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

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Abstract

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

Importance

Lagoviruses constitute a genus of the *Caliciviridae* family, comprising highly 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.

Introduction

High mutation rates, vast population sizes and short generation times make RNA viruses capable of rapidly adapting to a large number of hosts and thus prone to cross species boundaries (1, 2). Viruses are most likely to jump between closely related species (3) and this may result in unique spill-over infections or severe epidemics, depending on how successfully the virus adapts to the new host population. Host switching constitutes an important mechanism of virus evolution (4) and is important in several families of viruses, including *Caliciviridae*. These non-enveloped viruses have a single-stranded, positive-sense RNA genome. Caliciviruses comprise five recognized genera: *Norovirus*, *Sapovirus*, *Vesivirus*, *Nebovirus* and *Lagovirus* (5). The genus *Lagovirus* encompasses two presently recognized species: rabbit hemorrhagic disease virus (RHDV), highly fatal to the European rabbit (*Oryctolagus cuniculus*), and European brown hare syndrome virus (EBHSV), that affects European brown and mountain hares (*Lepus europaeus* and *L. timidus*, respectively) (6). Both RHDV and EBHSV emerged in the 1980s (7, 8) and are similar in terms of morphology, genome organization and epidemiological course of the respective diseases, rabbit hemorrhagic disease (RHD) and European brown hare syndrome (EBHS) (9, 10). For EBHSV, only a single serotype is known (11) and cases of non-pathogenic forms of the virus circulating in hare populations have just been described (12). In contrast, several serological subgroups are recognized for RHDV (13) including the antigenic variant RHDVa (14), and several non-pathogenic and moderately pathogenic strains also circulating in European rabbit populations from different parts of the world (15-19). In 2010, new pathogenic RHDV strains emerged and rapidly spread throughout Europe and Australia (20-28). Due to the large number of new lagovirus strains described, confusion in the extant nomenclature and close relationship between RHDV and EBHSV, a new classification of lagoviruses has recently been proposed distinguishing a single species with two genogroups and several genotypes

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

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

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

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

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

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

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

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

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

Results

Lagovirus GI strains attach to HBGA-type glycans

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

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

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

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

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

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

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

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

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

Variations in histo-blood group antigens expression across lagomorph species

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Virus-like particles (VLPs) and virus preparations

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

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

Antibodies to VLPs

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

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

Tissue sampling

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

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

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

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

Phenotyping ELISA

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

VLPs and virus binding assays

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

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

Printed glycan microarray assay

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

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

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

Immunohistochemistry

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

575 at 2 µg/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 µg/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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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

Figure legends

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

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

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

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

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

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

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

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

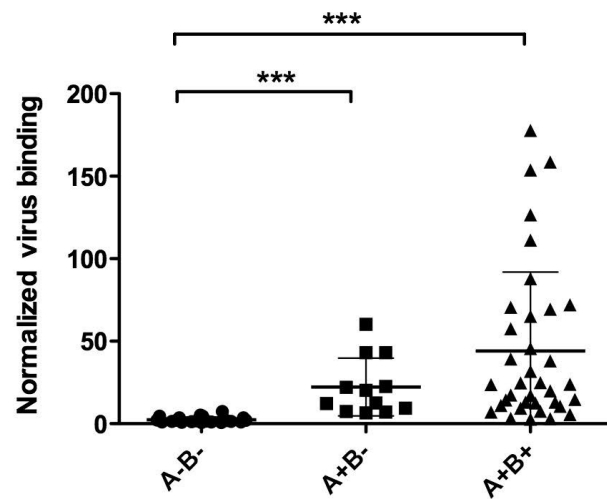
Figure 7: Blocking terminal GlcNAc residues decreases EBHSV binding. Upper panel: *Lepus europaeus* trachea tissue sections were either left untreated **(b)** or treated with β-hexosaminidase **(c)** prior to incubation with sWGA, showing a decrease of accessible terminal N-acetylglucosamine residues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

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

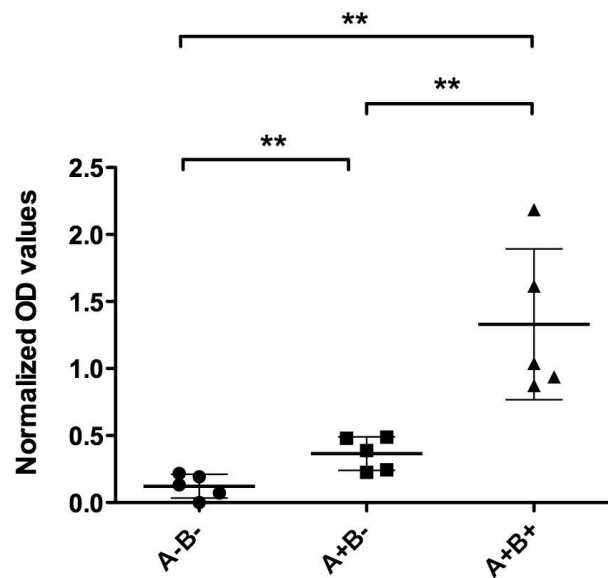
lectin I from *Ulex europaeus* (Ulex) for H antigen. Tissues from 21 *Oryctolagus cuniculus* individuals, 6 *Lepus europaeus* and 6 *Sylvilagus floridanus* were analyzed. Representative images corresponding to a x400 magnification (upper panel) or a x200 magnification (lower panel) from each species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

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

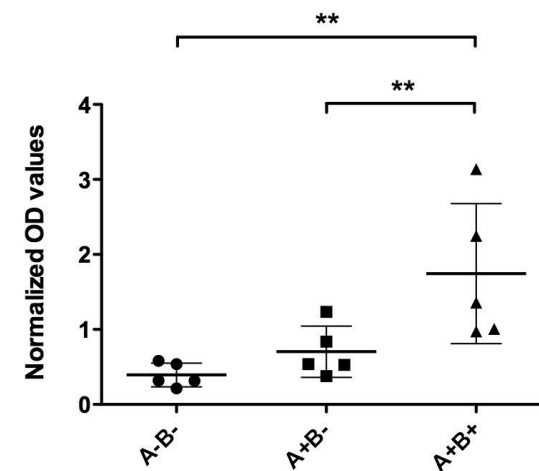




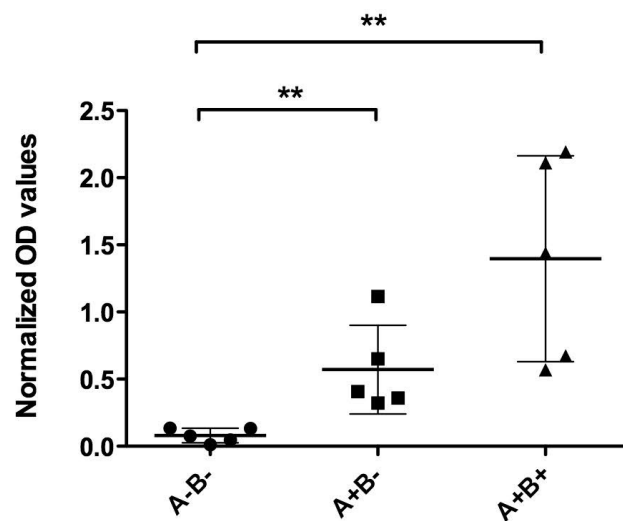
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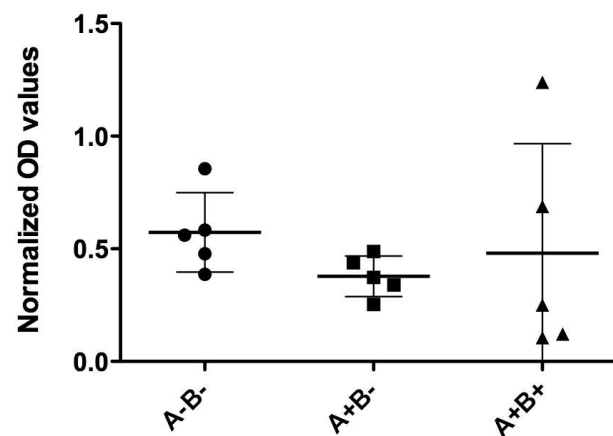
GI.4a (RCV-A1)



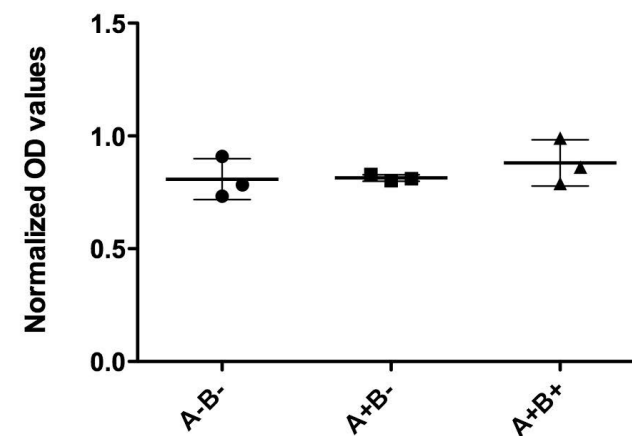
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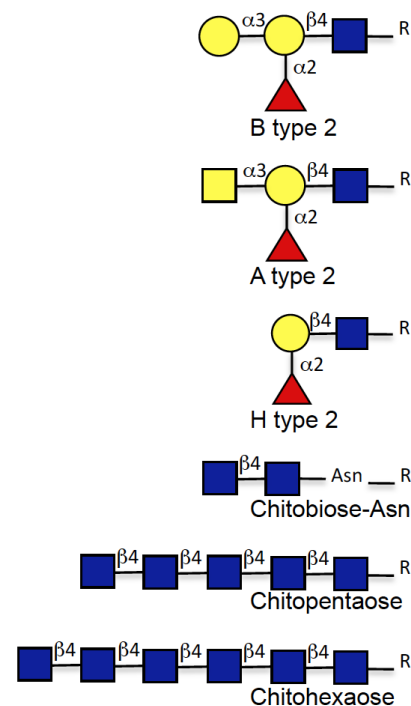
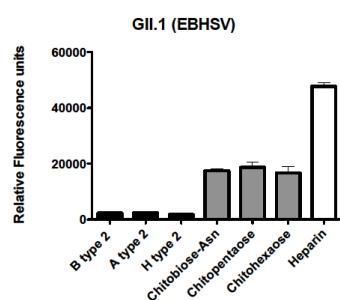
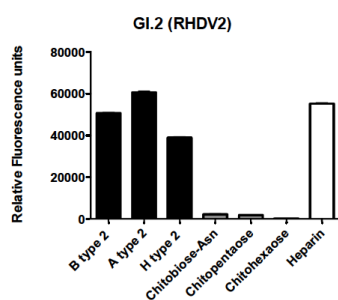
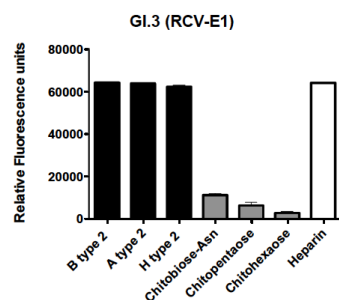
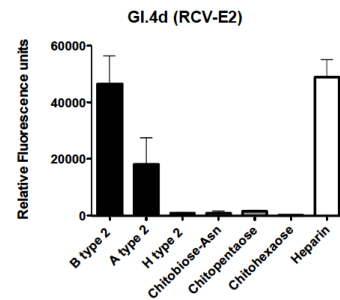
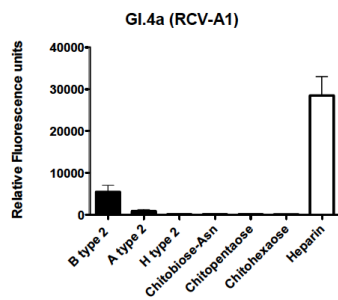
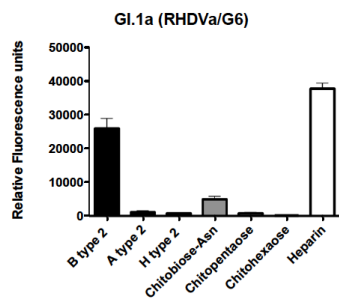
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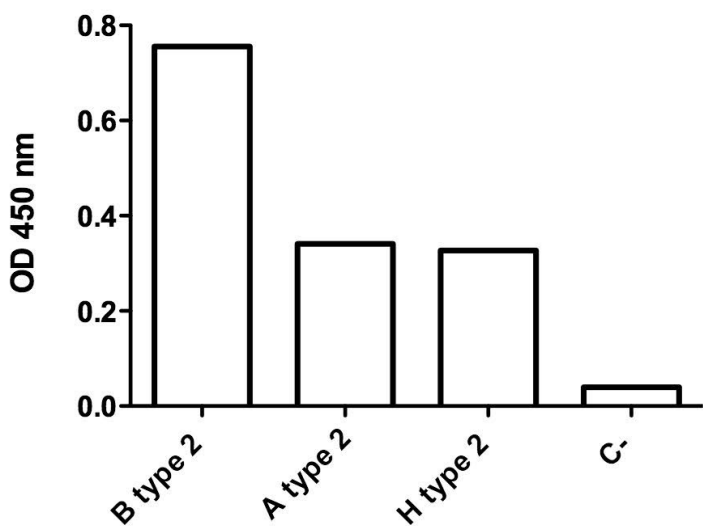
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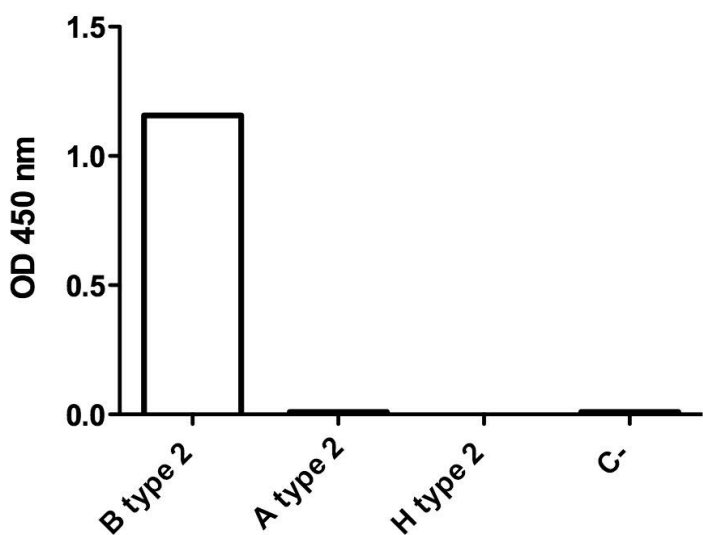
GII.1 (EBHSV)



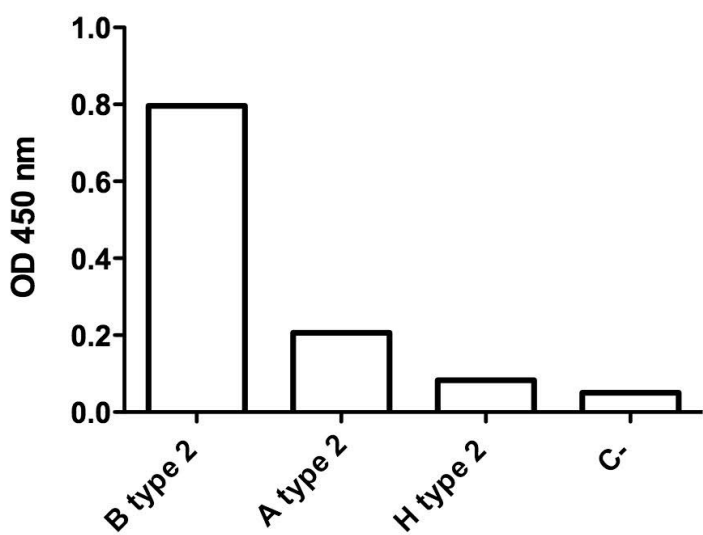
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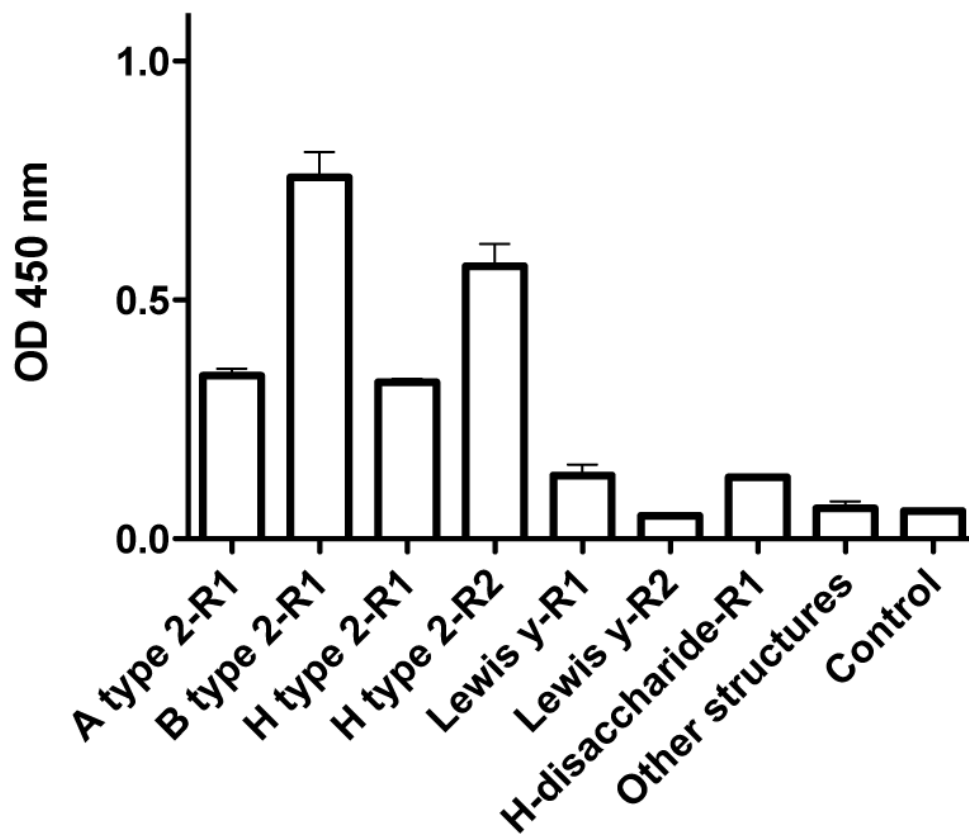


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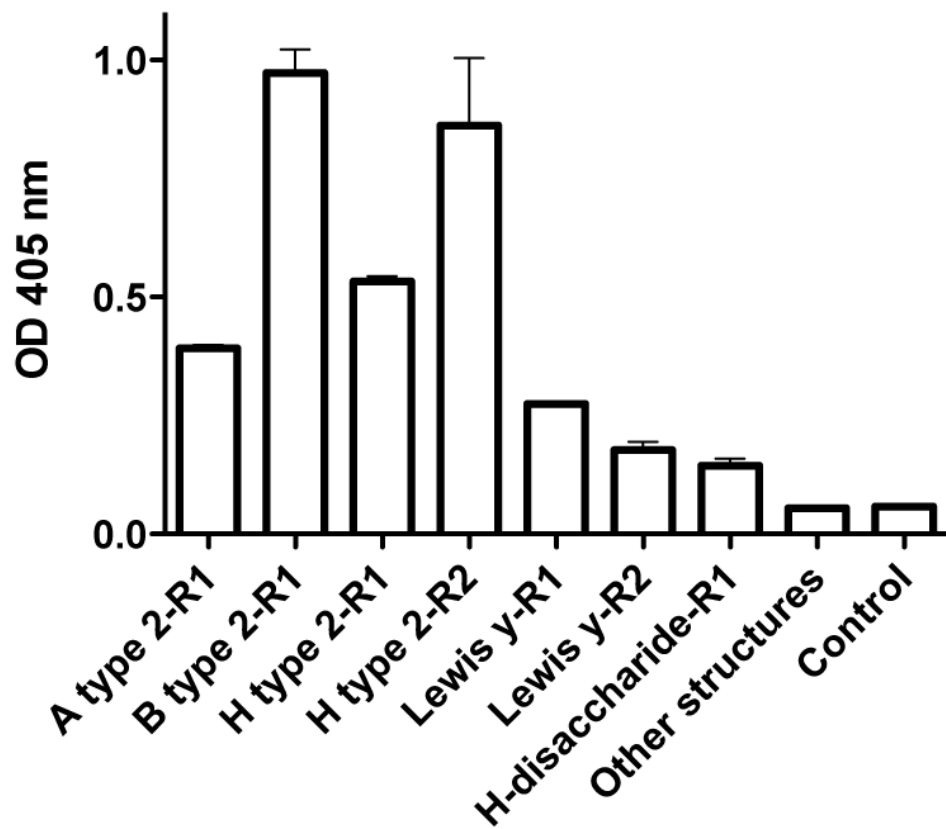


GI.4d (RCV-E2)

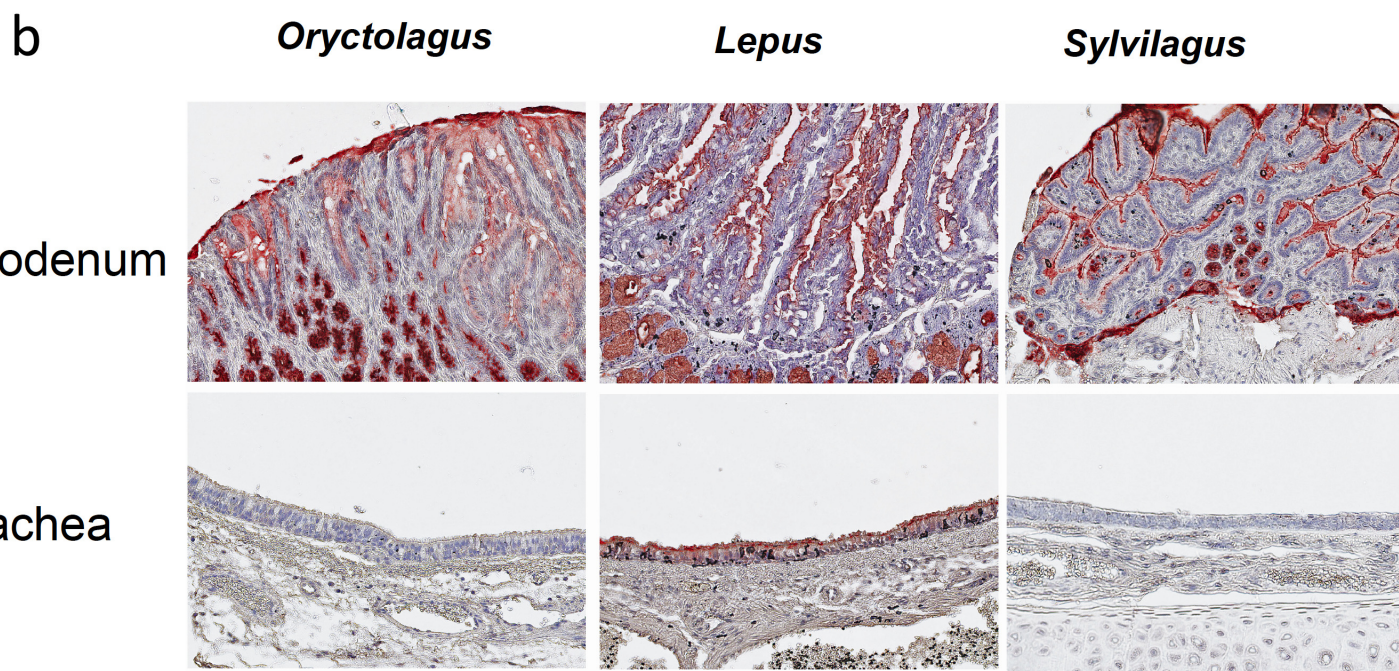
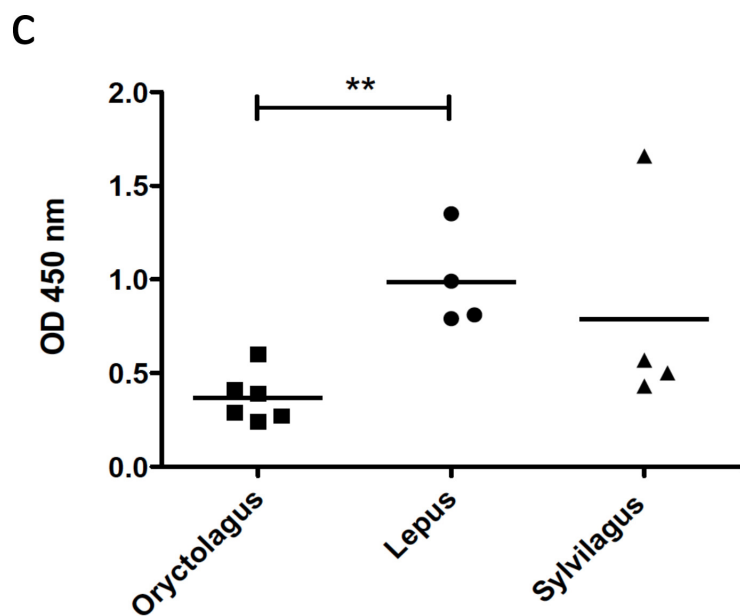
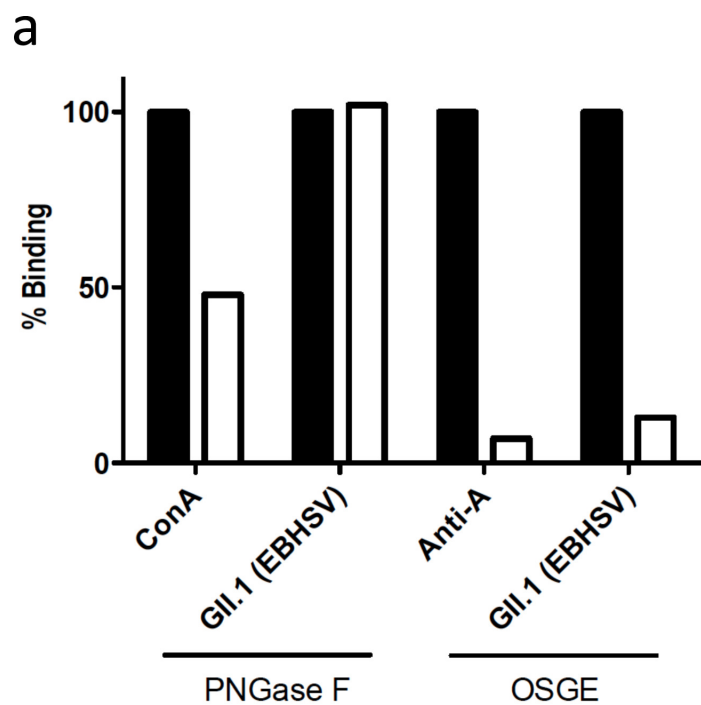




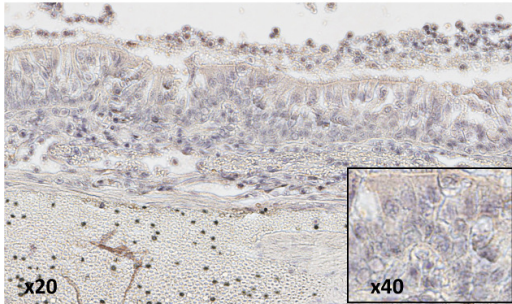
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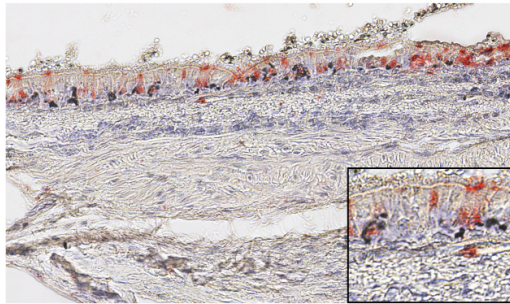
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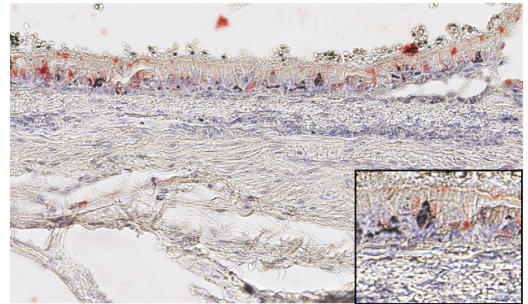
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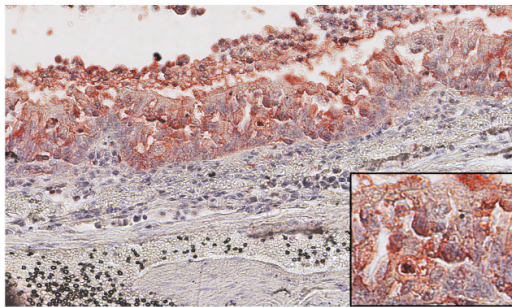
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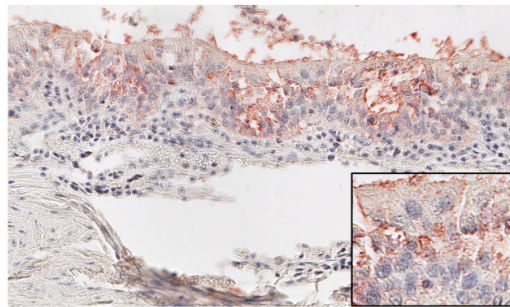
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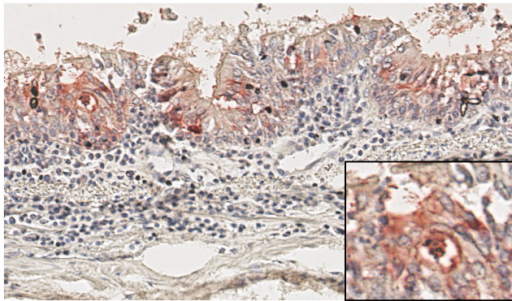
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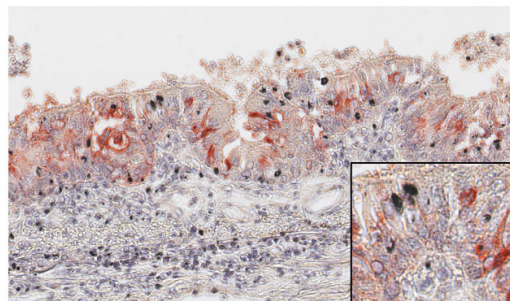
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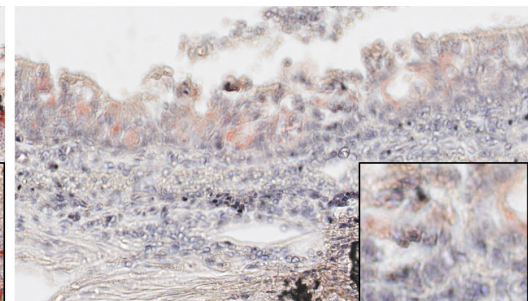
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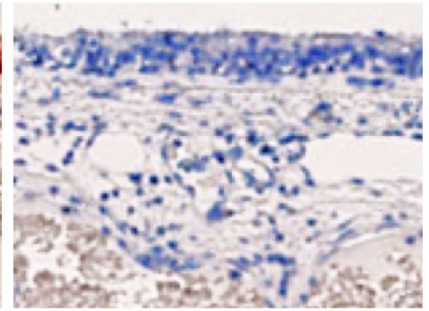
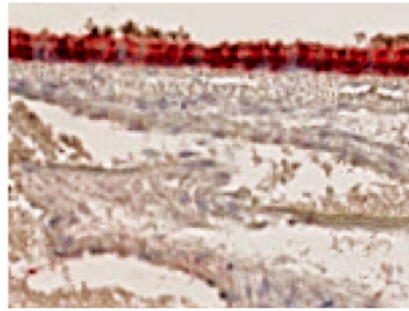
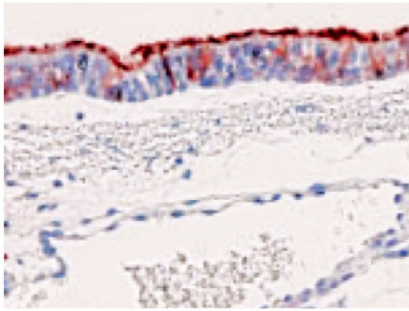


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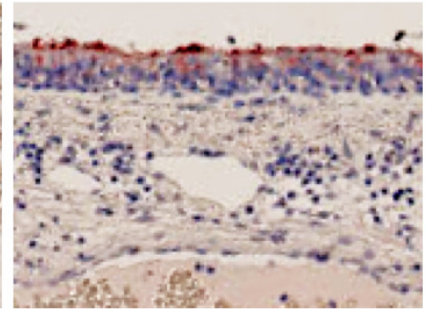
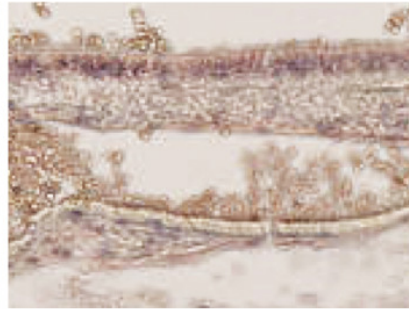
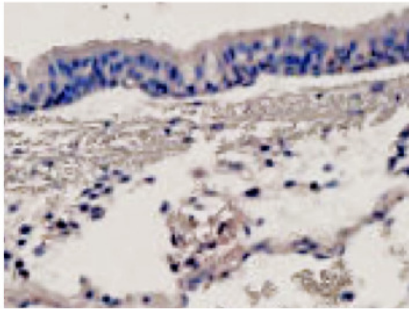
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Sylvilagus

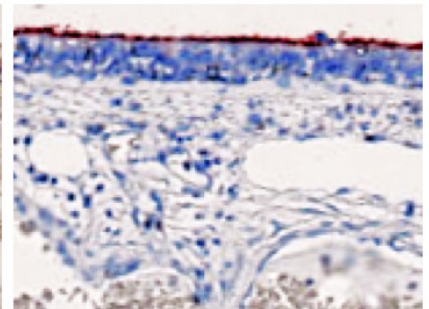
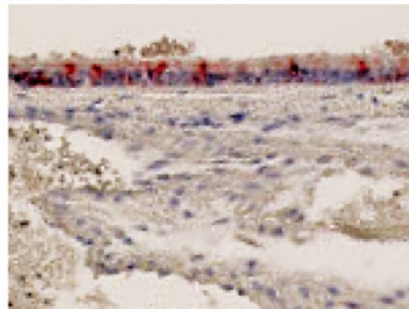
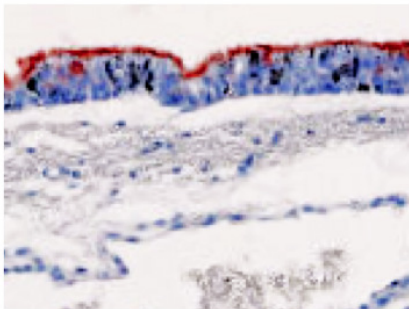
Anti-A



Anti-B



Ulex

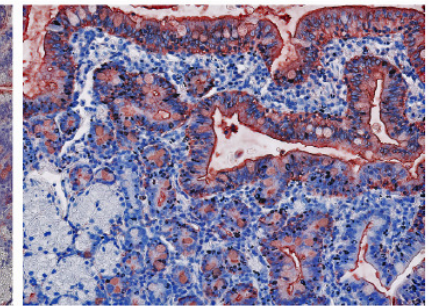
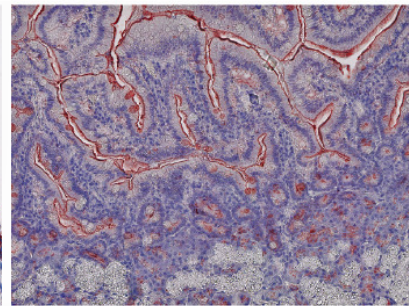
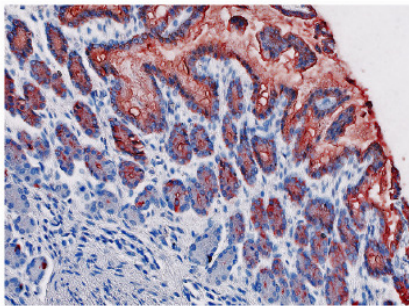


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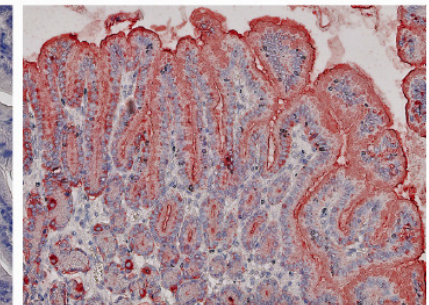
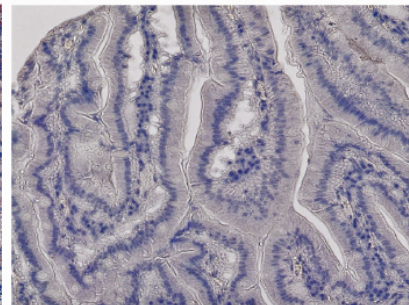
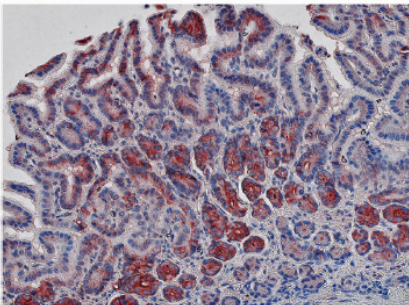
Lepus

Sylvilagus

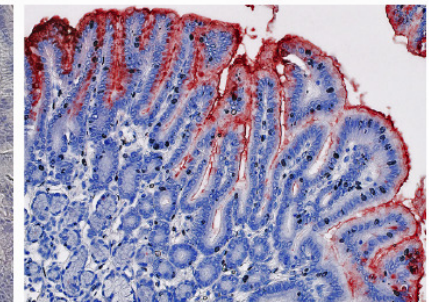
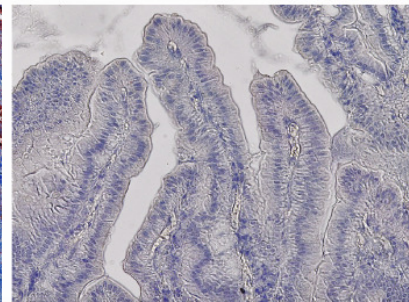
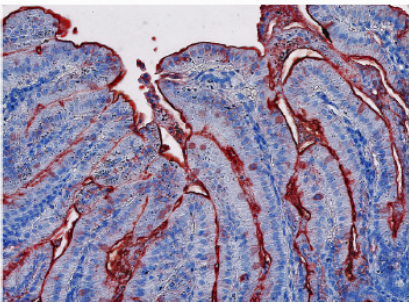
Anti-A



Anti-B



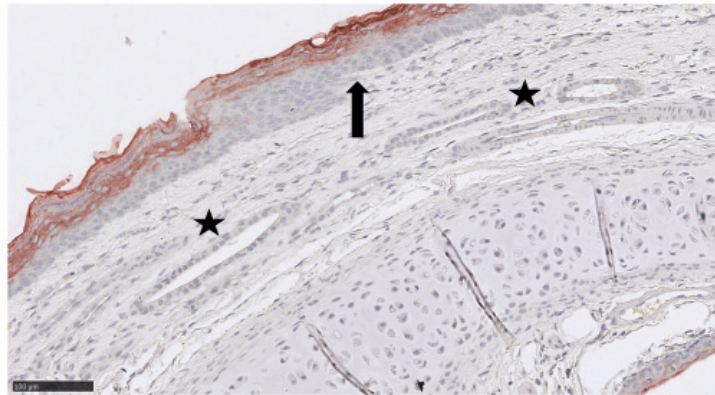
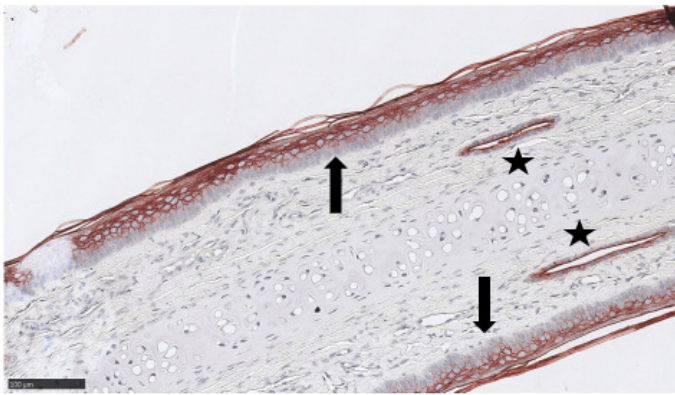
Ulex



Oryctolagus

Lepus

UEA-I



sWGA

