Polarisation of equine pregnancy outcome associated with a maternal MHC class I allele: preliminary evidence

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Abstract

Identification of risk factors which are associated with severe clinical signs can assist in the management of disease outbreaks and indicate future research areas. Pregnancy loss during late gestation in the mare compromises welfare, reduces fecundity and has financial implications for horse owners. This retrospective study focussed on the identification of risk factors associated with pregnancy loss among 46 Thoroughbred mares on a single British stud farm, with some but not all losses involving equid herpesvirus-1 (EHV-1) infection. In a sub-group of 30 mares, association between pregnancy loss and the presence of five common Thoroughbred horse haplotypes of the equine Major Histocompatibility Complex (MHC) was assessed. This involved development of sequence specific, reverse transcriptase polymerase chain reactions and in several mares, measurement of cytotoxic T lymphocyte activity. Of the 46 mares, 10 suffered late gestation pregnancy loss or neonatal foal death, five of which were EHV-1 positive. Maternal factors including age, parity, number of EHV-1 specific vaccinations and the number of days between final vaccination and foaling or abortion were not significantly associated with pregnancy loss. In contrast, a statistically significant association between the presence of the MHC class I *B2* allele and pregnancy loss was identified, regardless of the fetus / foal's EHV-1 status ($p=0.002$). In conclusion, this study demonstrated a significantly positive association between pregnancy loss in Thoroughbred mares and a specific MHC class I allele in the mother. This association requires independent validation and further investigation of the mechanism by which the mare's genetic background contributes to pregnancy outcome.

Introduction

Equid herpesvirus-1 (EHV-1) is an alpha herpesvirus which causes respiratory disease and leads to a cell associated viraemia which, *via* endothelial cell infection and thrombo-ischaemia results in late gestation abortion or neonatal foal death (Ma et al., 2013). Despite routine vaccination of pregnant mares, sporadic EHV-1 abortion outbreaks can still occur. Host risk factors for the development of herpes viral abortion, including the role of the mare's Major Histocompatibility Complex (MHC) class I genotype have received little attention to date. Classical equine MHC class I genes, also known as the Equine Leukocyte Antigen (ELA) complex, encode a large number of polymorphic alleles (Tallmadge et al., 2005). These allelic polymorphisms determine the range of peptides presented to cytotoxic T lymphocytes (CTL). Fifteen MHC class I serological ELA haplotypes have been identified at the ELA-A locus, but only a small number of haplotypes are common in the Thoroughbred breed, namely ELA-A2, ELA-A3, ELA-A5, ELA-A9 and ELA-A10 (Antczak, 1992). The sharing of MHC class I alleles between individuals can also be demonstrated functionally *in vitro* using an EHV-1 specific, cytotoxic T lymphocyte assay (Allen et al., 1995). One EHV-1 CTL target is the immediate early protein (ICP4, encoded by gene 64), which is restricted by the *B2* allele expressed on the ELA-A3 haplotype (Kydd et al., 2006) and an unknown allele on ELA-A2 (Kydd et al., 2014). Recently, the *B2* allele (Eqca-1*00101 using the current nomenclature) has been shown to bind a single 9 residue peptide, namely RDGARFGEL within the immediate early protein (Bergmann et al., 2015). The frequency of the *B2* allele is relatively common with estimates ranging from 13.4% to 25% in Thoroughbred horses (Bergmann et al., 2015; Antczak, D.F 1986).

The study described here exploited a field outbreak of multiple abortions in a herd of Thoroughbred mares to explore associations between several risk factors and pregnancy losses, some but not all of which were confirmed as EHV-1 positive. These preliminary

results suggested a significant association between pregnancy loss and the mare's MHC class I allele, regardless of the EHV-1 status of the feto-placental unit. No other significant associations were identified and vaccination provided partial protection.

Materials and methods

Groups of horses

Three separate groups of horses were used, namely the Cornell (n=9), Newmarket (n=40) and commercial pregnant Thoroughbred (n=46) herds. The Cornell horses were used to produce template cDNA for RT-PCR based tests to identify five known ELA haplotypes in the Thoroughbred horse. This herd consisted of 10 horses of known MHC class I haplotypes, previously characterised by serological typing and test matings. Nine homozygous horses included three carrying ELA-A2, three with ELA-A3, one ELA-A5 and one ELA-A9 Thoroughbreds and one ELA-A10 Standardbred horse. The heterozygote carried the ELA-A2 and ELA-A5 haplotypes. These horses were used for the optimisation of the allele-specific RT-PCRs using a panel of primer pairs. The second group (unrelated to the first) comprised 40 Newmarket Thoroughbreds of unknown haplotype that included a mixed family of half- and full-siblings, with eight foals by the same sire and six dams. Samples from these animals were used to validate the RT-PCR in an uncharacterised population. All horses were maintained according to national and institutional care guidelines, all samples were excess to requirements when horses were sampled for other reasons and standard ethical approval procedures were completed. The third group comprised 46 pregnant Thoroughbred mares which were used for commercial breeding purposes in which an EHV-1 outbreak occurred (details below).

RT-PCRs for MHC class I genotyping

Using cDNA extracted from peripheral blood mononuclear cells, RT-PCRs were developed (Supplementary File 1) to detect selected, sequenced alleles specific for five known ELA haplotypes in the Thoroughbred horse. To design allele-specific primers, sequence data of all the equine MHC class I genes (classical and non-classical) previously identified from the haplotypes ELA-A2, -A3, -A5, -A9, and –A10 were obtained from GenBank® and aligned using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html). Five MHC class I alleles, one per haplotype, were selected based on differences in sequence in the hypervariable regions (*8-9* (A2 haplotype), *B2* (A3.1), *5b* (A5), *9c* (A9) and *10a* (A10; Supplementary File 2). These common haplotypes represent approximately 90% of animals in the Thoroughbred breed (D.F. Antczak, personal communication). The genes from which the alleles originated had previously been assigned to four MHC class I loci (Supplementary File 3; see (Tallmadge et al., 2010) for nomenclature). To capture any additional alleles, generic RT-PCR primers were also used (adapted from (Chung et al., 2003)) that closely matched homologous regions of all the classical MHC class I genes but did not bind to known non-classical and pseudogene sequences. A sample of each RT-PCR amplicon was electrophoresed and visualized according to standard protocols. Following amplification, all RT-PCR products were cloned and sequenced using standard methods.

Commercial Thoroughbred broodmares, pregnancy losses and inclusion criteria

Pregnant Thoroughbred mares (n=46) were maintained on a well-managed stud farm in England and had been vaccinated against EHV-1 and EHV-4 as recommended with an inactivated, whole virus vaccine with carbomer adjuvant (Equip EHV-1,4, Zoetis UK Ltd., London). These mares shared a common paternal or maternal sire at the fourth or fifth generation. The herd also included five mares which were half-sisters out of two dams.

Following confirmation of two consecutive EHV-1 positive abortions, 46 mares were followed to determine their pregnancy outcome. Pregnancy losses which occurred at ≥100 days of gestation and ≤1 day *post partum* were included in the analysis. Potential risk factors including age, parity, number of EHV-1 specific vaccinations and the number of days between final vaccination and foaling or abortion were also assessed in 46 mares. Of these 46 mares, excess samples were available from 30 and these were studied retrospectively to determine any association between their MHC class I genotype and pregnancy loss (EHV-1 positive or any pregnancy loss). Fetuses or neonatal foals and placentae underwent full *post mortem* examinations and routine diagnostic tests by accredited pathologists. Twenty five foaling mares were typed by RT-PCR alone. Of 10 mares with pregnancy loss, two were typed by RT-PCR and cytotoxic T lymphocyte (CTL; see below) assay, two by RT-PCR alone, two by CTL assay alone, but samples were unavailable for RT-PCR or CTL from four mares.

Detection of cytotoxic T lymphocyte (CTL) activity

To confirm and extend RT-PCR results, CTL assays were performed using lymphocytes from selected Thoroughbred mares to determine the presence of a shared MHC class I allele (Allen et al., 1995). An MHC class I $B2^+$ pony which was hyper-immune to EHV-1 by previous experimental infection with strain Ab4, was used as: a) a source of effector cells with high frequencies of memory CTL activity (positive control) and b) a donor of $B2^+$ target cells. Other target cells included: a) autologous cells either infected with EHV-1 or medium or a recombinant vaccinia virus encoding EHV-1 gene 64 (NYVACC g64; Paillot et al., 2006), a known CTL target protein. Gene 64 encodes the immediate early (IE) protein of EHV-1, a CTL target when presented by products of the *B2* allele (ELA-A3) and potentially, the ELA-A2 haplotype (Kydd et al., 2014; Kydd et al., 2006). Thus effector CTLs' lysis of targets

which present an IE peptide on the *B2* allele or an allele on the A2 haplotype can confirm and extend RT-PCR results; b) $B2^+$ EHV-1 infected target cells were used to demonstrate sharing of the *B2* allele; c) EHV-1 infected targets from four allogeneic Thoroughbred stud farm mares with pregnancy loss, to determine any shared alleles. Insufficient Thoroughbred effector cells were available to test target cells for genetic restriction. A positive result was defined as infected target cells with a percent specific lysis greater than two standard deviations above that of the mock infected target cells.

Statistical analysis

Pregnancy loss was defined as any abortion or neonatal foal death during the study period following the initial EHV-1 positive abortion, regardless of the presence or absence of EHV-1. The risk factor variables were assessed statistically for the strength of their association with pregnancy loss. For the continuous measures of age of mare in years, number of previous foals and total number of vaccinations against EHV-1/4, the non-parametric Wilcoxon rank sum test was used on data from 46 mares. For the binary variable of presence/absence of the MHC class 1 *B2* allele, Fisher's exact test was applied to data from 30 mares in which the mares' genotype and pregnancy outcome had been determined. Statistical analyses were conducted using Stata12.0 statistical software (StataCorp, College Station, Texas) with statistical significance taken at $P \leq 0.05$.

Results

Characterisation of the MHC class I allele-specific RT-PCR

For each pair of primers, RT-PCR products of the predicted size were obtained from lymphocyte cDNA originating from the Cornell horses (Supplementary File 4). Each product

was present only in the specific predicted haplotype and there was no evidence that the allele-specific primers amplified any products from other haplotypes. Each RT-PCR product was sequenced and this matched the published database sequences, thus confirming the amplification of the allele of interest.

The five allele-specific primer pairs were then used to test cDNA samples from 40 Newmarket Thoroughbreds of unknown MHC haplotypes. Each sample produced an RT-PCR product of the expected size from at least one set of the allele-specific primers. A total of 28 cDNA samples generated an RT-PCR product from 2 sets of gene-specific primer pairs indicating a heterozygous genotype. The remaining 12 cDNA samples generated a RT-PCR product from just one allele-specific primer pair. This latter result may be interpreted as: i) that two copies of a single allele are present, demonstrating homozygosity or ii) that other alleles are present which are not recognised by any of the primer pairs, suggesting a heterozygous genotype. The 40 Newmarket Thoroughbreds included six mares which had eight offspring between them, sired by the same stallion (Table 1). In three cases (offspring 1, 2 and 4), it is certain that the sire transmitted ELA-A3. In two cases (offspring 6 and 8) the sire transmitted either ELA-A5 or ELA-A9 and in one case (offspring 3) either ELA-A5 or an unknown haplotype. The data show that the sire carried ELA-A3 and suggests that the sire's other MHC haplotype was ELA-A5. This fits the segregation pattern for all eight offspring and supports the conclusion that offspring 3, 5, and 7 are true MHC homozygotes. Dams PM and TJ are possible homozygotes.

To confirm homozygosity in the 12 putative homozygous Newmarket Thoroughbred samples, a generic RT-PCR (primers in Supplementary File 3) was performed on cDNA, to allow amplification and sequence analysis of as many of the classical MHC class I genes as possible from the A2, A3, A5, A9 and A10 haplotypes. Generic RT-PCR products were cloned from all 12 putative homozygotes. At least 25 clones from each sample were

sequenced across the binding site and the data compared with published nucleotide and deduced amino acid sequences of equine MHC class I genes. Sequences were considered a match to a published gene if they had ≤five deduced amino acid changes and those with one to five amino acid changes were classified as an allelic variant rather than a new gene (S.A. Ellis, Personal Communication). Of the 12 putative homozygous Newmarket Thoroughbred samples, 10 (including dams PM and TJ and foals 3, 5 and 7) did not result in the amplification of any additional nucleotide sequences showing homology with other published Thoroughbred horse-associated MHC class I alleles. These data provide further evidence of the specificity of the original allele specific RT-PCR and the unique presence of each selected allele within a single haplotype. In the remaining two putatively homozygous horses (1*00101, alias *B2* (ELA-A3) and N*00101, alias *8-9* (ELA-A2)), the generic classical MHC class I primers did amplify cDNA of a single additional gene that was homologous to a published MHC class I gene, 118 expressed on the W11 haplotype (Genbank Accession number AY176106). MHC class I haplotype information was not available for the sire or dam of these horses. However, these results indicate that these two horses may be heterozygous with 1*00101 / 118 (ELA-A3 / W11) and N*00101 / 118 (ELA-A2 / W11) genes respectively (118 allele frequency 5%; 2/40).

Outbreak of pregnancy loss in Thoroughbred mares and associated risk factor analysis

Following two EHV-1 positive pregnancy losses, the outcome of pregnancy was monitored over one season in a total of 46 Thoroughbred mares and risk factors for pregnancy loss investigated. The management included keeping mares in small isolated groups at pasture, but all were stabled together in a large American barn, with shared airspace, for one night during particularly bad weather. Thirty six mares produced a viable foal but 10 pregnancy losses (22%) were recorded and 10 fetuses underwent *post mortem* diagnostic tests, although

these were limited in one carcass (#29) due to predation of fetal tissues. Five of the 10 pregnancy losses were attributed to EHV-1 infection (Table 3). All but one of the losses were at >five months of gestation, including one mare (13) whose neonatal foal died at five hours *post partum*. One abortion (mare 38) occurred at 115 days (3.8 months) of gestation. There were no significant associations between pregnancy loss and the mares' age, parity, number of vaccinations or days since last vaccination (Supplementary File 5). The data confirmed previous field observations that even in vaccinated mares, EHV-1 infection can lead to pregnancy loss.

Cell mediated immune responses in Thoroughbred mares involved in the outbreak

MHC class I restricted, EHV-1 specific CTL activity was monitored once in PBMC from 5/10 mares with EHV-1 positive pregnancy loss (Table 3; Supplementary File 6). Three of these mares (13, 20 and 36) had not been MHC class I genotyped by RT-PCR. Maximum lysis of mock infected target cells was <9.9%. Effector CTL from all five mares showed positive lysis against autologous EHV-1 infected target cells, suggesting that virus specific, CTL memory cells were present. Sufficient cells were available for further tests from four of these five mares. Insufficient effectors were available from mares 5, 13 and 14 to include allogeneic, infected target cells and thus show genetic restriction. The absence of this control means that the data from this assay must be interpreted with caution. Effectors from three mares (5, 13 & 14) showed positive lysis of target cells from a known $B2^+$ horse, implying a shared expression of the *B2* allele. Effectors from these three mares, where sufficient cells were available, lysed EHV-1 infected target cells from each other, again suggesting the sharing of MHC class I antigens. Three of these mares lysed NYVACC gene 64 infected target cells (mares 5, 13 and 14), indicating that target peptides of the immediate early protein are presented to CTL in these animals (Kydd et al., 2006). These data confirmed and

extended RT-PCR results, suggesting the presence of a functional *B2* allele in mares 5, 13 and 14. Effector CTL from Mare 36 also showed positive lysis against NYVACC gene 64 infected target cells, but there were insufficient effectors to screen on $B2^+$ target cells. As peptides of the immediate early protein encoded by gene 64 may also be presented by an allele on the ELA-A2 haplotype (Kydd et al., 2014), no definitive conclusion about mare 36's haplotype could be drawn so she was excluded in further analyses.

Application of the MHC class I allele specific RT-PCR to commercial Thoroughbred broodmares

The presence of alleles *8-9* (A2 haplotype), *B2* (A3.1), *5b* (A5), *9c* (A9) and *10a* (A10) was detected by RT-PCR in 29 mares (25 foaling and four pregnancy losses) and only by CTL assay in 1 mare (13). One or two RT-PCR products were detected in each of the mares, of which eight appeared homozygous and 21 heterozygous (Table 2). The five half-sisters shared either allele *9b* or *10c*. A total of 30 genotyped mares were therefore included in the analysis. Of 25 mares that were genotyped and foaled normally, five mares (20%; mares 9, 32, 34, 35 and 39) had the *B2* allele. Of these five mares, four were heterozygous (80%) and one was putatively homozygous. In contrast, in four of the 10 mares which lost their pregnancy and were RT-PCR typed, the *B2* allele was detected in all four mares (100%; mares 5, 14, 29 and 38; Table 3). Of these four $B2^+$ mares, two showed putatively homozygous (*B2*) and two heterozygous (*B2*/*8-9* and *B2*/*9c*) genotypes.

To summarise the MHC class I alleles which were determined by RT-PCR and CTL assay in 30 mares, the percent allele frequency was calculated and the distribution according to

pregnancy outcome illustrated (Figure 1). To reduce bias in putatively homozygous animals, the allele detected was counted once and "x" was assigned to the potential unknown allele. The total of 16.7% *B2*$^{+}$ (Eqca-1*00101) horses in the current study is comparable with the 13.4% reported by Bergmann et al., (2015).

Statistical analysis of pregnancy outcome and MHC class I genotype

No statistically significant associations were found between pregnancy loss and mares' age, total number of previous foals and total number of vaccine doses. The relationship between the dam's MHC class I genotype and pregnancy outcome was analysed statistically in 30 mares (excluding mare 36). This demonstrated that of five pregnancies lost in which the MHC class I allele was determined conclusively, all five (100%) mares contained the *B2* allele and three of these mares had an EHV-1 positive abortion. In contrast, of the 25 mares which foaled successfully, only five (20%) animals carried the *B2* allele (four heterozygous and one putatively homozygous). Statistical analysis by Fisher's exact test comparing these proportions demonstrated a statistically significant association ($p=0.002$) between the presence of the *B2* allele and prevalence of pregnancy loss, where these criteria were known.

Discussion

This study described the characterisation of allele specific primer pairs in an RT-PCR to differentiate five common MHC class I haplotypes in the Thoroughbred horse. Application of the method to a population of Thoroughbred broodmares, combined with cross-reactive CTL activity, revealed a significant association between the presence of the MHC class I *B2* allele and pregnancy loss, regardless of the EHV-1 status of the fetus. This polarisation of clinical outcome highlights MHC class I as a potential risk factor associated with pregnancy loss in the Thoroughbred horse.

Risk factors associated with pregnancy loss are likely to be multifactorial, although only one was confirmed as statistically significant in the current study, namely the presence of the MHC class I *B2* allele in the dam. This association was also significant in EHV-1 positive losses. *In vitro,* MHC class I interacts with EHV-1 at several points in the pathogen's life cycle. Thus MHC class I acts as an entry receptor via viral glycoprotein D (Kurtz et al., 2010; Sasaki et al 2011a). Importantly, it appears that not all MHC class I alleles can act as entry receptors for EHV-1, but those with an alanine at position 173 in the α2 domain can aid viral entry (Sasaki et al., 2011b). The *B2* allele does have alanine at this position, in common with all known alleles at the two polymorphic loci (1 and 16; Tallmadge et al., 2010). However *in vitro,* although cells transfected with genes from the five common MHC class I haplotypes were susceptible to EHV-1 infection, amongst cells transfected with seven genes on the ELA-A3 haplotype, only cells transfected with the *B2* (3.1) allele permitted entry of EHV-1 (Azab et al., 2014). Bergmann et al (2015) have reported a single 9mer peptide binding motif in EHV-1's immediate early protein with affinity for the binding groove encoded by Eqca-1*00101 (*B2* allele). The same publication also noted interferon γ ELIspot responses to this peptide in PBMC from a single, multiply infected horse which contained the Eqca-1*00101 (*B2*) allele but not in horses vaccinated with an inactivated virus product. Additional interactions between EHV-1 and MHC class I have been identified. These include specific EHV-1 proteins which mediate partial downregulation of MHC class I molecules on the cell surface via dynamin-dependent endocytosis (Huang et al., 2015) and block the formation of peptide-MHC class I complexes in the transport associated pathway involved in antigen presentation (Koppers-Lalic et al., 2008). Bodo et al., (1994) has reported an association between the ELA-A10 haplotype and higher EHV-1 specific, virus neutralising antibody titres in peripheral blood, compared with other haplotypes. Thus all these observations point

to the importance of the host's individual MHC class I type and its emerging role in the pathogenesis and immune response to this virus.

Associations between MHC class I alleles and severity of clinical disease have been reported in people and animals (Blackwell et al., 2009; Dukkipati et al., 2006). For example, the development of post herpetic neuralgia following varicella zoster virus infection of Japanese people is associated with a higher frequency of three MHC alleles (Sato-Takeda et al., 2004). In contrast, a low prevalence of herpes simplex virus-1 infection and disease is associated with a high frequency of the Human Leukocyte Antigen (HLA-)-B*44 allele (Samandary et al., 2014). In veterinary species, MHC class I genes have been associated with susceptibility to disease, including mastitis in cattle (Park et al., 2004). In the horse, there is an association between MHC class I ELA-A9 haplotype and the development of uveitis (Deeg et al., 2004). The current data therefore add to the association between MHC class I and disease.

The association between a maternal *B2* allele and all pregnancy losses, regardless of EHV-1 status was unexpected. Thus the *B2* allele was also detected in two mares which suffered pregnancy loss and were diagnosed as EHV-1 negative using fetal and placental samples. A potential explanation is that regardless of the EHV-1 infection status, the *B2* allele in the mare is associated with an inadequate or different innate or adaptive immune response, leading to the loss of a viable feto-placental unit. For example, antimicrobial peptides in the human reproductive tract show a high degree of genetic variation and immune-modulatory function (Yarbrough et al., 2015), which may influence the innate immune response to pathogens and ultimately the outcome of pregnancy.

Intriguingly, the MHC class I *B2* allele was detected in some mares which foaled successfully. Closer interrogation of the data showed that four out of five of these mares were heterozygous for the *B2* allele. Thus it appears that heterozygosity of the mother may have an

overriding positive influence on successful pregnancy outcome. Maternal heterozygosity may dampen any immuno-dominant effect normally associated with the *B2* allele or, as discussed earlier, limit viral invasion and the subsequent immune response. Alternatively, it is possible that the allele inherited by the feto-placental unit from heterozygous mothers may have influenced pregnancy outcome, for example compromising the maternal tolerance to paternal antigens. The influence of individual alleles, such as *B2* on this tolerance remains to be investigated. In women, the placental unit has the capacity to modulate local maternal immune responses (Racicot et al., 2014). Thus inheritance of the *B2* allele may alter the immunological equilibrium at the feto-maternal interface and / or make it more vulnerable to challenge by pathogens.

The significant association between pregnancy loss and the MHC class I *B2* allele may be indicative of linkage with another unidentified locus or interaction with another risk factor. In women, genome wide association studies suggest that unexplained miscarriage may be associated with dysregulation of expressed genes involved in mitochondrial function and cytokine production (Lyu et al., 2013; Medica et al., 2009). In cattle, seven genes representing diverse pathways were moderately associated with fertility and early pregnancy loss (Minten et al., 2013). Thus using the equine genome sequence (Wade et al., 2009) as a basis for future genome wide association screening of horses, any genes and biological pathways involved in pregnancy loss may be elucidated.

In conclusion, this study provides new but preliminary evidence of an apparent positive association between the loss of an equine pregnancy and the presence of the MHC class I *B*2 allele in the mother. This newly identified association requires confirmation in other populations with more complete data sets, but nevertheless highlights an avenue meriting further research.

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Table 1. Inheritance patterns of predicted MHC class I haplotypes in a mixed half- and full-sibling Thoroughbred horse family. These animals were part of 40 Newmarket Thoroughbred horses of unknown MHC class I haplotypes on which the performance of the MHC class I, allele specific RT-PCR was tested.

Dam #	Dam Haplotype(s)	Offspring #	Offspring Haplotype(s)	Transmitted Sire Haplotype
AS	ELA-A2 / ELA-A5	1	ELA-A3 / ELA-A5	ELA-A3
AS	ELA-A2 / ELA-A5	2	ELA-A3 / ELA-A5	ELA-A3
AS	ELA-A2 / ELA-A5	3	ELA-A5	ELA-5 or Unknown
PM	ELA-A2	4	ELA-A2 / ELA-A3	ELA-A3
TJ	ELA-A3	5	ELA-A3	ELA-A3 or Unknown
YO	ELA-A5 / ELA-A9	6	ELA-A5 / ELA-A9	ELA-A5 or ELA-A9
SH	ELA-A3 / ELA-A9	7	ELA-A3	ELA-A3 or Unknown
MO	ELA-A5 / ELA-A9	8	ELA-A5 / ELA-A9	ELA-A5 or ELA-A9

Table 2. Details of the Thoroughbred mares' involved in the disease outbreak, their pregnancy outcomes and MHC class I genotype as determined by RT-PCR (n=29) or CTL assay (mares 13 and 36). The assumption was made that each allele was expressed on a single ELA-A haplotype. The haplotype was then deduced from the presence of DNA for that allele: A2 haplotype – allele *8-9*, A3.1 haplotype – allele *B2*, A5 haplotype – allele *5c*; A9 haplotype – allele *9c*; A10 haplotype- allele *10a*. Where only one RT-PCR product was amplified, the animal was likely to have been a homozygote, but to reduce bias, was assigned an *x* allele (X haplotype). * Genotypes of mares 13 and 36 were determined by CTL assay as *B2* (A3 haplotype) and an unknown allele on the A2 haplotype or *B2* respectively. Abbreviations: n/a= sample was not available for RT-PCR typing; no.= number; Pregnancy outcomes: F= viable foal; EHV Ab = EHV-1 positive pregnancy loss; Ab = EHV-1 negative pregnancy loss.

Mare no.	Pregnancy outcome	MHC class I allele(s) detected	Predicted ELA-A haplotype
1	F	*9c* / *x*	A9 / x
3	F	*8-9* / *10a*	A2 / A10
4	F	*8-9* / *10a*	A2 / A10
5	EHV Ab	*B2* / *x*	A3 / x
6	F	*8-9* / *9c*	A2 / A9
7	F	*8-9* / *5c*	A2 / A5
9	F	*B2* / *9c*	A3 / A9
11	F	*8-9* / *10a*	A2 / A10
12	F	*8-9* / *x*	A2 / x
13*	EHV Ab	*B2/ x**	A3* / x
14	EHV Ab	*8-9* / *B2*	A2 / A3
19	F	*5c* / x	A5 / x
21	F	*8-9* / *9c*	A2 / A9
23	F	*9c* / *10a*	A9 / A10
24	F	*8-9* / *5c*	A2 / A5
25	F	*9c* / *10a*	A9 / A10
26	F	*5c* / *9c*	A5 / A9

28	F	*5c* / *10a*	A5 / A10
29	Ab	*B2* / *9c*	A3 / A9
31	F	*5c* / *x*	A5 / x
32	F	*B2* / *9c*	A3 / A9
33	F	*8-9* / *5c*	A2 / A5
34	F	*B2* / x	A3 / x
35	F	*B2* / *9c*	A3 / A9
36*	EHV Ab	*B2* or *x**	A2 or A3*
38	Ab	*B2* / x	A3 / x
39	F	*8-9* / *B2*	A2 / A3
40	F	*9c* / *10a*	A9 / A10
41	F	*5c* / *x*	A5 / *x*
42	F	*5c* / *9c*	A5 / A9
44	F	*8-9* / *5c*	A2 / A5

Table 3. Details of the mares with pregnancy loss and determination of their MHC class I alleles. EHV-1 was detected in fetal and / or placental tissues using co-cultivation on Rabbit Kidney 13 indicator cells and amplification of DNA encoding glycoprotein B using specific primers. The MHC class I RT-PCR amplified the alleles *8-9* (A2 haplotype), *B2* (A3.1) and *9c* (A9). The Cytotoxic T Lymphocyte (CTL) column summarises the predicted MHC class I allele, as suggested by the ability of that mare's effector CTL to lyse defined target cells. nd= not done. *insufficient material due to predation of carcass.

Mare no.	Detection of EHV-1 in fetal and / or placental tissues		*Post mortem* diagnosis of the reason for pregnancy loss	MHC class I allele detected by the method shown	
	Virus isolation	PCR		RT-PCR	CTL
5	-	+	EHV-1	*B2*	*B2*
13	+	+	EHV-1	nd	*B2*
14	+	+	EHV-1	*8-9 / B2*	*B2*
20	-	+	EHV-1	nd	nd
36	+	+	EHV-1	nd	*B2*
27	-	-	Undetermined	nd	nd
29	nd	-	nd*	*B2 / 9c*	nd
30	-	-	Placentitis	nd	nd
37	-	-	Undetermined	nd	nd
38	-	-	Early placental separation	*B2*	nd

Supplementary File 1.

a) **First strand synthesis of RNA.** All incubations were performed in a thermocycler with heated lid. *Moloney Murine Leukaemia Virus Reverse Transcriptase

Step	Detail
Elution of RNA from column	50µl nuclease free water
RNA storage	65°C 10mins then cool on ice
First strand synthesis	25µl Master Mix 10µl 5x MMLV RT* buffer
	3.2µl 2mM dNTPs
	1.25µl (50units) recombinant RNAse inhibitor (RNase Out)
	1µl (0.5ug) oligo$(dT)_{12\text{-}18}$
	40u MMLV RT
	Nuclease free water to 25µl
Add master mix to the RNA	Incubate 37°C for 1.5hours

b) Details of the generic and allele specific MHC class I RT-PCRs.

a) Reaction mix for allele specific RT-PCR. Total reaction volume was 20µl

Step	Detail
Template cDNA	1.5µl
Amplitaq Gold DNA polymerase[1]	1 unit
PCR buffer II	1x
$MgCl_2$	2mM

Primers	0.5μM
dNTPs	200μM
dimethyl sulphoxide	2.5%

b) Reaction mix for generic RT-PCR. Total reaction volume was 50μl

Step	Detail
Template cDNA	3μl
Expand High Fidelity enzyme mix[2]	1.7 units
Expand high fidelity PCR buffer 2	1x
Primers	0.5μM
dNTPs	200μM
dimethyl sulphoxide	2.5%

c) Cycling conditions for allele specific RT-PCR

Step	Temperature (^{o}C)	Time (mins)
Denaturation	95^{o}C	5
Cycles (n=35)		
1 (denaturation)	95^{o}C	0.5
2 (annealing)	58^{o}C	0.5
3 (elongation)	72^{o}C	0.5
Final elongation	72^{o}C	1

d) Cycling conditions for generic RT-PCR

Step	Temperature (^{o}C)	Time (mins)
Denaturation	95^{o}C	5
Cycles (n=35)		
1 (denaturation)	95^{o}C	0.5

2 (annealing)	60^{o}C	0.5
3 (elongation)	72^{o}C	1
Final elongation	72^{o}C	1

[1] AmpliTaq Gold DNA polymerase, Applied Biosystems Warrington, Cheshire

[2] Taq polymerase and DNA polymerase with proofreading activity for high yield and fidelity, Expand High Fidelity Enzyme mix Roche, Welwyn Garden City, Hertfordshire.

Supplementary File 2. Alignment of DNA sequences (5’ to 3’) of selected regions for the five MHC class I alleles to show regions of forward and reverse primer binding. Underlined regions are primer sequences for the gene-specific PCR. Boxed regions show primer sequences for the generic PCR. Primers are 8-9 (gene N*00101; A2 haplotype), B2 (1*00101; A3), 5b (16*00201; A5), 9c (1*00201; A9) and 10a (N*00501; A10).

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5’          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   10         20         30         40         50         60         70         80         90        100
8-9        ATGCGGATCG TGATGCCCCC AACCTTCCTC CTGCTGCTCT CGGGGGCCCT GAGCCTGACC GAGACCCTGG CTGGCTCCCA CTCCATGAGG TATTTCTACA
3.1(B2)    ATGTGCGTCA TGATGCCGCC AACCTTCCTC CTGCTGCTGT CGGGGGCCCT GACCCTGACC GAGACCTGGG CGGGCTCCCA CTCCATGAGA TATTACAAAA
5b         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
9c         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
10a        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  110        120        130        140        150        160        170        180        190        200
8-9        CCGGCGTGTC CCGGCCCGGC CGCGGGGAGC CCCGCTTCGT CGCCGTCGGC TACGTGGACG ACACGCAGTT CGCGCGGTTC GACAGCGACG CCGCGAGTCC
3.1(B2)    CCGCCGTGTC CCGGCCCGGC CGCGGGGAGC CCCGCTTCAT CTCCGTCGGC TACGTGGACG ACACGCAGTT CGTGCGGTTC GACAGCGACG CCGCGAGTCC
5b         ---------- ---------- ---------- ---------- ---------- ---------- ---------- -GCGCGGTTC GACAGCGACG CCGCGAGTCC
9c         ---------- ---------- ---------- ---------- ---------- ---------- ---------- -GTGCGGTTC GACAGCGACG CCGAGAATCC
10a        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -GTGCGGTTC GACAGCGACG CCGCGGGTCC

           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  210        220        230        240        250        260        270        280        290        300
8-9        GAGGATGGAG CCGCGGGCGC CGTGGATGAA GCAGGAGAGG CCGGAGTATT GGGAGCGGAA CACGCGGATC TTCAAGGACA CAGCACAGAC TTTCCGAGTG
3.1(B2)    GAGGATGGAG CCGCGGGCGC GGTGGGTGGA GCAGGAGGGG CCGCAGTATT GGGAAGAAGA GACGCGGACC GCCAAGGGCC ACGCACAGAC TTTCCGAGGG
5b         GAAGGAGGAG CCGCTGGCGC CGTGGATGGA GCAGGAGGGG CCGGAGTATT GGGAGGAGAA CACGCGGATC TACAAGGGCA ACGCACAGAC TTTCCGAGAG
9c         AAAGGAGGAA CCGCGGGCGC CGTGGATGGA GCAGGAGGGG CCGGAGTATT GGGAAGAGAA CACGCGGCGC GCCAAGGGCC ACGCACAGAC TTCCCGAGTG
10a        GAGGATGGAG CCGCGGGCGC CGTGGGTGGA GCAGGAGGGG CCGGAGTATT GGGAAGAAGA GATGCGGACC GCCAAGGGCC ACGCACAGAC TTTCCGAGTG

           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  310        320        330        340        350        360        370        380        390        400
8-9        AGCCTGAACA ACCTGCGCGG CTACTACAAC CAGAGCGAGG CCGGGTCTCA CACCCTCCAG GACATGTATG GCTGCGACGT GGGGCCGGAC GGGCGTCTCC
3.1(B2)    AACCTGCGGA TCGCGCTCGG CTACTACAAC CAGAGCGAGG CCGGGTCTCA CACCTTCCAG TGGATGTCTG GCTGCGACGT GGGGCCGGAC GGGCGCCTCC
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5b          AACCTGAACA CCCTGCGCGG CTACTACAAC CAGAGCGAGG CCGGGTCTCA CACCCTCCAG TTGATGTACG GCTGCGACGT GGGGCCACAC GGGCGCCTCC
9c          AACCTGAACA CCCTGCGCGG CTACTACAAC CAGAGCGAGT CCGGGTCTCA CACCTACCAG GAAATGTATG GCTGCGACGT GGGGTCGGAC AGGCGCCTCC
10a         AACCTGAACA CCCTGCGCGG CTACTACAAC CAGAGCGAGG CCGGGTCTCA CACCCTCCAG GAAATGTATG GCTGCGACGT GGGGTCGGAC AGGCGCCTCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      410        420        430        440        450        460        470        480        490        500
8-9         TCCGTCGGGT ACAGACAGT- CGCCTACGAC GGCGCCGATT ACATCGCCCT GAACGAGGAC CTGCGCTCCT GGACCGCGGC GGACACGGCG GCTCAGATCA
3.1(B2)     TCCG-CGGGT ACAGTCAGTT CGCCTACGAC GGCGCCGATT ACATCGCCCT GAACGAGGAC CTGCGCTCCT GGACCGCGGC GGACACGGCG GCGCAGATCA
5b          TCAG-CGCAT CCTTTCAATA CGCCTACGAC GGCGCCGATT ACATCGCCCT GAACGAGGAC CGGCGCTCCT GGACCGCGGC GGACACGGCG GCTCAGATCT
9c          TCCG-CGGGT ACATTCAGTT CGCCTACGAC GGCGCCGATT ACATCGCCCT GAACGAGGAC CTGCGCTCCT GGACCGCGGC GGACGCGGCG GCGCAGATCA
10a         TCCG-CGGGT ATGAACAGTT CGCCTACGAC GGCGCCGATT ACCTCGCCCT GAACGAGGAC CTGCGCTCCT GGACCGCGGC GGACACGGCG GCTCAGATCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      510        520        530        540        550        560        570        580        590        600
8-9         CCCGGCGCAA GTGGGAGGCG GCCGGTGTGG CGGAGGACTT CAGGAACTAC CTGGAGGGCA CGTGCGTGGA GTCGCTCCTC AGATTCCTGG AGAACGGGAA
3.1(B2)     CCCGGCGCAA GTGGGAGGCG GCCGGTGAGG CGGAGCAGCA CAGGAACTAC CTGGAGGGCC GGTGCGTGGA GTGGCTCCTC AGATACCTGG AGAACGGGAA
5b          CCCGGCGCAA GTTGGAGGCG GCTGGTGTGG CGGAGGGCTA CAGGAACTAC CTGGAGGGGG AGTGCGTGGA GTGGCTCCTC AGATACCTGG AGAACGGGAA
9c          CCCGGCGCAA GTTGGAGGCG GCCGGTGTGG CGGAGCAGCG CAGGAACTAC CTGGAGGGGG AGTGCGTGGA GTGGCTCCTC AGACACCTGG AGAACGGGAA
10a         CCCGGCGCAA GTGGGAGACG GCCGGTGAGG CGGAGGGCTA CAGGAACTAC CTGGAGGGCA CGTGCGTGGA GTGGCTCCTC AGATACCTGG AGAACGGGAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      610        620        630        640        650        660        670        680        690        700
8-9         GGAGACGCTG CAGCGCGCGG ACTCGCCAAA GACACACGTG ACCCACCACC CCATCTCTGA CCGTGAGGTC ACCCTGAGGT GCTGGGCCCT GGGCTTCTAC
3.1(B2)     GGAGACGCTG CAGCGCGTGG ACCCTCCGAA GACACATGTG ACCCACCACC CCAGCTCTGA CCATGAGGTC ACCCTGAGGT GCTGGGCCCT GGGCTTCTAC
5b          GGAGACGCTG CAGCGCGTGG ATCCCCCAGA GACACACGTG ACCCACCACC CCATCTCTGA CCGTGAGGTC ACCCTGAGGT GCTGGGCCCT GGGCTTCTAC
9c          GGAGACGCTG CAGCGCGCGG ACCCCCCGAA GACACATGTG ACCCACCACC CCATCTCTGA CCGTGAGGTC ACCCTGAGGT GCTGGGCCCT GGGCTTCTAC
10a         CGAGACACTG CAGCGCGCGG ACGCCCCAAA GACACATGTG ACCCACCACC CCATCTCTGA CCATGAGGTC ACCCTGAGGT GCTGGGCCCT GGGCTTCTAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      710        720        730        740        750        760        770        780        790        800
8-9         CCTCCAGAGA TCAGCCTGTC CTGGCAGCGT GATGGGGAGG ACCTGACCCA GGACACGGAG TTTGTGGAGA CCAGGCCTGC AGGGGACGGG ACCTTCCAGA
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3.1(B2)     CCTGCGGAGA TCACCCTGAC CTGGCAGCGT GATGGAGAGG ACCTGACCCA GGACACGGAG TTTGTGGCGA CCAGGCCTGC AGGGGACGGG ACCTTCCAGA
5b          CCTGCGGAGA TCGCCCTAAC CTGGCAGCGT GATGGGGAGG ACATGACCCA GGACACGGAG CTTGTGGAGA CCAGGCCTGC AGGGGACGGG ACCTTCCAGA
9c          CCTGCGGAGA TCACCCTGAC CTGGCAGCGT GACGGGGAGG ACCTGACCCA GGACACGGAG TTTGTGGCGA CCAGGCCTGC AGGGGACGGG ACCTTCCAGA
10a         CCTGAGGAGA TCAGCCTGTC CTGGCAGCGT GACGGGGAGG ACGTGACCCA GGACACAGAG CTTGTGGAGA CCAGGCCTGC AGGGGACCGA ACCTTCCAGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      810        820        830        840        850        860        870        880        890        900
8-9         AGTGGGCTGC TGTGGTGGTG CCTTCTGGAG AGGAACAGAG CTACACGTGC CATGTGCAGC ACGAGGGGCT GCCTGAGCCT GTGACCCTGA GATGGGAGCC
3.1(B2)     AGTGGGCGGC TGTGGTGGTG CCTTCTGGGG AGGAGCAGAG ATACACGTGC CATGTGCAGC ACGAGGGGCT GCCTGAGCCT GTCACCCGGA GATGGGAGCT
5b          AGTGGGCGGC TGTGGTGGTG CCTTCTGGAG AGGAGCAGAG ATACACGTGC CATGTGCAGC ACGAGGGGCT GCCTGAGCCT GTCACCCGGA GATGGGAGCC
9c          AGTGGGCGGC TGTGGTGGTG CCTTCTGGGG AGGAGCAGAG ATACACGTGC CATGTGCAGC ACGAGGGGCT GCCTGAGCCC GTCACCCGGA GATGGGAGCC
10a         AGTGGGCGGC TGTGGTGGTG CCTTCTGGAG AGGAGCAGAG ATACACGTGC CATGTGCAGC ACGAGGGGCT GCCTGAGCCC CTCACCCTGA GATGGGAGCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      910        920        930        940        950        960        970        980        990       1000
8-9         GCCTCCTATG TCCACCATCC TCATCGTGGG CGTCCTTGCT GGCCTGGTTC TCCTTGTCGC TGTGGTGGCT GGAGCTGTGA TCTGGAGGAA GAAGCACTCA
3.1(B2)     GCCTCCTCAG TCCACCATCC TCATCGTGAG CATCATTGCT GGCCTGGTTC TCCTTGGAGC TGTGGTGGCT GGAGCTGTGA TCTGGAGGAA GAAGCGCTCA
5b          TCCTCCTCAG TCCACCATCC TCATCGTGGG CGTCCTTGCT GGCCTGCTTC TCCTTGTCGC TGTGGTGGCT GGAGCTGTGA TCTGGAGGAA GAAGCACTCA
9c          GCCTCCTCAG TCCATCATCC TCATCGTGGG CGTCCTTGCT GGCCTGGTTC TCCTTGGAGC TGTGGTGGCT GGAGCTGTGA TTTGGAGGAA GAAGCGCTCA
10a         GCCTCCTCAG TCCACCATCC TCATCGTGGG CGTCCTTGCT GGCCTGGGTC TCCTTGGAGC TGTGGTGGCT GGAGCTGTGA TCTGGAGGAA GAAGCGCTCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....| 3'
                     1010       1020       1030       1040       1050       1060       1070       1080       1090
8-9         GGTGAAAAAA GAGGGATTTA CGTGCAGGCT GCAAACAATG ACAGTGCCCA GGGATCTGAT GCGTCTCTCC CGCAGAAAGT GTGAGACGGT GGCCC
3.1(B2)     GGTGAAAAAA GAGGGATTTA CGTGCAGGCT GCAAACAGTG ACAGTGCCCA AGGCTCTGAT GCGTCTCTCC CACAGAAAGT GTGAGACAGT GGCCC
5b          GGTGAAAAAA GAGGGATTTA CGTGCAGGCT GCAAACAGTG ACAGTGCCCA AGGCTCTGAT GCGTCTCTCA CTCAGAAAGT GTGAGACAGT GGCCC
9c          GGTGAAAAAA GAGGGATTTA CGTGCAGGCT GCAAACAGTG ACAGTGCCCA AGGCTCTGAT GCGTCTCTCC CACAGAAAGT GTGAGACAGT GGCCC
10a         GGTGCAAAAA GAGGGATTTA CGTGCAGGCT GCAAACAGTG ACAGTGCCCA GGGATCTGAT GCGTCTCTCC CACAGAAAGT GTGAGACGGT GGCCC
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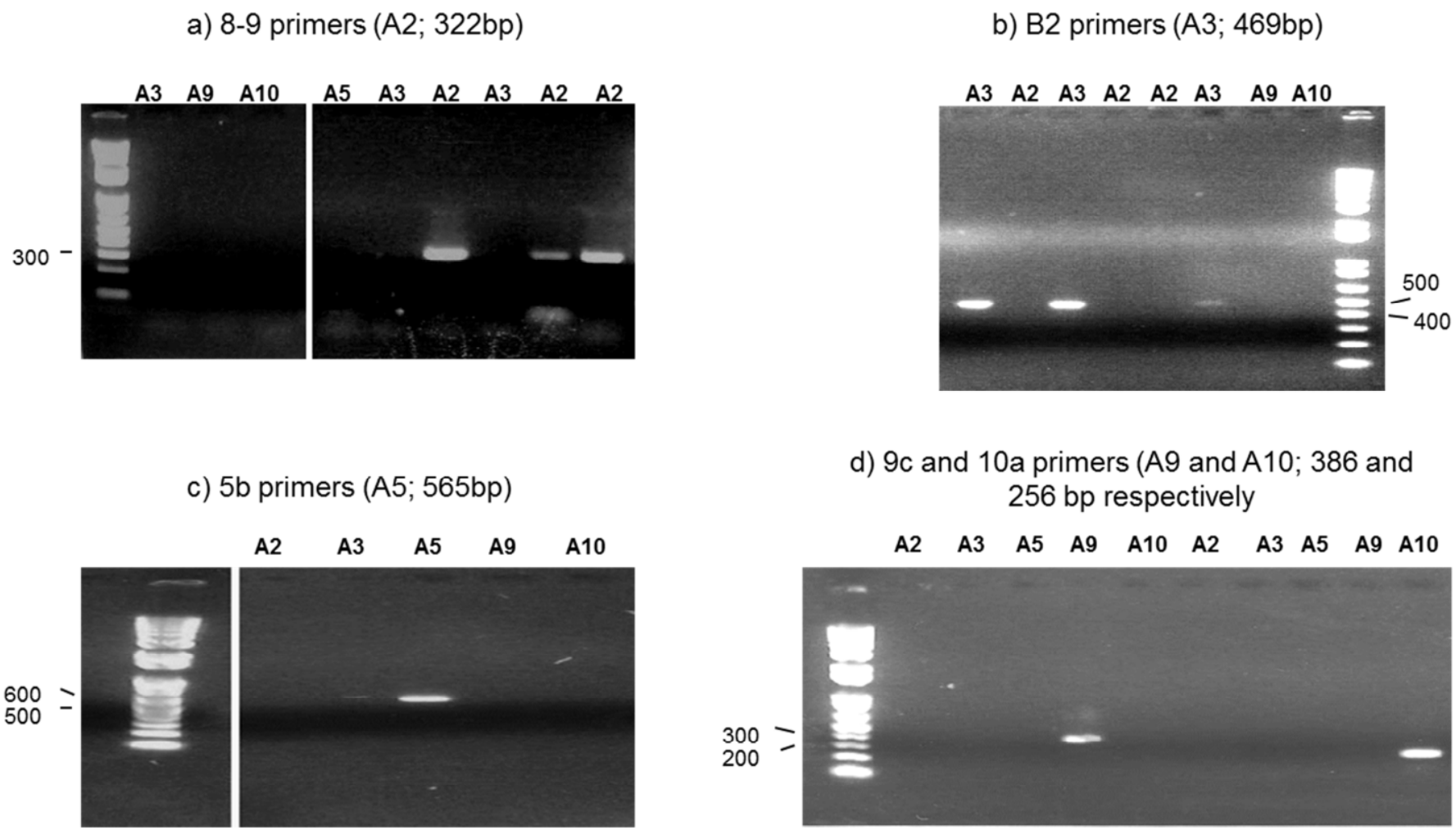
Supplementary File 3. Equine MHC class I genes and primers used to amplify specific alleles by RT-PCR.

Equine MHC Haplotype	Locus[#]	Gene Name[#] (Aliases)	GenBank Accession[#]	Primer name	Position relative to origin	Primer sequences	Predicted size (base pairs)
A2	N	N*00101 (8-9)	M95409	8-9F4 8-9R	406-425 707-727	TCCGTCGGGTACAGACAGTC CAGGACAGGCTGATCTCTGG	322
A3	1	1*00101 (3.1, B2)	DQ083407	B2Fext B2Rext	68-90 518-536	CCCACTCCATGAGATATTACAAA TTCCTGTGCTGCTCCGCCT	469
A5	16	16*00201 (5b)	DQ145594	5bF1 5bR	26-44 571-590	GTCCGAAGGAGGAGCCGCT AGCTCCGTGTCCTGGGTCAT	565
A9	1	1*00201 (9.1, 9c)	DQ145597	9cF 9cR	20-40 387-405	CCGAGAATCCAAAGGAGGAAC GAGCCACTCCACGCACTCC	386
A10	18	N*00501 (10a)	DQ145599	10aF5 10aR2	601-618 833-856	AGGCCTGCAGGGGACCGA CTGCACGTAAATCCCTCTTTTTG	256
Generic ELA^	N/A	N/A	N/A	Classical F Classical R	exon 3 exon 7/8	CGAGGCCGGGTCTCACACC GGGCCACTGTCTCACACTTTCTG	~760

[#]Loci, gene names, and GenBank Accession numbers from Tallmadge et al. (2010). ^primers adapted from Chung et al. (2003)

N/A = not applicable

Supplementary File 4. PCR products amplified from the Cornell horses' lymphocyte cDNA by allele-specific primers associated with the five selected Thoroughbred MHC class I haplotypes. The haplotypes of horses from which the cDNA was derived are shown at the top of each lane and a 1kb ladder is also illustrated. a) – d) Allele-specific primers used, their associated serological haplotype and predicted PCR product size: a) primers *8-9*, A2 haplotype; b) primers *B2*, A3.1 haplotype; c) primers *5b*, A5 haplotype and d) left side of gel, primers *9c*, A9 haplotype and right side of gel, primers *10a*, A10 haplotype.



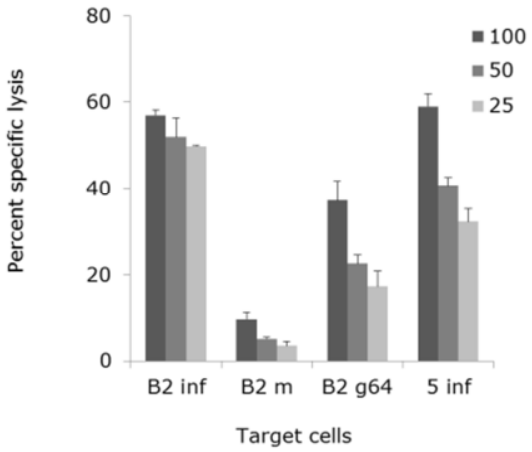
Supplementary File 5. Details of the mares' ages, reproductive and EHV-1 specific vaccination histories and pregnancy outcomes. Abbreviations: no.= number; Pregnancy outcomes: F= viable foal; EHV Ab = EHV-1 positive pregnancy loss; Ab = EHV-1 negative pregnancy loss.

Mare no.	Age (years)	No. of previous foals	Total no. of vaccine doses	Pregnancy outcome
1	9	5	22	F
2	13	8	30	F
3	6	1	15	F
4	19	12	35	F
5	9	4	23	EHV Ab
6	14	7	31	F
7	7	2	18	F
8	9	4	21	F
9	16	10	30	F
10	10	4	22	F
11	17	9	28	F
12	8	4	21	F
13	11	5	24	EHV Ab
14	6	1	14	EHV Ab
15	10	5	24	F
16	7	2	13	F
17	11	6	27	F
18	11	6	27	F
19	22	14	26	F
20	8	3	22	EHV Ab
21	5	0	5	F
22	13	7	28	

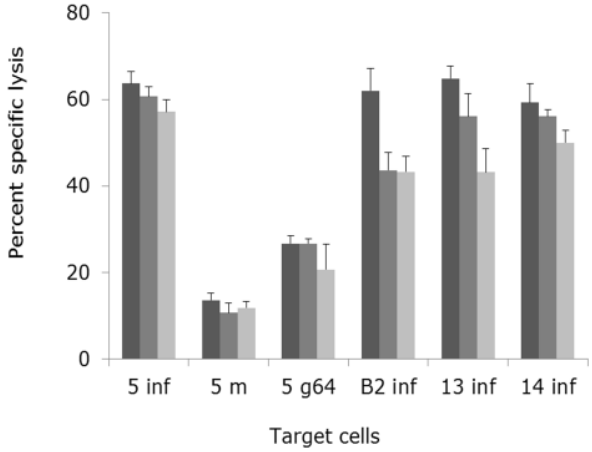
23	7	2	18	F
24	11	4	22	F
25	11	5	24	F
26	12	6	27	F
27	15	7	27	Ab
28	7	2	18	F
29	8	3	20	Ab
30	19	7	26	Ab
31	13	7	27	F
32	20	14	27	F
33	10	4	16	F
34	8	3	17	F
35	9	4	22	F
36	7	2	12	EHV Ab
37	10	4	22	Ab
38	5	0	4	Ab
39	6	1	17	F
40	10	5	23	F
41	11	5	26	F
42	9	3	20	F
43	8	2	12	F
44	17	10	28	F
45	13	7	28	F
46	13	7	29	F

Supplementary File 6. Cytotoxic T lymphocyte activity in EHV-1 stimulated, Thoroughbred PBMC against the target cells shown. Effectors were stimulated with EHV-1 strain Ab4: a) EHV-1 hyperimmune pony mare expressing the MHC class I *B2* allele was used as the positive assay control. b) to e) four Thoroughbred mares sampled once at 5-10 months after EHV-1 positive pregnancy loss. Target cells are shown on the x axis and are designated as either *B2* allele positive or the number of the mare, followed by target cells' status: inf = infected with EHV-1; m = mock infected; g64 = NYVACC gene 64 infected (Kydd et al., 2006; Paillot et al., 2006). Effector to target cell ratios 100:1, 50:1 and 25:1 as shown in a). Bars indicate standard error of mean of three replicate wells.

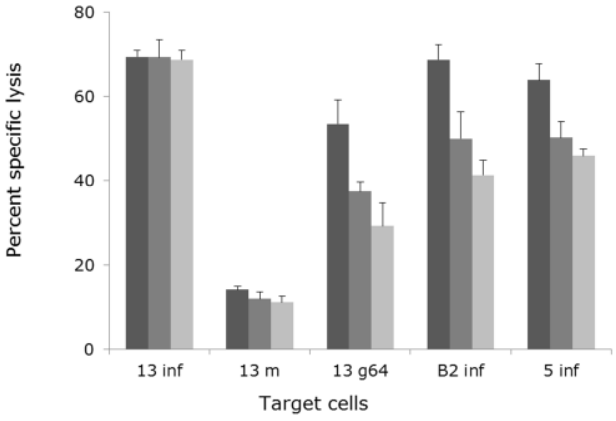
a) Effectors: MHC class I, B2 allele positive pony



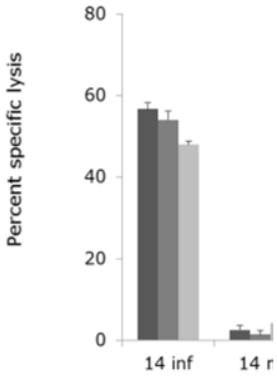
b) Effectors: Mare 5



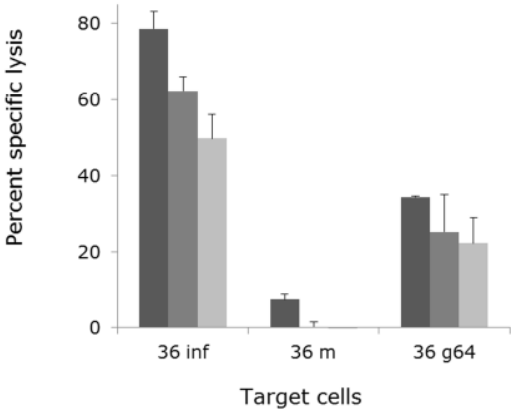
c) Effectors: Mare 13



d) Effectors: Mare 14



e) Effectors: Mare 36



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