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Genome-Wide Association Study among Four Horse Breeds Identifies a Common Haplotype Associated with *In Vitro* CD3$^+$ T Cell Susceptibility/Resistance to Equine Arteritis Virus Infection$^\nabla$

Yun Young Go,[1] Ernest Bailey,[1]* Deborah G. Cook,[1] Stephen J. Coleman,[1] James N. MacLeod,[1] Kuey-Chu Chen,[2] Peter J. Timoney,[1] and Udeni B. R. Balasuriya[1]*

Maxwell H. Gluck Equine Research Center, Department of Veterinary Science,[1] and Department of Molecular and Biomedical Pharmacology,[2] University of Kentucky, Lexington, Kentucky 40546-0099

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Previously, we have shown that horses could be divided into susceptible and resistant groups based on an *in vitro* assay using dual-color flow cytometric analysis of CD3$^+$ T cells infected with equine arteritis virus (EAV). Here, we demonstrate that the differences in *in vitro* susceptibility of equine CD3$^+$ T lymphocytes to EAV infection have a genetic basis. To investigate the possible hereditary basis for this trait, we conducted a genome-wide association study (GWAS) to compare susceptible and resistant phenotypes. Testing of 267 DNA samples from four horse breeds that had a susceptible or a resistant CD3$^+$ T lymphocyte phenotype using both Illumina Equine SNP50 BeadChip and Sequenom's MassARRAY system identified a common, genetically dominant haplotype associated with the susceptible phenotype in a region of equine chromosome 11 (ECA11), positions 49572804 to 49643932. The presence of a common haplotype indicates that the trait occurred in a common ancestor of all four breeds, suggesting that it may be segregated among other modern horse breeds. Biological pathway analysis revealed several cellular genes within this region of ECA11 encoding proteins associated with virus attachment and entry, cytoskeletal organization, and NF-κB pathways that may be associated with the trait responsible for the *in vitro* susceptibility/resistance of CD3$^+$ T lymphocytes to EAV infection. The data presented in this study demonstrated a strong association of genetic markers with the trait, representing *de facto* proof that the trait is under genetic control. To our knowledge, this is the first GWAS of an equine infectious disease and the first GWAS of equine viral arteritis.

Equine arteritis virus (EAV) is a small enveloped virus with a positive-sense, single-stranded RNA genome of 12.7 kb and belongs to the family *Arteriviridae* (genus *Arterivirus*, order *Nidovirales*) (16, 64). EAV is the causal agent of equine viral arteritis (EVA), a disease of equids (12, 13). The vast majority of EAV infections are inapparent or subclinical (5, 6, 68). However, some acutely infected horses may develop any combination of the following clinical signs: pyrexia, depression, anorexia, dependent edema (scrotum, ventral trunk, and limbs), conjunctivitis, lacrimation and swelling around the eyes (periorbital or supraorbital edema), respiratory distress, urticaria, and leukopenia (5, 6). During natural outbreaks of the disease, the virus can cause abortion in pregnant mares, with abortion rates varying from 10 to 71%, depending on the virus strain (5, 6). Following EAV infection, a variable proportion of stallions (30 to 70%) can become persistently infected and continuously shed the virus in their semen (55, 68). The mechanism of persistence of EAV in the male reproductive tract is not clear. However, studies have established that persistence of EAV in stallions is testosterone dependent (42, 52). Moreover, the prevalences of EAV infection differ markedly among different breeds of horses (68), strengthening the assumption of genetic influence on susceptibility to infection. The seroprevalences of EAV infection of horses vary between countries and among horses of different breeds and ages, with marked disparity between the prevalence of infection among Standardbred (STB) horses and that among Thoroughbred (TB) horses (49). EAV infection is considered endemic in STB horses but not in TB horses in the United States, with 77.5% to 84.3% of all STB horses but only up to 5.4% of TB horses being seropositive for the virus (5, 32, 49). Furthermore, the seroprevalence of EAV infection among Warmblood stallions is also very high in a number of European countries, as about 55 to 93% of Austrian Warmblood stallions are positive for antibodies to EAV (56). Although many of these differences are likely attributable to circulating virus strains and environmental or management conditions, inherent genetic differences may also play a significant role in determining clinical outcome and influence severity of disease following exposure to EAV.

In a recent study using dual-color flow cytometry, we demonstrated that the virulent Bucyrus strain of EAV (EAV VBS) can infect a small population of CD3$^+$ T lymphocytes *in vitro* from some but not all horses (28). The data suggested that the CD3$^+$ T lymphocyte subpopulation of individual horses varied in their susceptibilities to *in vitro* EAV VBS infection and they could be divided into susceptible and resistant groups (28). The susceptible/resistant CD3$^+$ T lymphocyte phenotypes

* Corresponding author. Mailing address: 108 Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099. Phone for U. B. R. Balasuriya: (859) 218-1124. Fax: (859) 257-8542. E-mail: ubalasuriya@uky.edu. Phone for E. Bailey: (859) 218-1105. Fax: (859) 257-8542. E-mail: ebailey@uky.edu.

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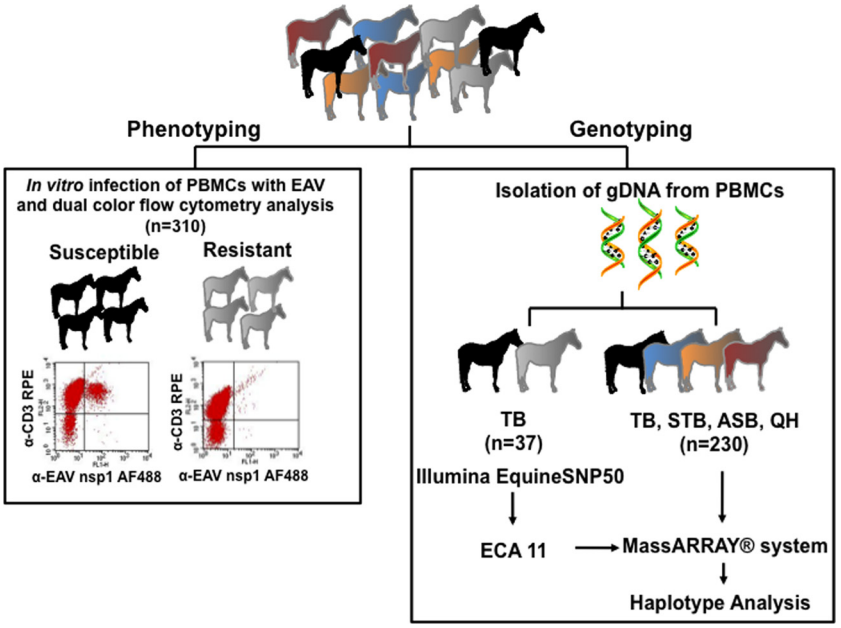

FIG. 1. Schematic representation of the study design. A total of 310 horses of different breeds, American Saddlebred (ASB; $n = 60$), Standardbred (STB; $n = 60$), Thoroughbred (TB; $n = 137$), and Quarter Horse (QH $n = 53$), were used in the study. Horses were phenotyped first and grouped into the susceptible or resistant phenotype group based on their $CD3^+$ T cell infectivity to EAV. To identify the region associated with EAV-susceptible/resistant phenotype, gDNA from 37 TB horses was isolated and analyzed using Illumina Equine SNP50. Results from the initial study were confirmed by genotyping 267 additional horses, including TB horses tested with Illumina Equine SNP50, with MassARRAY system technologies.

were not associated with age, prior exposure to EAV, or presence of antibodies to EAV but appeared to show an association with breed in preliminary studies. Therefore, we hypothesized that susceptibility and resistance of equine $CD3^+$ T lymphocytes to EAV reflect genetic differences between horses in their response to infection with the virus. The primary objective of this study was to identify chromosomal regions and candidate genes associated with susceptibility/resistance of $CD3^+$ T lymphocytes to EAV infection in horses by using a genome-wide association study (GWAS). Genetic factors contribute to host susceptibility and progression of disease, but the genes responsible for disease development are largely unknown. Until now, there was no evidence to indicate what role genetic factors play in determining the susceptibility to and outcome of EAV infection in horses. Availability of the equine genome sequence and development of genome-wide screening technologies offer unparalleled opportunities to identify variants associated with increased susceptibility/resistance to infectious disease agents such as EAV (20, 24, 31, 67, 70). In this study, we describe the identification of a distinct phenotypic trait that can be used as a marker to divide equine populations, regardless of breed, into susceptible and resistant groups based on an *in vitro* assay system. Using GWAS in combination with biological pathway analysis, we have identified for the first time several cellular genes that may be associated with the trait responsible for *in vitro* $CD3^+$ T lymphocyte susceptibility/resistance to EAV infection.

MATERIALS AND METHODS

Horses. Horses from four different breeds, Thoroughbred (TB), American Saddlebred (ASB), Standardbred (STB), and Quarter Horse (QH), a total of 310 horses, were randomly selected from farms in central Kentucky for this study. Blood samples were collected by jugular venipuncture into 10-ml Vacutainer tubes containing 15% EDTA (Monoject; Tyco Healthcare Group LP, Mansfield, MA) with short-term (<5 min) physical restraint of horses.

Virus and antibodies. The virulent Bucyrus strain of EAV (EAV VBS; ATCC VR-796, American Type Culture Collection, Manassas, VA) was used for *in vitro* infection of equine peripheral blood mononuclear cells (PBMCs) as previously described (28). The monoclonal antibody (MAb) to the equine CD3 surface molecule, UC F6G, was kindly provided by Jeff Stott, University of California, Davis. The R-phycoerythrin (R-PE)-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) was used as the secondary antibody. To detect EAV antigen in infected cells, Alexa Fluor 488-labeled MAb against nonstructural protein 1 (NSP1; MAb 12A4) was used (28, 71).

Phenotypic trait. The susceptible or resistant phenotype of each animal was defined by dual-color flow cytometric analysis of *in vitro* EAV-infected $CD3^+$ T lymphocytes as described previously (28). The horses were classified as susceptible or resistant to *in vitro* EAV infection based on their susceptible/resistant $CD3^+$ T lymphocyte phenotype.

DNA extraction. Genomic DNA (gDNA) was obtained from PBMCs of each animal by using the Puregene whole-blood extraction kit (Qiagen, Valencia, CA) by following the manufacturer's instructions. DNA quality and concentration were assessed using Nanodrop (Thermo Scientific, Wilmington, DE) at an absorbance ratio of optical density at 260 nm/280 nm ($OD_{260/280}$).

Genotyping and quality control. Samples were genotyped using Equine SNP50 BeadChip (Illumina, San Diego, CA) at the core facility for the Mayo Clinic in Rochester, MN. This array contains 59,355 single nucleotide polymorphisms (SNPs) derived from the EquCab2.0 SNP database of the horse genome (http://www.broadinstitute.org/mammals/horse), with an average probe spacing of 43.2 kb between adjacent variants. For the initial GWAS analysis ($n = 37$ horses), DNA samples from 16 TB horses known to be susceptible and 21 TB horses known to be resistant for the *in vitro* EAV infectivity trait were used for the Equine SNP50 BeadChip genotyping analysis (Illumina, San Diego, CA) (Fig. 1). Subsequently, additional SNPs from the region (Table 1) were selected using the Broad Institute Equine SNP database (http://www.broadinstitute.org/ftp/distribution/horse_snp_release/v2/) for further testing along with some of the original SNPs. The second SNP assay was conducted at Geneseek, Inc. (Lincoln, NE) using the Sequenom MassARRAY system (Sequenom Inc., San Diego,

TABLE 1. ECA11 SNPs selected for the MassARRAY system

SNP[c]	Position on ECA11 (bp)
BIEC2-157847	49389493
BIEC2-157867[a]	**49424839**
BIEC2-157892	49446386
BIEC2-157899[b]	49459247
BIEC2-165951[b]	49473877
BIEC2-165967[b]	49475165
BIEC2-157944	49509504
BIEC2-157945	49514091
BIEC2-165988	49529433
BIEC2-157947	**49529680**
BIEC2-165991	49533085
BIEC2-165992	49537432
BIEC2-157953[b]	49541824
BIEC2-165997	49545584
BIEC2-165998	49549767
BIEC2-165999	49558533
BIEC2-166001	49564266
BIEC2-166003	49569444
BIEC2-166005	49572804
BIEC2-166006	49580531
BIEC2-166009	49584513
BIEC2-166011	49589952
BIEC2-166013[b]	49594275
BIEC2-166014[b]	49604594
BIEC2-166015	49613954
BIEC2-166016	49621050
BIEC2-166018	49627471
BIEC2-166020	49634340
BIEC2-166021	49637772
BIEC2-166027	49643932
BIEC2-166028[b]	49649380
BIEC2-166029	49660962
BIEC2-157989	**49663560**
BIEC2-166033	49665617
BIEC2-166034	49676359
BIEC2-166036	49682023
BIEC2-166040	49684089
BIEC2-157998	**49684299**
BIEC2-166042[b]	49688617
BIEC2-158000	**49691181**
BIEC2-166044	49693957
BIEC2-166047	49696679
BIEC2-166049	49700501
BIEC2-166050	49707607
BIEC2-166052[b]	49711761
BIEC2-166056	49716340
BIEC2-166060	49724419
BIEC2-166065	49729958
BIEC2-166066	49747467
BIEC2-158024[b]	49757978
BIEC2-166449	50863263
BIEC2-158587	**51188148**
BIEC2-159231	**52379156**

[a] Most highly associated SNP in the initial assay.
[b] SNPs included on the assay but excluded due to failure to type or to low minor allele frequency.
[c] The SNPs included on the Illumina Equine SNP50 chip are shown in bold font.

CA). Horses tested in this second assay included a second population of TB horses ($n = 57$) as well as horses of several different breeds (60 ASB, 60 STB, and 53 QH horses) plus the 37 TB horses tested in the original Illumina assay.

Data analyses. Data from both assays were analyzed using the Golden Helix SNP & Variation Suite 7 (Bozeman, MT). Of the 59,355 SNPs, 53,747 had call rates above 95%. The rest were excluded from the analyses. Filtering for minor allele frequencies (removing SNPs with minor allele frequencies below 55%) and genotyping (excluding SNPs with genotypes involving less than 90% of the horses), 42,506 SNPs were evaluated. Data from both studies were evaluated using the association (chi-square) in the additive model and linkage disequilibrium (LD).

Gene ontology clustering and pathway analysis. Unsupervised analysis was performed using the Ingenuity Pathways Analysis (IPA) tool (Ingenuity Systems Inc., Redwood City, CA) to investigate biological and molecular networks associated with genes identified within the selected region (3, 14). The IPA builds networks relevant to queried genes based on their functional annotation and their known molecular interactions supported by previously published peer-reviewed scientific articles which are then stored in Ingenuity Pathways Knowledge Base (IPKB). A list of interactions between genes identified in the GWAS (focus genes) and all other genes stored in the IPKB was obtained, with a maximum network size of 35 genes. Networks of direct or indirect interactions among genes are displayed graphically. In addition, IPA computes a score for each network, the $-\log$ (P value), which indicates the likelihood of the focus genes in a network from IPKB being found together due to random chance. To investigate the molecular and biological functions, genes identified within the region were categorized using the PANTHER classification system (www.pantherdb.org). In addition to the above-mentioned database analyses, functional information was also obtained from a literature search.

RESULTS

Phenotype analysis based on *in vitro* susceptibility of CD3$^+$ T lymphocytes to EAV infection. In an attempt to define the susceptible or resistant CD3$^+$ T lymphocyte phenotype, blood was collected from each horse ($n = 310$) and PBMCs were isolated. PBMCs were infected with EAV VBS and subsequently subjected to dual-color flow cytometric analysis to identify horses with the susceptible or resistant CD3$^+$ T lymphocyte phenotype to EAV infection (Fig. 1, left). Cells from horses with a susceptible CD3$^+$ T lymphocyte phenotype were stained with antibodies to both the CD3$^+$ T lymphocyte surface molecule and intracellular EAV antigen (NSP1). In contrast, cells from horses with a resistant CD3$^+$ T lymphocyte phenotype were stained with antibody to the CD3$^+$ T lymphocyte surface molecule but not to intracellular EAV antigen. Consistent with previously reported results, horses were divided into two distinct groups, those with CD3$^+$ T cells susceptible and resistant to EAV infection. Of the 310 horses, 167 horses had the susceptible CD3$^+$ T lymphocyte phenotype, and 143 horses had the resistant CD3$^+$ T lymphocyte phenotype. Subsequently, data were analyzed by individual horse breed. In the susceptible phenotype group, there was no significant difference in the percentage of the EAV-infected CD3$^+$ T lymphocyte subpopulation, regardless of breed. The percentages of the CD3$^+$ T cell subpopulation susceptible to EAV were 4.0% ± 0.95% in TB horses, 5.4% ± 0.4% in STB horses, 7.2% ± 0.6% in ASB horses, and 3.9% ± 0.4% in QH horses (Fig. 2A). The results confirm that determination of the EAV-susceptible/resistant CD3$^+$ T cell phenotype is consistent in horses, regardless of breed. Interestingly, there was a clear difference in prevalence of the susceptibility/resistance phenotype among breeds (Fig. 2B). Ninety-five percent of STB horses tested in this study were of the susceptible phenotype (57 out of 60). Similarly, 53 out of 60 ASB horses had the CD3$^+$ T lymphocyte susceptible phenotype (~90%). The lowest prevalence of the susceptible phenotype was in TB horses, 32 out of 137 (23%), and QH horses had both phenotypes evenly distributed, with approximately 50% prevalence of each phenotype (Fig. 2B).

GWAS. To investigate the possibility of genetic influence on susceptibility/resistance of CD3$^+$ T lymphocyte to *in vitro* EAV infection, we applied the GWAS to identify the genetic ele-

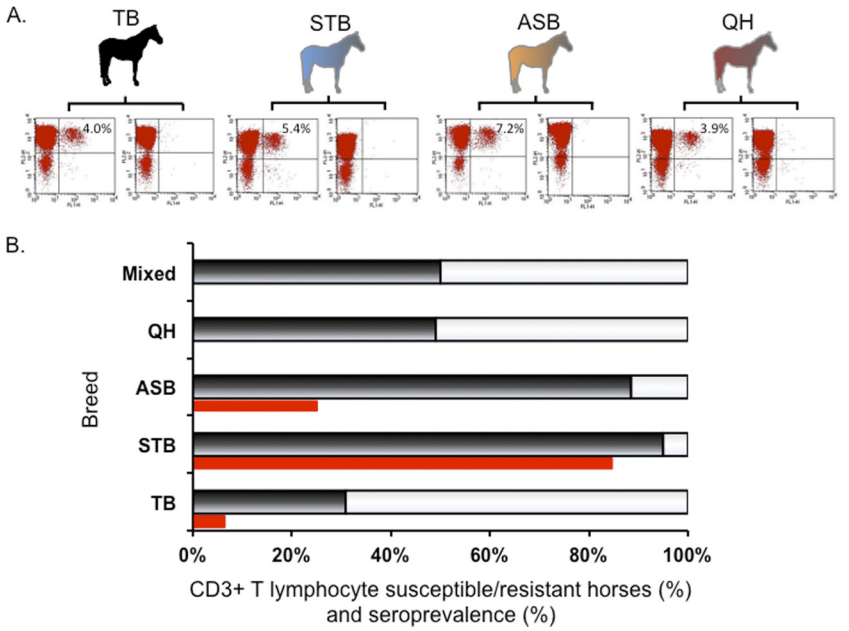

FIG. 2. Effect of breeds in prevalence of T cell susceptible/resistant phenotypes. (A) Representative dot plots from flow cytometry analysis for each breed are shown. The mean percentage of $CD3^+$ T cells with intracellular EAV NSP1 antigen is indicated in the right upper quadrant for the susceptible phenotype. (B) The percentages of susceptible (black) and resistant (white) phenotypes for each breed are indicated in a bar graph. Seroprevalences of each breed (red) are indicated below the bar, representing phenotypic prevalence where available. TB, Thoroughbred (n = 137); STB, Standardbred (n = 60); ASB, American Saddlebred (n = 60); QH, Quarter Horse (n = 53).

ment(s) associated with this phenotypic trait. In order to simplify the process of defining any genetic differences between positive and negative groups, we initially focused on TB horses since they have less diversity for genetic markers than most other breeds of horses. A group of 80 TB yearlings, serologically negative for EAV, were tested for the phenotype described above. Only 16 out of 80 TB yearlings had the susceptible $CD3^+$ T lymphocyte phenotype. Thus, 37 horses were selected for the GWAS, including 16 with the susceptible phenotype and 21 with the resistant phenotype. DNA from these horses (Illumina TB horses) was genotyped for SNPs using the Equine SNP50 BeadChip (Illumina Inc., San Diego, CA) (Fig. 1, right). Using the option for association studies, the distributions of SNPs were compared between the susceptible horses and the resistant horses. The results from this study are represented graphically in a Manhattan plot (Fig. 3) showing the P values when comparing the distributions of markers for susceptible and resistant horses for each SNP. The values from a

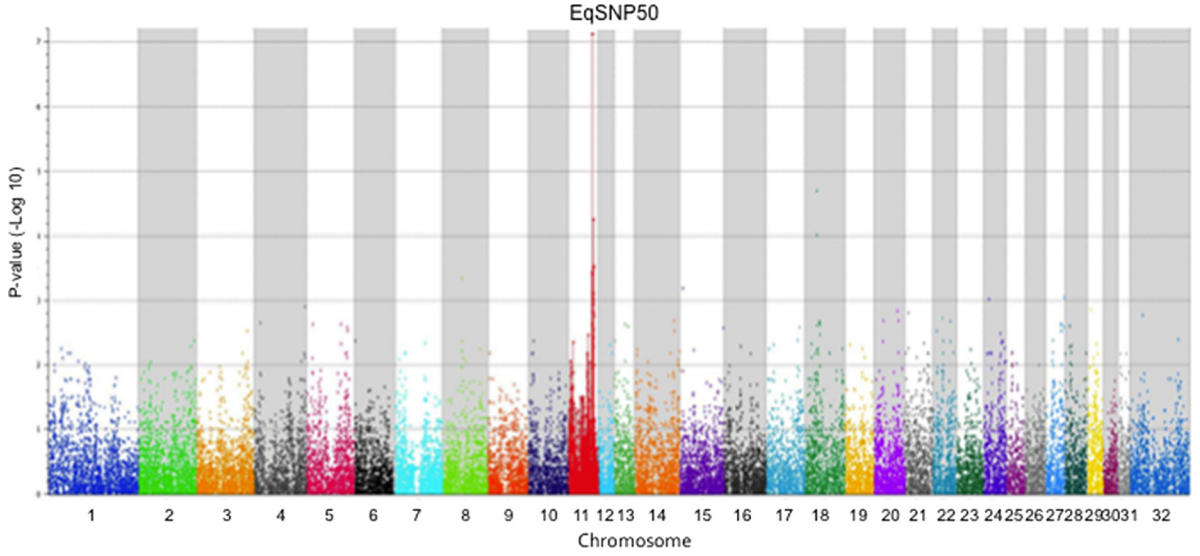

FIG. 3. Manhattan plot showing the distribution of probability values ($-\log_{10}$ transformed) for the 42,506 SNPs investigated using the 37 Thoroughbred horses (16 were susceptible and 21 were resistant). Genomic positions are indicated by chromosomes with different colors.

TABLE 2. Results from the GWAS study identifying the SNPs, chromosome location, and *P* values for the 10 highest associations found using the Illumina Equine SNP50 BeadChip based on 37 TB horses exhibiting the *in vitro* infection phenotype ($n = 16$ susceptible) and the *in vitro* resistance phenotype ($n = 21$ resistant)

SNP no.	Chromosome no.	Position	Uncorrected *P* value
BIEC2_157867	11	49424839	7.42×10^{-8}
BIEC2_409206	18	24674961	2.00×10^{-5}
BIEC2_158932	11	51677777	5.42×10^{-5}
BIEC2_409219	18	24736336	9.69×10^{-5}
BIEC2_159231	11	52379156	2.90×10^{-4}
BIEC2_158587	11	51188148	3.06×10^{-4}
BIEC2_158588	11	51188190	3.06×10^{-4}
BIEC2_157160	11	48595739	3.63×10^{-4}
BIEC2_157720	11	49039853	3.98×10^{-4}
BIEC2_1046042	8	40043641	4.47×10^{-4}

region of equine chromosome 11 (ECA11) showed a distinctive peak. The 10 highest associations that were found included 7 SNPs in one region on ECA11 (Table 2). The single highest association was found for SNP marker BIEC2-157867 from ECA11, which had statistical significances (*P* values) of 7.42×10^{-8} (uncorrected) and 0.003 (using the Bonferroni correction for the number of assays). None of the other markers achieved statistical significance when the correction to the probability value was factored in. These data clearly suggested that susceptibility/resistance of $CD3^+$ T lymphocytes to *in vitro* EAV infection is highly associated with ECA11.

Study validation and haplotype analysis. To confirm and extend the results from this initial study, additional SNPs were selected from the EquCab2.0 SNP database (Table 1) along with some of the original SNPs for the region on ECA11 and tested using the Sequenom MassARRAY system (Sequenom Inc., San Diego, CA). The Illumina-tested TB horses served as controls, being tested in both SNP assays, and had identical results for the 12 SNPs in common in the two assays. Table 3 provides the results from comparing the distributions of SNPs among susceptible and resistant horses for all horses (combined), TB horses alone, QH horses alone, ASB horses alone, and STB horses alone. The SNP showing the strongest association in the Illumina study, BIEC2-157867, had a significant and slightly higher *P* value of 5.84×10^{-5} for this new set of TB horses. The most significant value was found for BIEC2-166050 with the combined set of horses ($P = 1.73 \times 10^{-22}$). For the group of TB horses, the most significant *P* value was for BIEC2-166027 ($P = 4.96 \times 10^{-7}$). All breeds except STB horses showed statistically significant associations for the trait with SNPs in this region.

Next, linkage disequilibrium (LD) among the SNPs in this region was investigated and compared to the distribution of susceptible and resistant phenotypes in the different populations. A haplotype defined by 8 SNPs, BIEC2-166005, -166006, -166009, -166011, -166015, -166018, -166021, and -166027, spanning 71 kb from ECA11 positions 49572804 to 49643932, had the highest association across all breeds. The SNP BIEC2-166016 did not contribute to the definition of the haplotype and was therefore not included in the haplotype definition. The haplotype associated with the susceptibility phenotype was defined by the presence of nucleotides GGGGAGGT at those 8 SNP sites. Figure 4 shows a schematic representation of LD for the haplotype among all groups of horses tested. Figure 5 provides the frequencies for this haplotype among susceptible and resistant horses as well as the statistical significance for the differences in each group. In each breed, including STB horses, this haplotype was associated with susceptible horses. The haplotype was also present among the resistant horses but at a much lower frequency. Among the 13 TB horses with susceptible phenotype, all but one horse were heterozygous for the haplotype, possessing one copy of the haplotype; the remaining susceptible TB horses did not possess the haplotype (data not shown). Among the 44 resistant TB horses, 8 horses possessed

TABLE 3. Probability values comparing cases to controls for the combined set of horses ($n = 267$) and for the individual groups of TB ($n = 57$), QH ($n = 53$), ASB ($n = 60$), and STB ($n = 60$) horses, showing the SNPs in ECA11 with the lowest 17 *P* values

BIEC2 SNP no.	Position	*P* value				
		Combined	TB	QH	ASB	STB
166050	49707607	1.73×10^{-22}	6.11×10^{-4}	3.00×10^{-3}	6.18×10^{-11}	N/A[b]
166027	49643932	1.49×10^{-21}	4.96×10^{-7}	1.62×10^{-3}	1.24×10^{-5}	0.61
166011	49589952	1.17×10^{-19}	1.46×10^{-4}	1.07×10^{-3}	1.74×10^{-6}	N/A
165991	49533085	1.70×10^{-19}	7.64×10^{-5}	6.26×10^{-4}	2.00×10^{-4}	0.72
166065	49729958	3.44×10^{-19}	7.02×10^{-5}	9.40×10^{-4}	9.48×10^{-5}	N/A
166015	49613954	4.99×10^{-19}	2.74×10^{-4}	9.40×10^{-4}	4.11×10^{-5}	N/A
166021	49637772	5.97×10^{-19}	1.87×10^{-4}	9.40×10^{-4}	4.11×10^{-5}	N/A
166009	49584513	6.30×10^{-19}	1.87×10^{-4}	9.40×10^{-4}	3.24×10^{-5}	N/A
166047	49696679	3.23×10^{-18}	7.02×10^{-5}	9.40×10^{-4}	9.48×10^{-5}	0.61
166018	49627471	6.03×10^{-18}	3.72×10^{-4}	1.64×10^{-3}	4.11×10^{-5}	N/A
157944	49509504	5.75×10^{-17}	5.78×10^{-4}	3.02×10^{-3}	2.00×10^{-4}	0.56
166001	49564266	3.10×10^{-13}	2.69×10^{-3}	0.01	3.89×10^{-4}	0.44
157867[a]	49424839	1.12×10^{-9}	5.84×10^{-5}	0.50	1.66×10^{-5}	0.48
157847	49389493	3.60×10^{-9}	0.01	0.17	2.97×10^{-6}	0.93
165999	49558533	5.45×10^{-9}	0.10	0.18	0.10	0.34
165997	49545584	2.28×10^{-5}	0.26	0.23	0.69	0.80
166056	49716340	6.52×10^{-5}	3.93×10^{-3}	0.07	0.02	0.46

[a] The most highly associated SNP in the initial assay.
[b] N/A represents no variation observed between the resistant and susceptible groups.

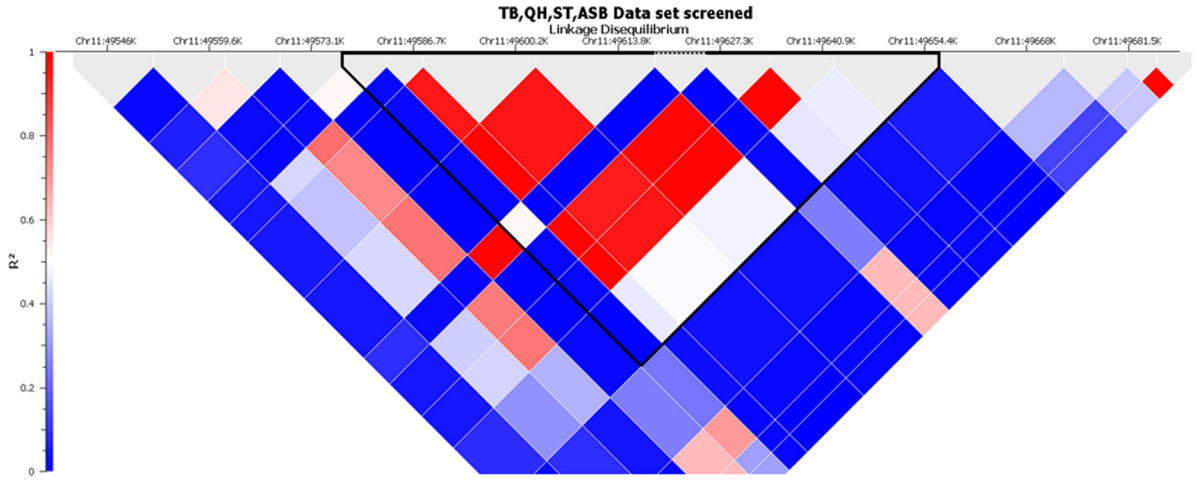

FIG. 4. Linkage disequilibrium (LD) plots for all breeds, showing the region used for defining the EAV susceptibility haplotype (ECA11 positions 49572804 to 49643932). The haplotype block is highlighted with a black line.

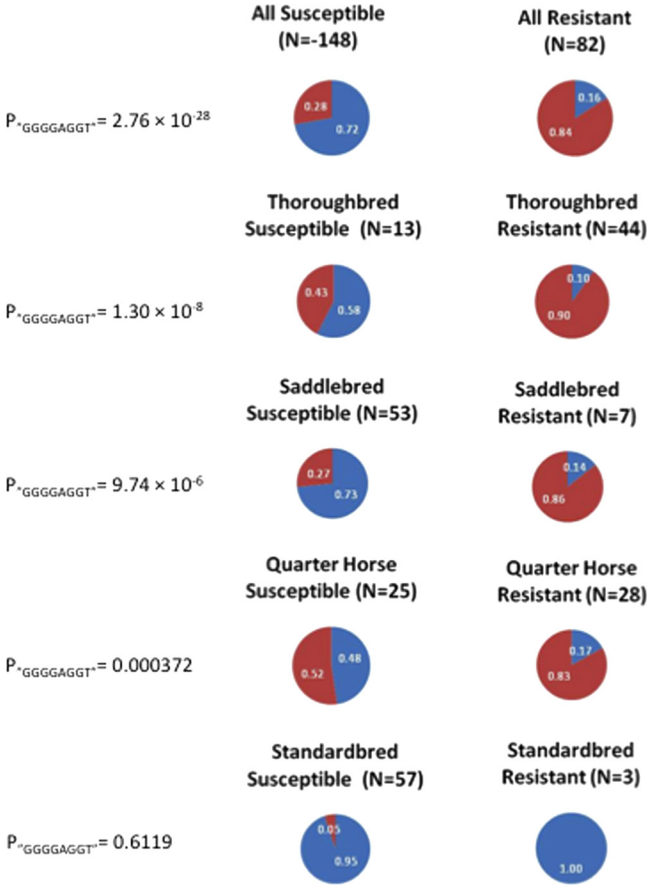

FIG. 5. Frequency of the GGGGAGGT haplotype found for selected SNPs between ECA11 positions 49572804 to 49643932 among horses susceptible and resistant for the EAV *in vitro* infection phenotype. The blue area in the pie chart represents the proportion of the GGGGAGGT haplotype, and the red area represents the allelic haplotypes. The frequency represented by each section of the pie chart is shown on the pie chart in white. Data are represented for all horses together (All) and for the individual breeds (Thoroughbred, American Saddlebred, Quarter Horse, and Standardbred). The statistical significance for the frequency differences of the GGGGAGGT haplotype between susceptible and resistant horses is shown to the left of each set of pie charts ($P_{\text{"GGGGAGGT"}}$).

one copy of the haplotype, while two horses were homozygotes (data not shown). Similar results were found for the distribution of the haplotype in other breeds.

Gene network analysis. The EAV-associated haplotype is located in a block with strong LD, expanding ~71 kb. This LD block includes five genes: the SPAG7, ENO3, PFN1, CHRNE, and MINK1 genes. For the purpose of analyzing possible functional relationship among genes using the IPA (Ingenuity Systems) and the PANTHER classification system, we have included those genes in high LD and genes located outside this particular LD block (500 kb upstream and downstream of the most significantly associated gene, the KIF1C gene). Unsupervised IPA analysis retrieved three top networks that were merged into a single interaction network (Fig. 6A). The network contained 22 genes showing significant correlation with networks implicated in cell morphology and cellular movement. Similarly, genes categorized by biological functions were mostly involved in cellular movement ($P = 1.2 \times 10^{-5}$), cell death ($P = 1.45 \times 10^{-3}$), cell morphology ($P = 1.45 \times 10^{-3}$), cellular assembly and organization ($P = 1.45 \times 10^{-3}$), and cellular function and maintenance ($P = 1.45 \times 10^{-3}$). We also classified the genes from the region by their molecular function and biological process categories using the PANTHER classification database. The enriched molecular function categories included binding, catalytic activities, and receptors, suggesting that these genes might mediate virus-cell interaction and facilitate viral entry (Fig. 6B). Furthermore, there was a predominance of genes regulating metabolic and cellular processes as well as cell communication and immune responses (Fig. 6C; Table 4).

DISCUSSION

The data presented in this study demonstrate a very strong association of genetic markers with the susceptible phenotype described for *in vitro* infection of $CD3^+$ T lymphocytes with EAV VBS. Association of genetic markers with a trait is *de facto* proof that the trait is under genetic control. To our knowledge, this is the first GWAS of an equine infectious disease and the first GWAS of EVA. We have developed an

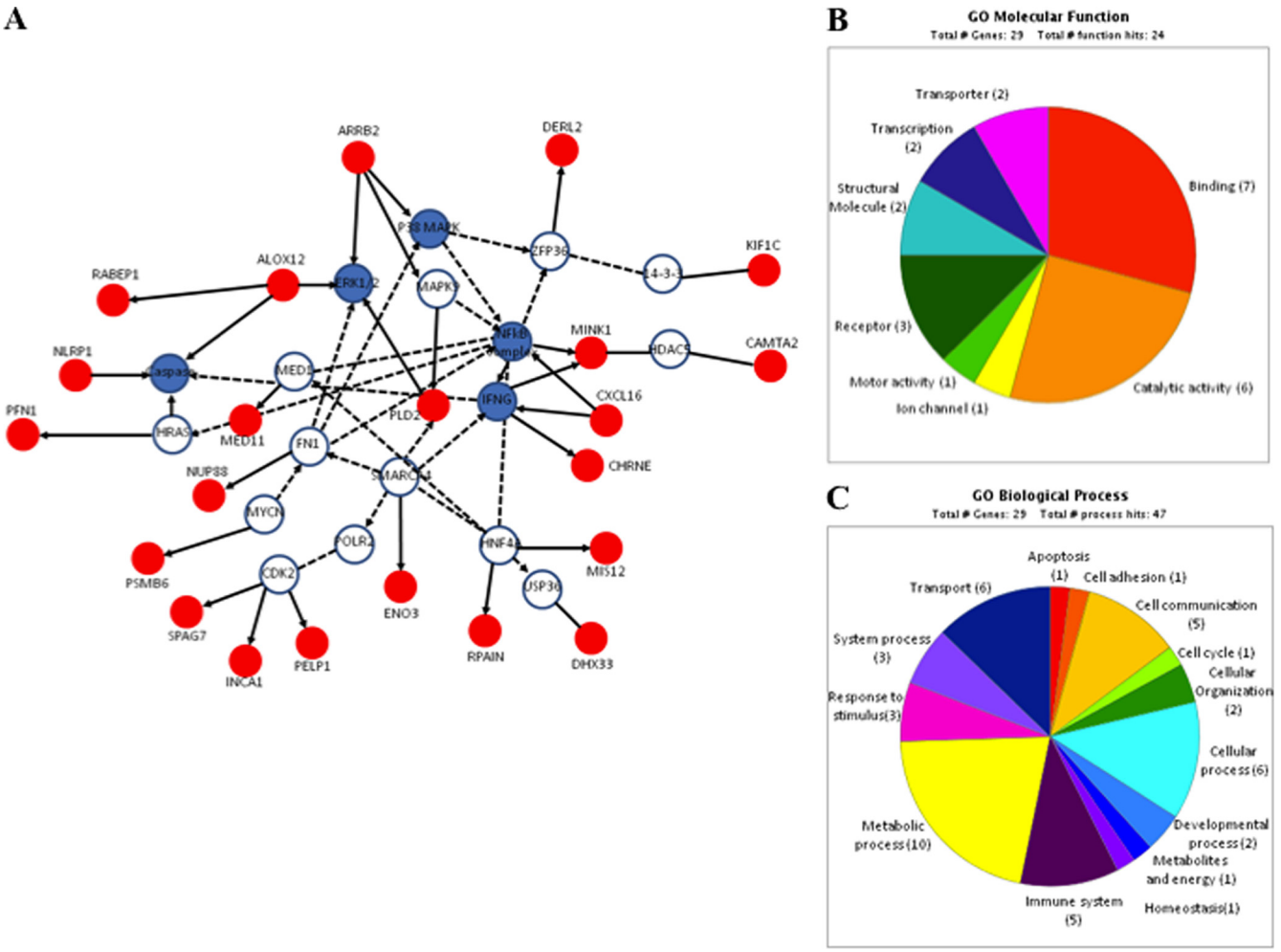

FIG. 6. (A) Three top networks retrieved using unsupervised IPA were merged into a single interaction network. Red nodes represent molecules encoded by genes found in the region of ECA11; white nodes are genes identified by IPA with direct or indirect interactions with genes of ECA11. The blue nodes represent molecules/complexes identified by IPA involved in canonical pathways. Classification of candidate molecules based on their molecular functions (B) and biological processes (C).

easy and reliable *in vitro* assay based on dual-color flow cytometry to phenotype an equine population. The data presented in this study clearly demonstrated that there was a genetic difference within and among the horse populations based on the susceptibility of their $CD3^+$ T lymphocytes to *in vitro* infection with EAV VBS. The *in vitro* assay described is currently the most precise method for determining the phenotype. However, it is expensive, time-consuming, and cumbersome to run on a routine or large-scale basis. The association of the ECA11 haplotype with the phenotype will allow us to develop a molecular test in large-scale studies to determine the impact of this phenotype on the consequences of infection.

The association of the haplotype with $CD3^+$ T lymphocyte susceptibility in all four breeds of horses (TB, ASB, STB, and QH) is remarkable. This implies that the trait first appeared in a common ancestor of these breeds. Although these horses are products of four different breed registries, they are known to have common ancestors within the last 100 years (11). The use of the four breeds together also produced the strongest associations for the trait using both the haplotype and individual SNPs (Table 3). This was a product of the wide differences among the selected horse breeds for the *in vitro* susceptibility/resistance trait. The choice of TB horses for the initial stage of the study was fortuitous since the susceptibility/resistance trait is highly polymorphic among TB horses. Had the initial study been conducted among STB horses, the association would not have been uncovered, because almost all STB horses have the susceptibility phenotype. Indeed, STB horses appear almost fixed for the genes and the trait. Therefore, the results of this study suggest that preliminary studies of such traits might best be conducted using pools of different horse breeds when that trait is manifested across breed lines.

Interestingly, distinct phenotypic prevalence of each breed found in this study was similar to EAV seroprevalence that has been reported previously (Fig. 2B) (32, 49). Similar distributions of both seroprevalence and the susceptible $CD3^+$ T lymphocyte trait strengthened our hypothesis that genetic factors might play a role in determining the $CD3^+$ T lymphocyte susceptibility/resistance of horses to EAV infection. The haplotype associated with the trait was distributed among horses in

TABLE 4. List of genes located in close vicinity with the highest associated haplotype block to the EAV-susceptible/resistant phenotypes

Type	Gene symbol[a]	NCBI gene ID	Description	Known molecular function	Virus (reference)
Receptor activity	CHRNE	100146223	Similar to nicotinic acetylcholine receptor epsilon	Acetylcholine receptor activity, ligand-gated ion channel activity	HIV-1 (8)
	ARRB2	100072892	Similar to beta-arrestin 2	Receptor internalization, endocytosis	HIV-1 (4, 54)
	PELP1	100072897	Similar to proline, glutamic acid, and leucine-rich protein 1	Estrogen receptor coactivator, receptor activity	
	RABEP1	100072805	RAB GTPase binding effector protein 1	Endocytic pathway, fusion of early endosome, and regulation of recycling vesicle formation	
	CXCL16[b]	100061442	Similar to SR-PSOX	Scavenger receptor family, NKT cell, and macrophage (MØ) activation	HIV, severe acute respiratory syndrome coronavirus (SARS-CoV), Kaposi's sarcoma-associated herpesvirus (KSHV) (38, 76, 77)
Intracellular vesicle trafficking	DERL2	100061027	Derlin-2 (Der1-like protein 2)	Removal of misfolded protein from endoplasmic reticulum	Murine polyomavirus (41)
	KIF1C	100072836	Kinesin family member 1C	Vesicle transport, redistribution of Golgi membrane, microtubule motor activity	
Actin and cytoskeleton	SPAG7	100061215	Similar to sperm-associated antigen 7	Nucleic acid binding, cytoskeleton	Human parvovirus B19 (34, 35)
	PFN1	100061295	Similar to profilin 1	Actin polymerization, cytoskeletal signaling pathway	Respiratory syncytial virus (RSV) (7)
Innate and adaptive immunity	NLRP1		NLR family, pyrin-containing domain 1	Regulation of inflammatory response, induction of apoptosis	KSHV/human herpesvirus 8 (HHV-8), Myxoma virus, HIV-1 (30, 61)
	DHX33	100072797	DEAH (Asp-Glu-Ala-His) box polypeptide 33	Putative RNA helicase	HIV-1 (9)
	NUP88	100061117	Nucleoporin, 88 kDa	Innate immunity, nuclear export	Hepatitis C and B viruses (36, 74)
	PSMB6	100072870	Proteasome subunit beta type 6 precursor	Peptidase activity, involved in antigen presentation by major histocompatibility complex I (MHCI)	
	ALOX12	100072916	Similar to 12-lipoxygenase	Oxidoreductase activity, MØ activation, B cell-mediated immunity	
	CXCL16[c]	100061442	Similar to SR-PSOX	Scavenger receptor family, NKT cell, and MØ activation	HIV, SARS-CoV, KSHV (38, 76, 77)
Transcription factors	RPAIN	100061089	RNase protection assay (RPA)-interacting protein	Nuclear transporter of RPA	
	ZFP3	100061153	Zinc finger protein 3 homolog	DNA binding, transcription factor	
	CAMTA2	100072844	Similar to calmodulin-binding transcription activator 2	Transcriptional coactivator, DNA binding	
	MED11	100072890	Mediator complex subunit 11; similar to HSPC296	Component of pol II transcription machinery, transcription initiation complex stabilization	Measles virus (63)
Enzyme activity	ENO3	100061187	Hypothetical protein LOC100061187	Lyase activity	
	MINK1	100061370	Misshapen-like kinase	Stress-activated protein kinase (SAPK)/Jun N-terminal protein kinase (JNK) signal pathway, kinase activity	
	PLD2	100072863	Similar to phospholipase D2	ERK1/2 signaling pathway, hydrolase activity	HIV-1 (59)
Others	MIS12	100060987	MIND kinetochore complex component	Chromosome segregation	
	C17orf87		Transmembrane protein C17orf87	Transmembrane protein	
	L11_049426896	100072818	Hypothetical protein		
	L11_049471914		Hypothetical protein		
	INCA1	100072840	Similar to HSD45	Regulation of cell proliferation, spermatogenesis	
	GLTPD2	100072874	Glycolipid transfer protein domain-containing protein 2	Lipid transport	
	VMO1	100072878	Vitelline membrane outer layer protein 1 homolog precursor		
	TM4SF5	100072881	Transmembrane 4 L6 family member 5		
	ZMYND15	100061402	Zinc finger MYND domain-containing protein 15		
	L11_049822588		Hypothetical protein		

[a] Based on consensus equine protein-coding gene annotation (19).
[b] Transmembrane form.
[c] Secretory form.

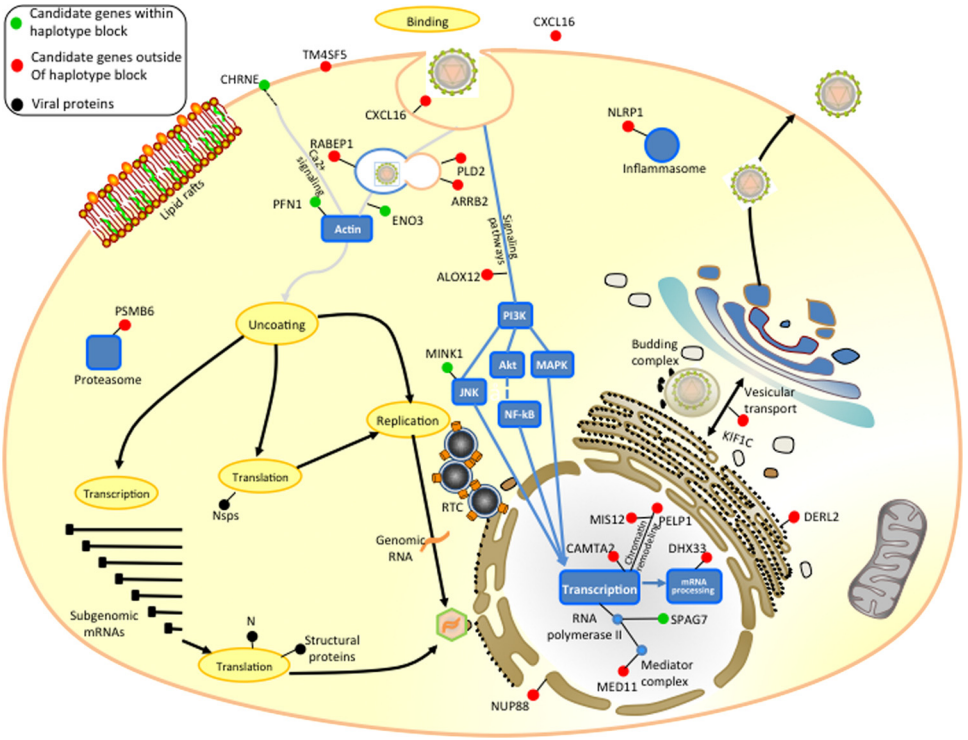

FIG. 7. Subcellular location of candidate genes and EAV life cycle. The function and subcellular location of candidate molecules were manually curated based on PANTHER, IPA, and published literature search. Subsequently, molecules were mapped along with stages of the EAV life cycle at the position most likely to interact with the virus life cycle. Green circles, molecules encoded by genes located in the haplotype block; red circles, putative molecules encoded by genes found within 500 kb upstream and downstream of the haplotype block; blue circles, molecules in the vicinity interacting with genes located in the region retrieved in the network analysis; black circles, viral proteins.

a manner characteristic for that harboring a gene with a dominant mode of action. However, the association of the trait with the genetic markers was imperfect, since few susceptible horses were found without the implicated genetic markers implicated for susceptibility, and conversely, some resistant horses were found with susceptibility markers. However, this is not surprising since the 53 SNPs under study represented a very small proportion from the 3 million-bp-long region, and the chances of having selected the SNP defining the actual mutation defy expectations. Although additional work is needed to investigate candidate genes and resequencing of candidate regions, it is clear that genes within the region of ECA11 positions 49572804 to 49643932 play a major role in determining the $CD3^+$ T lymphocyte susceptibility phenotype. In a parallel study, we have experimentally inoculated both groups of horses and assessed the severity of clinical disease, virus shedding, and production of proinflammatory/immunomodulatory cytokines in PBMCs using real-time quantitative reverse transcription (RT)-PCR (Y. Y. Go, R. F. Cook, J. Q. Fulgêncio, J. R. Campos, P. Henney, P. J. Timoney, and U. B. Balasuriya, submitted for publication). The data showed that there was a significant difference between the two groups of horses in terms of cytokine mRNA expression and increased clinical signs in horses possessing the *in vitro* resistant $CD3^+$ T cell phenotype. Thus, there was a clear correlation between the *in vitro* susceptibility or resistance phenotype of $CD3^+$ T lymphocytes and the different clinical/cytokine expression responses observed in horses following infection with EAV. Therefore, further studies on genes located in ECA11 will enhance our understanding of the pathogenesis of EAV infection and variation in susceptibility or resistance to establishment of the carrier state in stallions and will hopefully lead to the identification of a putative EAV cell receptor(s).

In this study, we have identified for the first time a significant number of candidate genes that might be associated with $CD3^+$ T lymphocyte susceptibility to EAV infection, and these genes may also play a major role in pathogenesis and persistent infection in the stallion. We used IPA in an unsupervised manner, allowing identification of gene-gene relationships without *a priori* assumptions. This analysis linked 22 of 28 genes to other nodes in a highly significant network (Fig. 6A). These genes might be functionally interrelated and may play a direct or indirect role in susceptibility to EAV infection and pathogenesis of EVA. The proteins encoded by these genes are involved in virus attachment and entry pathway (putative cellular receptors for viral entry, molecules associated with cytoskeleton and endocytosis), various transcription factors, cell-signaling molecules, and molecules associated with inflammatory response and innate and adaptive immunity (Fig. 6A and B). Interestingly, some of these proteins are well-known cellular factors involved in other virus infections (Table 4) (4, 7–9, 30, 34–36, 38, 41, 54, 59, 61, 63, 74, 76, 77).

We found several proteins, such as CHRNE, CXCL16, RABEP1, ARRB2, and PELP1, that may be involved in EAV attachment and entry into the cell. Although the cellular receptor for EAV is not yet known, it is plausible that some of

these molecules (e.g., CHRNE, CXCL16, and PELP1) may act as putative EAV receptors or serve as essential components in viral attachment and entry processes. In addition, proteins involved in the endocytic pathway, such as multifunctional proteins ARRB2 (17, 18, 60) and RABEP1 (69), may also play a critical role in the resistance or susceptibility of cells to EAV since it has been demonstrated that EAV is taken up via clathrin-dependent endocytosis and is delivered to acidic endosomal compartments during entry into the cell (57). Interestingly, the endocytic recycling receptor molecules, ASGPR1 and ASGPR2 (65), were located in the same chromosome approximately 800 kb downstream of RABEP1, making them attractive receptor candidates in line with clathrin-dependent endocytosis.

The molecules involved in intracellular trafficking, cell movements, cell-to-cell communication, as well as cell-substrate adhesion pattern and cell polarization are critical in relation to the viral life cycle (2, 10, 27, 33, 40, 47, 58). It has become apparent that numerous viruses interact with cytoskeletal components at various stages of their life cycles, disrupting or remodeling cytoskeletal networks to their own advantage (73). Some viruses can directly or indirectly hijack cytoskeletal cell component organization to their own advantage, often altering cell behavior and cell fate. In EAV-infected cells, cytopathic effect (CPE) is characterized by rounding, vacuolation, increased optical density, refraction, and detachment from the supporting surface, indicating viral modulations of the actin cytoskeleton (15, 37, 50, 51). In the region of ECA11 identified in this study, there are several genes that encode proteins determined to be involved in cellular movement function (e.g., ARRB2, PLD2, and PFN1 genes) as well as in actin cytoskeleton reorganization (Fig. 7) (7, 29).

Finally, our network analysis also included some genes encoding proteins associated with apoptosis and modulating innate and adaptive immune responses to virus infections (Fig. 7) (25, 39, 62, 66). Proteins such as ARRB2 have been increasingly implicated in the modulation of nuclear factor kappa B (NF-κB) signaling and interacting with I kappa B alpha (IκBα) and extracellularly regulated kinases 1 and 2 (ERK1/2) (21–23, 26, 44–46, 53, 66, 72, 75). In addition, some genes in the region were associated with the host inflammatory response and innate immunity. For example, CXCL16, a member of the CXC chemokine family, is expressed as a soluble form and a transmembrane protein (38, 43). While the transmembrane form may have receptor activity, the soluble CXCL16 induces inflammatory responses (1, 38, 48). EAV may interact with some of these cellular proteins to evade the equine immune response and establish persistent infection in the cells of the reproductive tract of the stallion. However, further studies are needed to investigate potential mechanisms that involve the roles of proteins encoded by genes located in this region of ECA11 in the virus life cycle and host response to EAV infection, as well as to identify the mechanism of establishment of persistent infection in some but not all stallions infected with the virus. The findings from this study can help to develop working hypotheses to decipher novel mechanisms of viral immune evasion, establishment of persistent infection, and viral pathogenesis.

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