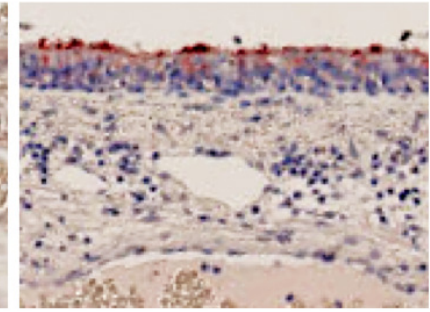
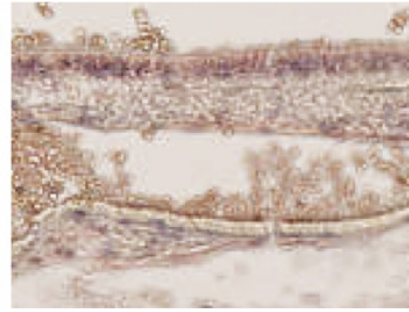
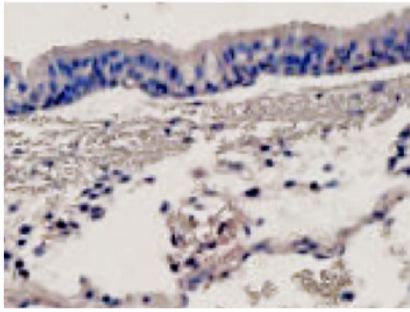
Lepus

Sylvilagus

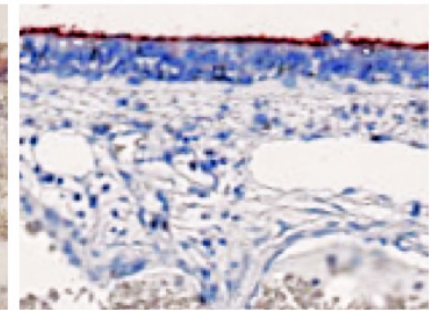
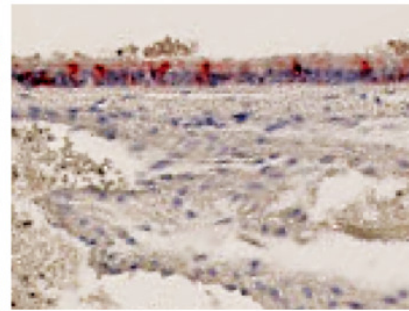
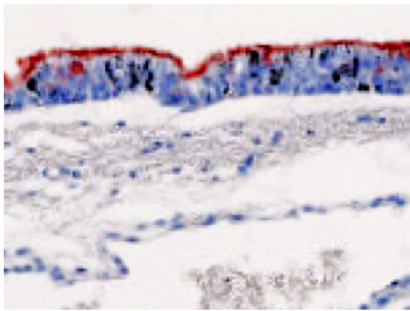
Anti-A



Anti-B



Ulex

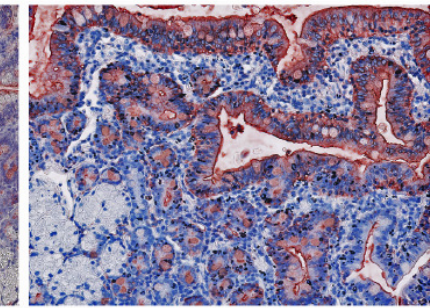
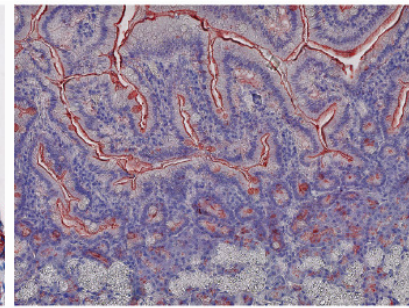
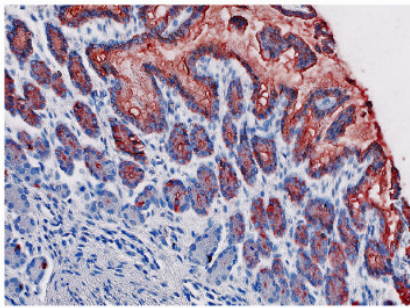


Oryctolagus

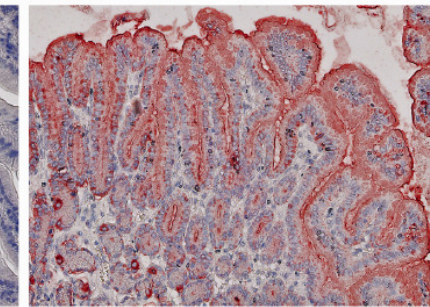
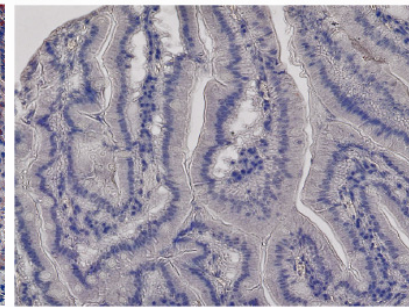
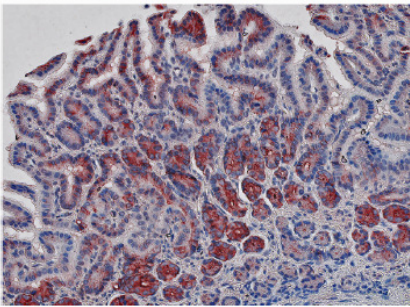
Lepus

Sylvilagus

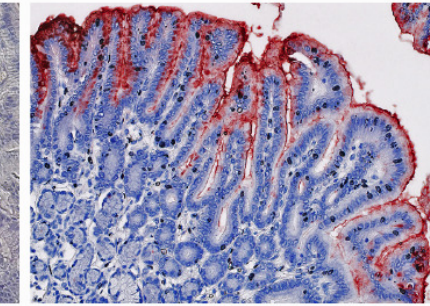
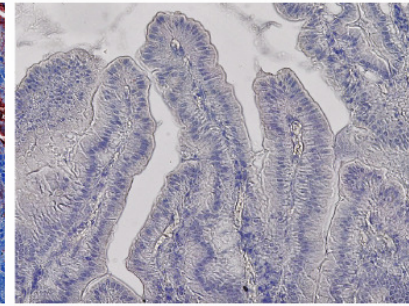
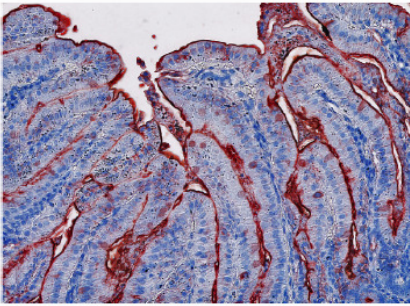
Anti-A



Anti-B



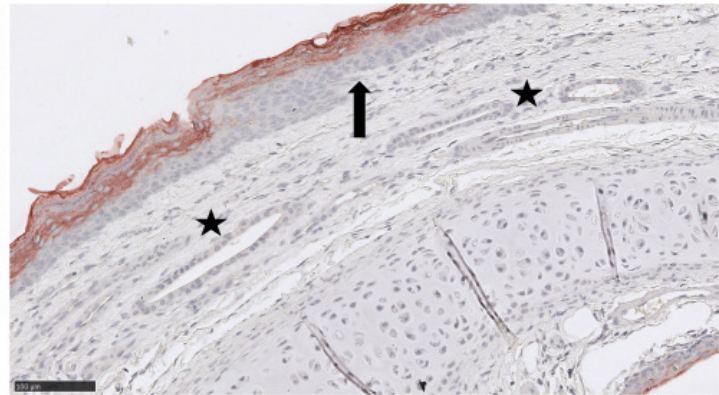
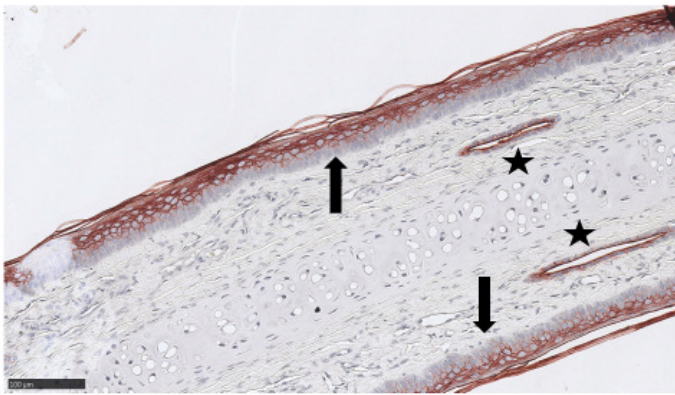
Ulex



Oryctolagus

Lepus

UEA-I



sWGA

